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REVIEW

Mycoplasma pneumoniae from the Respiratory Tract and Beyond

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Published 24 May 2017

Citation Waites KB, Xiao L, Liu Y, Balish MF, Atkinson TP. 2017. *Mycoplasma pneumoniae* from the respiratory tract and beyond. Clin Microbiol Rev 30:747–809. https://doi.org/10.1128/CMR.00114-16.

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SUMMARY *Mycoplasma pneumoniae* is an important cause of respiratory tract infections in children as well as adults that can range in severity from mild to life-threatening. Over the past several years there has been much new information published concerning infections caused by this organism. New molecular-based tests for *M. pneumoniae* detection are now commercially available in the United States, and advances in molecular typing systems have enhanced understanding of the epidemiology of infections. More strains have had their entire genome sequences published, providing additional insights into pathogenic mechanisms. Clinically significant acquired macrolide resistance has emerged worldwide and is now complicating treatment. *In vitro* susceptibility testing methods have been standardized, and several new drugs that may be effective against this organism are undergoing development. This review focuses on the many new developments that have occurred over the past several years that enhance our understanding of this microbe, which is among the smallest bacterial pathogens but one of great clinical importance.

KEYWORDS *Mycoplasma pneumoniae*, macrolide resistance, pneumonia, respiratory pathogens

INTRODUCTION

Since the last review on *Mycoplasma pneumoniae* was published in *Clinical Microbiology Reviews* in 2004 (1), considerable new information has been reported concerning the disease spectrum and clinical manifestations of infections caused by this organism. The first exotoxin (community-acquired respiratory distress [CARDS] toxin) has been described; acquired macrolide resistance has emerged worldwide; antimicrobial susceptibility testing methods have been standardized; several new molecular tests for *M. pneumoniae* detection have been described; more strains have had their entire genome sequences published, providing new insights into pathogenic mechanisms; and advances in molecular typing systems have enhanced understanding of the epidemiology of *M. pneumoniae* infections. Much of the older, historical information concerning taxonomy, cell biology, histopathology of lung infections, immunology, and clinical microbiological aspects of *M. pneumoniae* infections described in earlier reviews (1–4) is still relevant for understanding this organism and the diseases it causes. Therefore, the present review focuses on the new developments that have occurred over the past several years.

MYCOPLASMA PNEUMONIAE INFECTION EPIDEMIOLOGY

M. pneumoniae can induce both upper and lower respiratory infections and occurs both endemically and epidemically worldwide. Although tracheobronchitis is a more common clinical manifestation, pneumonia is the most clinically important illness associated with *M. pneumoniae* infections. *M. pneumoniae* may be responsible for about 4 to 8% of community-acquired bacterial pneumonias (CABP) during periods of endemicity. However, this organism can cause up to 20 to 40% of CABP in the general population during epidemics, rising to as much as 70% in closed populations (5, 6). An estimated 2 million cases occur annually, resulting in about 100,000 hospitalizations of adults in the United States (7, 8). However, due to the relatively mild nature of many *M. pneumoniae* infections, their similarity in presentation to other causes of pneumonia, and the lack of reliable point-of-care diagnostic tests to confirm the microbiological diagnosis, many infections as well as clusters and outbreaks are likely to be undetected (8). The proportion of CABP caused by *M. pneumoniae* varies according to age, with school-age children and adolescents being the most common age groups affected, but this organism can cause infections in persons from infancy up through old age (7–11). Lack of an organized surveillance program for *M. pneumoniae* infections in the United States and most other countries makes it impossible to assess the true impact of this organism on public health, but data are available from limited surveillance studies and clinical trials that can provide some insight into the magnitude of disease burden. Although numerous prospective studies designed to assess etiologies of pneumonia have been published over the past several years, many of them have significant

shortcomings, such as limiting investigation only to bacteria, excluding viruses, and limiting diagnostic testing to suboptimal methods such as serology in lieu of more accurate techniques such as molecular-based methods.

Global and Regional Epidemics

M. pneumoniae occurs endemically worldwide in many different climates. Infections tend to be more common in summer or early fall, but can occur at any time of the year. Japanese researchers have reported that there is a positive correlation between increases in temperature and the occurrence of *M. pneumoniae* infections, which might help explain the increased numbers that may occur during warmer months (12, 13). Epidemics that can encompass broad geographic ranges tend to occur every few years. Since 2010, several European countries have experienced increased numbers of *M. pneumoniae* infections, particularly those in the northern regions (e.g., Denmark, Sweden, Norway, England, Germany, Scotland, Finland, the Netherlands, and France) (5, 11, 14–24). Additional epidemics have been reported in Chile, Brazil, Israel, South Korea, Japan, and China during the same time period (24–30).

Even though there are no organized surveillance programs for *M. pneumoniae* in the United States, there are data from focused surveillance programs in some countries and regions. Surveillance data from England and Wales obtained during 2011 to 2012 using quantitative real-time PCR on nasopharyngeal (NP) and oropharyngeal (OP) swabs showed that the incidence of *M. pneumoniae* infection in children aged less than 16 years was 9%, rising to 14.3% in the 5- to 14-year-olds. Only 1 of 60 (1.6%) children less than 5 years of age was PCR positive (31). In contrast, in an analysis of 1,232 PCR-positive laboratory samples from Scotland obtained between 2008 and 2011, Gadsby and colleagues noted that the highest incidence of *M. pneumoniae* occurred in the youngest children. About 29% of the PCR-positive samples were from children aged 4 years and younger, and an additional 18% were from children between 5 and 9 years of age (11). However, among laboratories that reported serological data, *M. pneumoniae* infections were more common among children in the 5- to 9-year age group than among those in the 0- to 4-year group (18% versus 10.4%, respectively).

M. pneumoniae isolates can be classified into 2 major genetic groups, designated subtype 1 and subtype 2, based on sequence differences in repetitive elements RepMP2/3 and RepMP4 in the P1 protein gene (32). Parts of these repetitive elements are considered a reservoir for recombinative processes that have resulted in several subtype variants (33–41), distinguishable by techniques such as real-time PCR followed by high-resolution melt analysis (HRM) (41, 42). It has been speculated that the cyclical *M. pneumoniae* epidemics that tend to occur every few years could be related to a shift from one P1 subtype to the other, since the two major subtypes are immunologically distinct and exposure to one subtype may induce transient herd immunity that suppresses infections with that subtype while allowing the other one to reemerge (43). Indeed, alternating predominance of subtype 1 or subtype 2 strains in a population has been documented (44–46). However, this is not always the case, as there can be cocirculation of both subtypes and multiple variants in the same setting (5, 29, 42, 47, 48).

An analysis of the French epidemic in 2011 found that 82% of isolates were subtype I, with 18 different multilocus variable-number tandem-repeat analysis (MLVA) subtypes (discussed further in Molecular Typing below), indicating that the epidemic was polyclonal, as were the epidemics in Israel and in England and Wales that occurred at about the same time (18, 24). However, clonal dissemination of *M. pneumoniae* within families and communities clearly occurs (49, 50).

Based on serological assays, the multinational Asian Network for Surveillance of Resistant Pathogens (ANSORP) reported that *M. pneumoniae* was responsible for 11% of CABP in 955 adults and *Chlamydia pneumoniae* for 13% in eight Asian countries. *Streptococcus pneumoniae*, detected in 29% of patients, was the most common organism identified in that study (51). In a study conducted in Australia during 2004 to 2005 in which 885 adults with pneumonia were recruited from emergency departments, *S.*

pneumoniae was the most common pathogen detected (14%) and *M. pneumoniae* was second using serology, appearing in 9%. No pathogen was detected in more than half of the patients. A prospective study conducted in Hong Kong among urban adults hospitalized with pneumonia in 2004 to 2005 (52) found that 29% had infections with atypical pathogens. *M. pneumoniae* was detected by serology in 78/1,193 patients (6.5%), and *C. pneumoniae* was detected in 55/1,193 (4.6%). A Chinese study of children with a clinical diagnosis of pneumonia demonstrated *M. pneumoniae* using a commercial PCR assay in tracheal aspirates in 26/176 (15%) children, making it the third most common pathogen detected, following *Haemophilus influenzae* and *S. pneumoniae*. However, over half of the patients had no pathogen detected (53). Samransamruajkit et al. (54) reported the prevalence of *M. pneumoniae* and *C. pneumoniae* in severe CABP among hospitalized pediatric patients in Thailand in 2005 and 2006. Among 52 patients, 13 (25%) were positive for *M. pneumoniae*, 8 (15%) were positive for *C. pneumoniae*, 4 (8%) were serologically positive for both organisms, and 27 (52%) were negative. Three patients infected with *M. pneumoniae* and 2 with *C. pneumoniae* developed respiratory failure. Significant limitations to this study were reliance solely on serology and failure to include detection methods for other microorganisms, including respiratory viruses. A Chinese study conducted in 2008 to 2009 (55) used PCR and culture to detect *M. pneumoniae* in 62/215 (29%) adult outpatients with pneumonia. As reported in some of the other studies, no microbiological diagnosis was possible in more than half of all patients tested. In a prospective study of adult outpatients with CABP in Germany between 2002 and 2006, there were 7% (307/4,532) patients who were PCR positive for *M. pneumoniae* by real-time PCR and/or positive for elevated IgM antibodies (56). Another German study (57) used real-time PCR to test adult patients with confirmed CABP for infection with *M. pneumoniae* during 2011 to 2012. Overall, 12.3% (96/783) of samples were PCR positive. The results from a large international clinical trial of CABP conducted in 16 countries in adults with radiologically demonstrated pneumonia suggested a microbiological etiology in 54% of patients enrolled, with *M. pneumoniae* detected by PCR and/or culture in 9% (58).

The U.S. Centers for Disease Control and Prevention (CDC) performed surveillance for radiologically confirmed community-acquired pneumonia in hospitalized children and adults in 2010 to 2012 (EPIC Study), using real-time PCR for detection of *M. pneumoniae* (59, 60). A viral or bacterial pathogen was detected in 81% (1,802/2,222) of children, with multiple pathogens detected in 26%. However, bacteria alone were found in only 175 (8%). Respiratory viruses were the most commonly detected pathogens in children. *M. pneumoniae* was detected in 8%, compared to 4% for *S. pneumoniae*. *M. pneumoniae* was more common in children 5 years of age or older than in younger ones (60). *M. pneumoniae* was detected in only 43/2,247 (2%) of adult patients (59). No pathogen was detected in the majority of adult patients. The relatively low frequency of detection for *M. pneumoniae* in some of the aforementioned studies in comparison to others probably reflects variable virulence factors, including the fact that some were done during periods when there was a low level of *M. pneumoniae* circulating in the respective communities where the studies took place as opposed to an epidemic period. In addition, the ages of the patients being sampled, as well as the laboratory methods used in identification of the pathogen, varied among the studies.

Coexistence of *M. pneumoniae* with other agents of CABP is also common. Jain and colleagues from the CDC (60) reported coinfections in 28% of patients. Another recent study of hospitalized patients with CABP from the CDC (61) found one or more coinfections in 125/209 (60%) NP or OP specimens that were positive for *M. pneumoniae* using a TaqMan Array. They noted that such coinfections were found predominantly in children. There were 34 (16%) coinfections with bacteria and viruses, 17 (8%) infections with viruses alone, and 74 (45%) with bacteria alone. Using the Biofire FilmArray, Zheng and coworkers (62) reported that 26/80 (33%) respiratory specimens from symptomatic children had a viral pathogen detected along with *M. pneumoniae*. A study from China using only serology demonstrated that *M. pneumoniae* was present along with other bacteria or viruses in 46 of 77 (60%) children (63). Whether such coinfection involving *M. pneumoniae* with multiple

other pathogens is related to severity of illness in these patients is not known, but Mandell has previously noted that mixed infections with bacteria and viruses tend to be associated with more severe illnesses (64).

Local Outbreaks in the United States

The CDC has described several clusters and outbreaks of *M. pneumoniae* infections in various states over the past several years, using serology, molecular-based detection, and strain typing techniques to characterize these events (65–70). Between 2006 and 2013, there were 17 CDC-assisted investigations of sporadic cases, clusters, and local outbreaks (47). *M. pneumoniae* caused an outbreak of pneumonia involving 57 students in 4 Rhode Island schools in 2006 to 2007 and was associated with the development of life-threatening encephalitis in 2 children. Investigators demonstrated that infection from the schools also spread in households, further amplifying the outbreak (70). An outbreak in West Virginia in 2011 involved all 8 schools in two counties, in which there were 125 cases of pneumonia. Among those, 23/43 (53%) were PCR positive. Eighty-three cases of pneumonia were identified in a 2012 outbreak at a Georgia college, making it the largest outbreak since 1979 (66, 71). There were 12 of 19 persons (63%) who were PCR positive. Five persons required hospitalization, indicative of the potential seriousness of these infections and the needs for prompt outbreak recognition and implementation of public health control measures to limit transmission and complications (66). The potential severity of *M. pneumoniae* infection was also illustrated in another CDC investigation of a healthy South Carolina woman who developed pneumonia requiring mechanical ventilation for 13 days in 2013. In addition, her 55-year-old uncle and 26-year-old cousin were also hospitalized with similar symptoms and required mechanical ventilation for respiratory failure. *M. pneumoniae* was identified by PCR from the cousin of the index case (69). In 2014, an outbreak occurred in a long-term-care facility in Nebraska. The illness eventually involved 55 persons with 7 deaths, resulting in closure of the facility to new patients for a period of time (65). There were 41 probable and 14 PCR-confirmed cases of pneumonia due to *M. pneumoniae*. Even though institutional outbreaks of *M. pneumoniae* are well known, this one was unusual because of the type of facility and the number of fatalities. In another outbreak, the primary manifestation in several children hospitalized in Colorado during 2013 was Stevens-Johnson syndrome (SJS) with mucosal and cutaneous bullous lesions, which are well known complications of *M. pneumoniae* infections that rarely occur in epidemiological clusters (67). Strain typing during CDC-assisted investigations confirmed the cocirculation of both major subtypes and multiple MLVA types, with strain diversity within individual outbreaks. MLVA types 4-5-7-2, 3-5-6-2, and 3-6-6-2 accounted for 97% of all infections analyzed between 2006 and 2013 (47). MLVA type 4-5-7-2 strains were subtype 1, while type 3-5-6-2 isolates were subtype 2 (72). Additional community and institutional outbreaks of macrolide-resistant *M. pneumoniae* (MRMP) have been reported, and those are described in "Macrolide Resistance" below. Due to the long incubation period and prolonged carriage of *M. pneumoniae* after resolution of illness, outbreaks can last for several months (47). In some of these outbreaks *M. pneumoniae* was not suspected initially, resulting in a delay of diagnostic testing and implementation of strict infection control measures needed to be implemented in a timely manner to contain the illness and reduce morbidity and mortality. Another interesting outbreak of respiratory illness occurred on a United States navy vessel in 2006 to 2007. There were 179 cases of acute respiratory illness reported in military personnel over a 4-month period, including 50 cases of radiographically confirmed pneumonias. Using PCR and cultures from a subset of the affected persons, *M. pneumoniae* was confirmed to be the etiological agent (73). This is the first documented *M. pneumoniae* outbreak on a ship, although outbreaks on military bases are well known (4).

Infection and Carriage in the Upper Respiratory Tract

Although most epidemiological and clinical studies involving *M. pneumoniae* have focused on CABP, some prospective investigations have assessed its role in upper

respiratory tract infections. Using serology and/or a positive PCR assay, Esposito et al. reported that among 127 children with acute pharyngitis aged 6 months to 14 years, *M. pneumoniae* was the most common bacterial etiology (25 patients, 19.7%) (74). These investigators also performed a prospective, randomized study of children with pharyngitis due to *M. pneumoniae* or *C. pneumoniae* comparing azithromycin with acetaminophen to acetaminophen alone, which showed benefit of treatment for pharyngitis caused by *M. pneumoniae* (75). In contrast, a recent study of 312 students aged 15 to 30 years presenting to a university student health clinic with an acute sore throat found that *Fusobacterium necrophorum* was detected by real-time PCR in 21% of patients, *Streptococcus pyogenes* was detected in 10% of patients, group C/G beta-hemolytic streptococcus was detected in 9% of patients, and *M. pneumoniae* was detected in only 1.9% of patients, suggesting that it is not an important cause of pharyngitis in this population (76). A study of children undergoing adenotonsillectomy for recurrent upper respiratory infections reported that tonsil or adenoid tissue from 6 of 55 (11%) of them were PCR positive for *M. pneumoniae*. However, there was no control group for comparison (77). *M. pneumoniae* is also uncommon in other upper respiratory conditions, such as otitis media, rhinosinusitis, and the common cold (78, 79).

Natural immunity to *M. pneumoniae* infections is usually short-lived as evidenced by the frequency of reinfections in the same persons over time, and organisms continue to be shed for variable periods after resolution of clinical illness. This observation indicates a failure of natural immunity to eliminate the organisms, leading to prolonged carriage in some instances. Moreover, macrolide and tetracycline antibiotics commonly used to treat mycoplasmal infections are bacteriostatic agents and thus may contribute to prolonged carriage. A study from the Netherlands (80) found *M. pneumoniae* DNA in 21% of asymptomatic children versus 16% of symptomatic children and in similar bacterial loads. They also found that the organisms can persist for up to 4 months in the absence of clinical illness and that carriage rates were variable between seasons and sampling years. A study from the United States reported that 56% of healthy children carried *M. pneumoniae* in the upper respiratory tract, and carriage rates of 6.7% to 13% were described during an outbreak investigation (81). However, another study that included students with no symptoms of pharyngitis or other respiratory infection attending a university health clinic found 0 of 180 individuals were positive for *M. pneumoniae* by PCR on OP swabs (76). Other studies also reported low rates of carriage in healthy persons. Palma et al. (82) found that only 4/185 asymptomatic children in Chile were PCR positive. Reasons for different carriage rates among published studies are not readily apparent, other than they could be related to the prevalence of the organisms in the local community at a given time. It appears that no single detection method or interpretation is capable of reliably differentiating colonization from infection (83).

NEW DEVELOPMENTS IN PATHOGENESIS AND HOST DEFENSE

M. pneumoniae is an obligate parasite. Therefore, it can be quite difficult to distinguish *bona fide* virulence factors from general cellular processes. The ability to grow *M. pneumoniae* in culture axenically and to obtain mutants with reduced ability to cause disease can provide useful measures of what constitutes virulence factors and also provide insights regarding interactions between *M. pneumoniae* and host cells. Considerable knowledge concerning pathogenesis of *M. pneumoniae* infections has also been derived from use of animal models, primarily chimpanzees, hamsters, guinea pigs, and mice, and from cell cultures *in vitro* (84). Murine models have been especially useful to understand host-microbe interactions because of the availability of transgenic mice. However, unlike chimpanzees, in which mycoplasmal disease is very similar to that of humans, infections in mice may differ in terms of pathology, disease progression, and host-pathogen interactions (84). Animal studies have also shown *M. pneumoniae* infection to be influenced by gender, age, genetic background, and environmental stress, factors which may contribute to the wide range of clinical manifestations that occur in

human disease (85, 86). Against a host-derived onslaught of proinflammatory cytokines, such as interleukin-8 (IL-8), IL-6, IL-10, tumor necrosis factor alpha (TNF-α), and IL-1-β, and cellular elements, including neutrophils, lymphocytes and macrophages, and mast cells, *M. pneumoniae* adheres in close proximity to the epithelial cell surface, where it uses toxic molecules to damage host cells, inducing ciliostasis and epithelial desquamation, in an effort to acquire the critical nutrients that, unlike bacteria with less streamlined genomes, it cannot make for itself (4, 87).

Cytadherence

Adherence of *M. pneumoniae* to host cells is absolutely critical for virulence, as evidenced by the absence of virulence in strains deficient for this property (88). This requirement for cytadherence is likely to be related to access to nutrients available from the host cell and might also promote evasion of the host immune response. Binding of *M. pneumoniae* to the host cell surfaces relies on proteins modified with sialic acid (89) and sulfated glycolipids (90). Adherence is itself inherently required for gliding motility of *M. pneumoniae* cells on surfaces (91), a function linked to both cell division (92) and spreading during infection (93). Gliding motility of *M. pneumoniae* is unidirectional (91), not known to be associated with chemotaxis, and mechanistically distinct from similar processes in distantly related organisms, including *Mycoplasma mobile* (94). Intriguingly, a nearly nonmotile *M. pneumoniae* mutant, II-3R, which retains most of its cytadherence capability, is avirulent (95), suggesting that at least part of what makes cytadherence essential for virulence is its ability to confer motility. Invasion of host cells occurs in some tissue culture models but not with normal human bronchial epithelial (NHBE) cells (96). Studies with NHBE cells suggest that once *M. pneumoniae* cells traverse the mucus layer, cilia are an initial attachment site, followed by translocation to the host cell surface, presumably via gliding motility (93). These cells respond by stimulating mucin production at the transcriptional level, exacerbating disease symptoms (97). *M. pneumoniae* cells can organize on surfaces as biofilms (98) with strain-specific phenotypic differences (99), suggesting a further role for adherence in the disease process and perhaps promoting resistance to host defenses, antibiotics, or both.

Both cytadherence and motility require an attachment organelle, a polar cellular projection about 290 nm in length that is continuous with the cell membrane (100). Biogenesis of the attachment organelle, which appears to occur by template-mediated duplication of its internal cytoskeletal core starting at the distal end and proceeding toward the base of the structure, occurs coincident with onset of DNA replication (101). Two broad classes of proteins associated with motility and cytadherence are present in the attachment organelle: those directly associated with these functions and those that provide underlying structural and organizational support in the form of the cytoskeletal core. Control of phosphorylation of many of these proteins by the kinase and phosphatase pair PrkC and PrpC is poorly understood at the regulatory level but essential for function (102). However, phosphorylation of *M. pneumoniae* proteins is widespread even in the absence of PrkC, suggesting the existence of other means by which proteins become phosphorylated (103). Although undiscovered protein kinases may exist, at least some proteins, such as enolase, are labeled with phosphate because of their covalent linkage to phospholipids, representing a recently discovered means by which proteins may be targeted to the plasma membrane. P1 is considered to be the primary protein adhesin of *M. pneumoniae* because antibodies against P1 block cytadherence (104) and a mutant lacking P1 is nonadherent and avirulent (105). P1 from *M. pneumoniae* cultures copurifies with protein B but not protein C (106), which is derived proteolytically from a common precursor with protein B, called open reading frame 6 (ORF6) (107–109), and colocalizes with it and P1. The purified P1-B complex, which appears to be the principal component of the nap layer lining the outer surface of the attachment organelle, consists of two molecules of protein P1 and two of protein B and has been visualized as a slightly ovoid structure measuring 19 by 22 nm along its axes (106). The amino acid sequences of P1 and the ORF6 products are variable among

isolates (34, 36, 110, 111). This variation likely stems from recombination of the genes with a series of repeat elements designated RepMP2/3, RepMP4, and RepMP5 (32, 112), which are scattered throughout the genome (113).

Transmembrane proteins P1, B (P90), C (P40), and P30 are most directly implicated in cytadherence. There is no evidence that sequence variation leads to functional changes in the affected proteins, supporting the idea that the variation in their sequence principally serves to generate antigenic variation. Another repeat element, RepMP1, is also present and associated with complex changes in an expression site unrelated to P1 and ORF6, potentially constituting an independent source of antigenic variation. Protein P30 is a transmembrane protein localized to the very distal tip of the attachment organelle (114). It contains an unusually long leader sequence which must be processed for protein function, a cytoplasmic N-terminal domain, and an extracellular domain following a single transmembrane domain, (115, 116). Loss of the cytoplasmic domain results in loss of cytadherence without impacting other visible phenotypes, suggesting an important role for P30 in conveyance of signals from the cell interior to the exterior to activate key steps in cytadherence and motility (117). The transmembrane domain of P30 cannot be replaced with that of another protein, suggesting that control of cytadherence and motility occurs at least partly through intramembrane interactions (116). The surface-exposed C-terminal portion of P30 is highly enriched in imperfect proline-rich repeats (115). Reduction in the number of repeats compromises P30 stability and gliding speed (107–109).

The cytoskeletal core of the *M. pneumoniae* is required for attachment organelle formation and localization of the adhesins to this cell pole (100). Proteins HMW1 and HMW2 appear to constitute at least some of the mass of the thin and thick parallel plates, respectively (118), that define the cytoskeletal rod structure (34, 36, 110, 111). Proteins HMW3 and P65 localize to the region of the terminal button at the distal end of the core (118). Although transmembrane protein P30 is required for stability of P65 (119), a truncated P65 results in weakened association of P30 with cells (120). Proteins at the proximal base of the core include P200, MPN387, P41, TopJ, and P24 (121, 122). P200 (123) and MPN387 (118, 120) are both specifically implicated in motility. P41 acts as a linchpin in whose absence attachment organelles are prone to detaching from cells (124). Attachment organelle cores are fully assembled in the absence of TopJ, a DnaJ family cochaperone protein (121), but have a high rate of failure to protrude from the cytoplasm to make a visible attachment organelle (125). Attachment organelles are nonfunctional in the absence of TopJ, because of either the mispositioned cores or the alterations to P1. Both the P1 proteolytic susceptibility and the irregularities associated with the core are recapitulated by alteration of amino acid residues known in DnaJ to be involved in protein folding functions (125, 126). The coupling between attachment organelle duplication and DNA replication onset is severed in the absence of protein P24 (127). Considering that there is a physical association of chromosomal DNA with the base of the attachment organelle core in species closely related to *M. pneumoniae* (128, 129), it is likely that P24 lies at the heart of the mechanism controlling timing and location of attachment organelle duplication.

X-ray crystallography has revealed the structures of some attachment organelle components in *M. pneumoniae* and its close relatives, including MPN387 (130), P41 (131), and a protein-protein interaction motif, the EAGR box (132), found only in a few attachment organelle proteins (133). Electron cryotomographic imaging has also revealed information about the architecture of the cytoskeletal core of the attachment organelle (134, 135). In combination with immunogold electron microscopy and proteomics, these advancements have led to models about how motility occurs. Differences between samples in the orientation or spacing of elements within the thick plate of the core have led to the suggestion that stretching and contraction of the rod drive movement (134). On the other hand, the variation in rod length within samples was measured to be minimal (128). In *Mycoplasma genitalium*, absence of a core, which normally leads to immotility, can be partly overcome by overproduction of adhesins, arguing for a primary role of the adhesins and a limited role for the core (136).

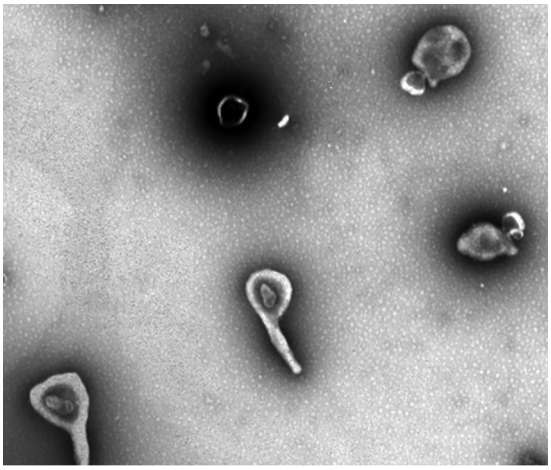


FIG 1 Electron micrograph of *Mycoplasma pneumoniae* cells, demonstrating flask-shaped morphology and a prominent attachment tip structure.

Conceivably, the role of the core is to concentrate and organize the adhesins in one place so that they can cooperate to drive motility in a catch-and-release manner, similarly to eukaryotic cytoskeletal motors and the unrelated motor of *M. mobile* (94).

Although primary cytadherence requires the attachment organelle, *M. pneumoniae* also has attachment organelle-independent adherence factors that likely do not come into play until after the attachment organelle has initiated binding. Generally speaking, these factors are moonlighting proteins, which are proteins with more than one nonoverlapping function (137). Many of these proteins present on the cell surface, despite having better-characterized roles in the cytoplasm and a lack of described export signals, have related activities in other bacteria. Fibronectin-binding proteins of *M. pneumoniae* include elongation factor Tu and pyruvate dehydrogenase subunit E1β (138, 139). Various pyruvate dehydrogenase subunits and a number of glycolytic enzymes also bind to plasminogen (140–142), and glyceraldehyde-3-phosphate dehydrogenase has a moonlighting role in binding to fibrinogen (143). It is likely that the individual role of each of these proteins in binding extracellular matrix components is small, but together they contribute to robust binding to host cells. Figure 1 shows an electron micrograph of *M. pneumoniae* cells demonstrating the prominent attachment organelle. Figure 2 illustrates the organization of the electron-dense core.

Community-Acquired Respiratory Distress Syndrome Toxin

The discovery of community-acquired respiratory distress syndrome (CARDS) toxin displaced the dogma that mycoplasmas are devoid of exotoxins. Initially identified as a surfactant protein A (SP-A)-binding protein, CARDS toxin, with sequence homology with the S1 subunit of pertussis toxin over part of its length, was subsequently found to have ADP-ribosyltransferase activity (144, 145). Its overall structure as revealed through X-ray crystallography is unique relative to that of other ADP-ribosylating bacterial toxins (146). Similar proteins are encoded only in the genomes of a small number of other mycoplasmas, making CARDS toxin a mycoplasma-specific molecule. CARDS toxin is strongly implicated as a significant virulence factor of *M. pneumoniae* by a variety of lines of experimentation. Transcription of the CARDS toxin-encoding gene (MPN372) is induced and protein levels increase upon exposure of *M. pneumoniae* to host cells, supporting roles for this protein in host cell interactions. Administration of purified recombinant CARDS toxin to model animals reproduces substantial features of *M. pneumoniae* disease, including increased cytokine production, eosinophilia, and hyperreactivity of the airway closely resembling asthma (147–149). Just as occurs in tissue culture cells treated with CARDS toxin, infected areas of animal respiratory tracts exhibit ciliostasis (145, 147). Positive correlation between CARDS toxin production and

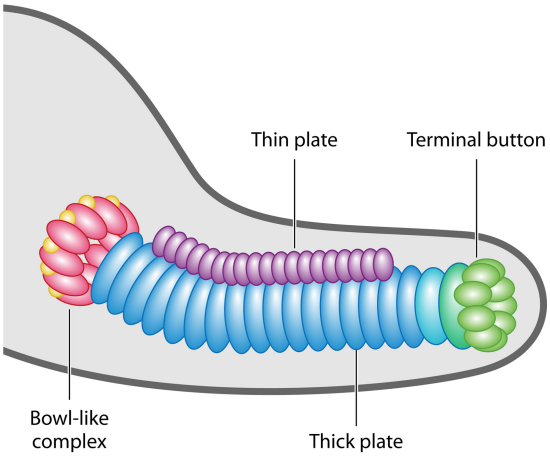


FIG 2 Organization of the electron-dense core of the *M. pneumoniae* attachment organelle. Within the attachment organelle membrane, which is enriched for the transmembrane adhesin P1 and its binding partners, proteins B (P90) and C (P40) (106, 108), is an electron-dense core about 290 nm in length with a characteristic bend (134, 135). The core consists of several substructures. At the distal end, likely interacting with the transmembrane adhesin P30, which is restricted to the tip of the attachment organelle (114), is the terminal button, containing proteins P65 and HMW3 (118). The terminal button is in contact with the thick plate, containing protein HMW2 (118, 473). Parallel to the thick plate is the thin plate, which contains HMW1 (118). Adjacent to the plates at the end close to the cell body, and likely in contact with the chromosomal DNA (128), is the bowl complex, which contains proteins P200, TopJ, P41, and P24 and a protein that arises from an internal translational start within the gene coding for HMW2, called P28 or HMW2-S (118, 474). The bowl complex may be in contact with the membrane. Between the core and the attachment organelle membrane is an electron-lucent space of uncertain composition. This figure was generated with the assistance of Natalie Clines and Patrick Lane.

severity of disease among *M. pneumoniae* strains in an animal model reinforces the significance of this toxin as a disease determinant (150). Additionally, antibody responses to CARDS toxin can be demonstrated in humans with *M. pneumoniae* infection, further supporting its role in human disease (145). Recombinant CARDS toxin also induces considerable formation of Rab9-associated vacuoles in tissue culture cells (151).

It is not known what all of the host cell substrates of CARDS toxin are, but one likely significant cellular target is nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3), a cytoplasmic sensor for diverse microbial pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) that triggers assembly of the best known of four subunits of the inflammasome complexes, macromolecular assemblages of proteins whose roles are, upon stimulation, to activate a protease that itself activates interleukin-1β, mediating inflammation (152, 153). Whether ADP-ribosylation of NLRP3 by CARDS toxin affects NLRP3 activity is unknown at present, but it is tempting to speculate that by modifying this protein, *M. pneumoniae* may be able to downregulate host innate inflammatory responses. To reach its substrates, CARDS toxin is translocated from the *M. pneumoniae* cell to the interior of the host cell by clathrin-mediated endocytosis (154). CARDS toxin lacks conventional export signals, but up to 10% of total CARDS toxin is located on the surface of *M. pneumoniae* cells (155). The receptors for CARDS toxin appear to be not only surfactant protein A but also annexin A2 (156), and the protein also has phosphatidylcholine- and sphingomyelin-binding activity, presumably fostering interactions with the outer leaflet of the host cell membrane. Increased production of annexin A2 in tissue culture cells also increased CARDS toxin binding and toxicity (156), underscoring the importance of this receptor. It relies on its unusual C-terminal domain to enter host cells through a clathrin-mediated mechanism, although the mechanism by which endocytosis is triggered is unknown.

Other Virulence Factors

During infection, hydrogen peroxide is used by both pathogens and hosts to cripple the opposing member of the interaction. Production of hydrogen peroxide is impli-

cated in *M. pneumoniae* pathogenesis based principally on two observations. First, *Mycoplasma mycoides* subsp. *mycoides* SC, a very significant animal pathogen, is considerably attenuated as a pathogen in a strain in which hydrogen peroxide production is compromised at the genetic level, and there is further strong evidence that there is a causative relationship between hydrogen peroxide and disease for this organism (157, 158). Second, cytotoxicity of *M. pneumoniae* to tissue culture cells is markedly reduced in mutant strains unable to produce hydrogen peroxide (159).

Most hydrogen peroxide is generated in *M. pneumoniae* by glycerol-3-phosphate (G3P) oxidase, referred to in the literature as either GlpD or GlpO (159, 160). Its substrate, G3P, can be derived from free glycerol, whose phosphorylation is catalyzed by glycerol kinase (GlpK) after transport into the cytoplasm by the glycerol facilitator (GlpF) (161). G3P can also be generated by the action of glycerophosphodiesterase (GlpQ) on glycerophosphocholine (GPC) (161), taken up via the GlpU transporter (162). *M. pneumoniae* is quite likely to encounter GPC during infection, as it arises from the action of lipases, whether derived from *M. pneumoniae* or from other inhabitants of the respiratory tract on membrane phospholipids. GlpO is also capable of using the glycolytic intermediate glyceraldehyde-3-phosphate as a peroxigenic substrate with low affinity (163). Given that all ATP-generating pathways in *M. pneumoniae* flow through the three-carbon stage of glycolysis, this relaxed substrate specificity ensures that as long as *M. pneumoniae* is metabolically active, a basal level of hydrogen peroxide is produced. The fact that loss of GlpO in *Mycoplasma gallisepticum*, which carries out the pathway for metabolizing free glycerol but lacks the GPC metabolic pathway, has little impact on virulence in an animal infection model raises the possibility that the GPC pathway is more physiologically relevant for hydrogen peroxide generation *in vivo* than the pathway for metabolism of free glycerol in *M. pneumoniae*.

Lysis of red blood cells *in vivo* has been attributed to hydrogen peroxide, but evidence has suggested that although this molecule contributes significantly to hemoxidation, it does not cause hemolysis. The observation that hemolysis by *Mycoplasma penetrans* increases in the presence of cysteine (164) was possibly explained by the discovery in *M. pneumoniae* of an enzyme, cysteine desulfurase/desulfhydrase, designated HapE, with unusual properties linked to hemolysis (165). Acting as a desulfurase, it converts cysteine to alanine, but in its capacity as a desulfhydrase, HapE converts cysteine to pyruvate and hydrogen sulfide. Recombinantly produced HapE causes hemolysis in the presence of cysteine, implicating hydrogen sulfide in hemolysis and making HapE a potential virulence factor of *M. pneumoniae*.

The absence of catalase from most mycoplasma species, including those that, like *M. pneumoniae*, produce hydrogen peroxide, has raised questions of how mycoplasmas protect themselves from this toxic molecule. The activities of enzymes of *M. genitalium* that control the damage done by reactive oxygen species, such as peptide methionine sulfoxide reductase (166), organic hydroperoxide reductase (167), and hydroperoxide peroxidase (168), have been described. However, direct reduction of hydrogen peroxide by catalase among mycoplasmas is described only in *Mycoplasma iowae* (169). The fact that *M. gallisepticum* engineered to produce catalase is strongly attenuated for toxicity in the invertebrate animal model *Caenorhabditis elegans* (169, 170) not only underscores the pathogenic value of reactive oxygen species but also might explain the absence of catalase in *M. pneumoniae*.

Another potential virulence factor of *M. pneumoniae*, encoded by MPN133, is a lipoprotein with two distinct functions attributed to it. MPN133 is implicated in uptake of free glycerol (162). However, recombinant MPN133 is also a calcium-dependent nuclease that degrades not only RNA but also DNA (171). This cytotoxic protein binds to, becomes internalized by, and reaches the nuclei of tissue culture cells in a manner dependent upon an unusual motif enriched in hydrophilic amino acids. Interestingly, the critical motif for host cell entry, which is dispensable for nuclease activity, is absent in the *M. genitalium* homolog of this protein (171), suggesting that if the nucleolytic activity of MPN133 is a virulence factor, its function might be limited to *M. pneumoniae*. It is unclear how these two functions are related, suggesting that MPN133 is a

moonlighting protein with two distinct roles in virulence. Additional recent evidence of nucleases as a virulence factor in *M. pneumoniae* comes from a study by Yamamoto et al. (172), who demonstrated that when heat-killed *M. pneumoniae* is coincubated with neutrophils, the formation of neutrophil extracellular traps (NETs) occurs. However, when live *M. pneumoniae* is coincubated with neutrophils, the viability of the bacteria is not affected and few NETs are detected. Using transposon mutagenesis coupled with experiments in a mouse model of infection, MPN491 was identified as a magnesium-dependent nuclease secreted by *M. pneumoniae* that is responsible for degradation of NETs and enhancement of bacterial survival.

Importance of Host Factors

As in some other types of infections, the characteristics and magnitude of the host immune response and the immunocompetence of the host can dramatically affect the clinical outcome of *M. pneumoniae* respiratory disease and the myriad of extrapulmonary complications that can occur. Host immunity involving macrophages, mast cells, neutrophils, and natural killer (NK) cells, as well as T and B lymphocytes and humoral immune responses, have been studied extensively for many years. Even though the humoral response to infection does not provide complete immunity to future infections, the importance of an intact humoral immune system in the containment of disease is apparent, since persons with antibody deficiency can develop severe and prolonged respiratory illness due to *M. pneumoniae* (173) and the risk of extrapulmonary complications such as meningitis and arthritis also increases (1–3, 84, 174, 175).

Once the organisms reach the lower respiratory tract, they are opsonized by antibody and complement and then phagocytized by activated macrophages. Subsequently, an inflammatory exudate comprised of neutrophils and lymphocytes develops. A critical importance of pulmonary macrophages in controlling *M. pneumoniae* infection was demonstrated in a murine model by Lai et al. (176), who found that macrophage depletion, but not neutrophils, impairs organism clearance, consistent with studies of the rodent pathogen *Mycoplasma pulmonis*, in which macrophages determine the extent of lung infection (84). Macrophage activation and subsequent killing of *M. pneumoniae* are dependent on Toll-like receptor (TLR), particularly TLR2, recognition (177, 178), results that are further confirmed by deficient clearance seen in mice deficient in the essential TLR signaling protein MyD88 (176). Macrophage-derived cytokines IL-18 and IL-8 are directly related to severity of mycoplasmal pneumonia (179). The proinflammatory cytokine IL-18 activates T cells, while the chemokine IL-8, also produced by pulmonary epithelial and smooth muscle cells, is a potent neutrophil chemoattractant (180). Following cytadherence, *M. pneumoniae* stimulates mast cells to produce IL-4 and also activates and induces cytokine production by peripheral blood leukocytes, respiratory epithelial cells, and macrophages. One study utilized a murine model to demonstrate that cathelicidin-related antimicrobial peptide (CRAMP) reduced the growth of *M. pneumoniae in vitro* by 100- to 1,000-fold and that the presence of the organisms stimulated the release of this peptide from murine neutrophils. These results suggested that release of CRAMP from neutrophils in *M. pneumoniae* infection in mice (and, thus, potentially the related LL-37 cathelicidin in humans) may serve an important role in the host innate immune response (181).

Research using both mouse models and airway tissue culture models has demonstrated that resistance to infection by *M. pneumoniae* is facilitated by a number of host-produced factors. SPLUNC1 was previously characterized as a highly positively charged, bactericidal protein produced in the respiratory tract that binds lipopolysaccharide, supporting a role in protection against Gram-negative bacteria (182). An additional role for SPLUNC1 in protection against *M. pneumoniae* infection was suggested by the ability of the recombinant protein to reduce growth of the bacteria *in vitro* (182, 183), partially through activation of neutrophils and induction of human neutrophil elastase (184). Reduction in expression of SPLUNC1 is accompanied by increased growth of *M. pneumoniae* in tissue culture models (183). Conversely, SPLUNC1 expression is stimulated by *M. pneumoniae* infection through

TLR2-mediated signaling of mitogen-activated protein kinase (MAPK) and activator protein-1 (AP-1) (185) Interestingly, the increased production of SPLUNC1 is impaired in the presence of IL-13, an allergy-associated cytokine, and IL-13 also reduces the activity of albuterol and formoterol in decreasing *M. pneumoniae* levels in tissue culture cells, suggesting that this defense mechanism is disrupted during an allergic response (186). zTLR2 and NF-κB also protect against *M. pneumoniae* infection through stimulation of the heat shock response (187, 188).

The results of microbial virulence determinant interactions with host bronchial epithelium cells, and the ensuing host inflammatory and immune responses, are cytopathic effects characterized by reduction in oxygen consumption, glucose utilization, amino acid uptake, and macromolecular synthesis with loss of cilia, vacuolation, exfoliation, and the production of pneumonic infiltrates (84). These gross structural, cellular, and subcellular changes translate into the typical clinical manifestation of a persistent hacking cough which is well known in *M. pneumoniae* infections. Recent studies suggest that the severity of illness is related to the degree of ciliary damage (189). Cytokine production and lymphocyte activation may either minimize disease and eliminate the mycoplasmas or exacerbate disease through immunological hypersensitivity and worsening damage to the respiratory epithelium. The more vigorous the cytokine stimulation and cell-mediated response, the more severe pulmonary injury becomes.

NEW INSIGHTS INTO CLINICAL MANIFESTATIONS AND IMMUNOLOGICAL COMPLICATIONS

Clinical Presentation

The clinical presentation of *M. pneumoniae* infection varies widely. The most common manifestation is tracheobronchitis, with a cough that may either be dry or productive of mucoid or mucopurulent sputum. Many patients may have nonspecific symptoms resembling those of an upper respiratory infection, which may include headache, sore throat, coryza, and otitis media. Those patients complaining of sore throat do not typically have exudates or lymphadenopathy. Chest auscultation may reveal coarse rales and rhonchi if disease is limited to tracheobronchitis and fine inspiratory rales and dullness at lung bases if pneumonia has developed. Experimental infection has shown that the patients with more severe symptoms following infection are those without preexisting antibody (190). Since several different bacteria as well as viruses can produce lower respiratory infections with similar clinical manifestations, attempting to achieve a precise microbiological diagnosis requires additional laboratory testing at considerable expense. In a large study from China evaluating more than 12,000 children hospitalized for respiratory infections, the investigators were unable to identify any particular pattern of symptoms or laboratory findings to distinguish children with evidence of *M. pneumoniae* infection (191). However, the Japanese Respiratory Society (JRS) has developed a set of clinical parameters to predict whether adults have pneumonia due to *M. pneumoniae* in order to guide patient management and antimicrobial therapy. Application of these parameters in a recent evaluation of patients who had mycoplasmal infection confirmed by serology and/or culture indicated that the sensitivity of the JRS scoring system was 83% (192).

This disease often spreads slowly through families, with an incubation time of approximately 23 days. The index case is likely to be an older child, 5 to 14 years of age, and children under five are more likely to have milder disease. In one study, *M. pneumoniae* infection typically resulted in a prolonged cough that lasted 3 to 4 weeks in older children and adolescents and an average of 54 days in adults (193).

Additional respiratory-related illnesses caused by *M. pneumoniae* include lung abscesses, bronchiolitis obliterans with organizing pneumonia, cellular bronchiolitis, bronchiectasis, pulmonary embolism, pleural effusion with empyema, chronic interstitial fibrosis, and adult respiratory distress syndrome (194). *M. pneumoniae* IgM antibody

was detected in 10 of 42 children with postinfectious bronchiolitis obliterans, second only to adenovirus in association with this condition (195).

Occurrences of fulminant and/or fatal cases of *M. pneumoniae* have been known for 50 years but are quite uncommon. Although the pathogenesis is incompletely understood, persons with severe illness may experience a hyperimmune response in the lung as a result of prior infections priming the immune system. They may also be unable to eradicate the organism from the lung, resulting in a prolonged infection which further stimulates the immune system. Finally, they may have overactive macrophages and lymphocytes, adversely affecting cellular immunity (196). Deaths have occurred in otherwise healthy younger persons, as well as older persons with underlying diseases (197). Two studies reported that the bacterial load of *M. pneumoniae* in OP specimens is higher in patients with more severe illness requiring hospitalization than in patients with less severe infections who could be treated as outpatients (48, 198), and a third study found that the same was true for bronchoalveolar lavage (BAL) fluid specimens (199). In fatal cases, death has been associated with diffuse pneumonia, adult respiratory distress syndrome, vascular thrombosis, and disseminated intravascular coagulation, often with multiorgan failure (194). In some instances, death has occurred from extrapulmonary complications such as SJS without a prominent respiratory component (196, 200). Kannan and colleagues described an extraordinary cluster of *M. pneumoniae* infections in a single Texas family in which 5 previously healthy siblings developed respiratory infection requiring hospitalization; 2 of them, a 15-year-old male and a 13-year-old female, experienced acute respiratory failure and died (194). *M. pneumoniae* infection was confirmed by serology and PCR on lung tissue. The autopsy of the 15-year-old male showed hypoxic ischemic encephalopathy and acute and organizing pneumonia with bronchiolitis obliterans. Lung and serum samples from the female sibling were PCR positive. Her autopsy showed bronchiolitis and focal sites of bronchiolitis obliterans with alveolar edema and intra-alveolar hemorrhages. Immunostaining and immunoelectron microscopy demonstrated the organisms localized on the surface of bronchiolar epithelium, as well as the presence of CARDS toxin. *M. pneumoniae* was also isolated from lung tissue from sibling 2. Muir and colleagues suggested that the CARDS toxin may cause significant pulmonary inflammation, cytokine release, and airway dysfunction that may be responsible for respiratory failure in critically ill patients such as these fatalities (201). This hypothesis is supported by experiments in mice and baboons (147).

Laboratory evaluation of patients with *M. pneumoniae* infection is typically nondiagnostic in patients with mild disease with normal complete blood counts, electrolytes, hepatic function, and renal function. C-reactive protein (CRP) may be mildly elevated. In patients with more severe disease, particularly primary atypical pneumonia, inflammatory markers (CRP and erythrocyte sedimentation rate) may be markedly elevated, and about 50% of patients are positive for cold agglutinins. Serum IL-18 levels correlate with those of lactate dehydrogenase (LDH), and both analytes have some value as predictors of more severe or refractory illness (202). Miyashita et al. suggested that elevation of LDH can be used as a parameter on which to base initiation of corticosteroid therapy for severe mycoplasmal pneumonias because it is more easily measured in clinical laboratories than IL-18 (202).

Radiographically, *M. pneumoniae* infection may be indistinguishable from a viral lower respiratory infection, with patchy airspace consolidation and γround glass opacities. The more pronounced radiographic abnormalities are usually in the lower lobes. Unilateral pleural effusions are particularly common in children. In one recent study, 41% of patients with cough for at least 1 week who were diagnosed with *M. pneumoniae* infection using serology and/or culture were found to have abnormalities on chest computed tomography (CT) that were absent on chest films, including bronchial wall thickening (95%), centrilobular nodules (50%), lymphadenopathy (40%), ground-glass attenuation (31%), and reticular or linear opacities (9%) (203).

Asthma

The prevalence of asthma has been increasing over the past several decades, and determining the causes of the disorder in order to identify strategies for prevention and more effective treatment has been the subject of intense investigation. The majority of asthma begins early in childhood and is associated with eosinophilic infiltration of the airways and both atopy and recurrent respiratory infections. A genetic predisposition toward both allergies and asthma has been known for decades, but the proportion of the disease attributable to heredity is only about 50% (204). Interestingly, of the genes that have been identified with a linkage to asthma, many are related to host defense, which supports the close association of asthma with infection and microbial products (205).

A number of studies have examined the possibility that infection with specific viral or bacterial pathogens might be a causal factor in the development of asthma (206). Data from the Copenhagen Prospective Study on Asthma in Childhood birth cohort have demonstrated that the immune response to pathogenic bacteria is already distinctive in infants destined to develop asthma by 6 months of age (207). Further analyses from this cohort have shown that early colonization of the infant airway by 1 month of age with *S. pneumoniae*, *H. influenzae*, or *Moraxella catarrhalis* is highly correlated with the development of asthma. It has recently been proposed that patients with atopic disease and asthma have defective responses to infection as a result of their Th2-biased immune responses. Thus, the propensity of asthmatics to suffer exacerbations due to infection appears to be the result of inherited factors combined with early predisposing environmental influences, including gastrointestinal dysbiosis and reduced exposure to environmental microbial products that are important in the development of protective pulmonary immune responses.

M. pneumoniae has evolved to create persistent infection and seems to be a formidable microbial opponent for patients with asthma or atopic disease, who appear to have less robust immune responses to respiratory infections. There are several questions related to the relationship of *M. pneumoniae* to asthma to be considered: first, whether *M. pneumoniae* is related to asthma pathogenesis; second, whether *M. pneumoniae* infection plays a significant role in asthma exacerbations; third, whether chronic *M. pneumoniae* infection is related to ongoing airway inflammation in chronic asthma; and fourth, whether the use of antibiotics such as macrolides can show a beneficial effect in the subset of asthmatics who harbor *M. pneumoniae* in their airways. For the purposes of this review, the studies that will generally be considered to answer these questions are those employing either PCR or acute- and convalescent-phase serology with or without culture results. The reason for this, as discussed elsewhere, is that we believe that single positive serological tests for *M. pneumoniae*, while useful in the diagnosis of individual patients, are too fraught with potential error to be useful for population studies to determine acute or ongoing infection. We will also consider results obtained by one group using a novel antigen capture assay for the *M. pneumoniae* CARDS toxin, a test that appears to offer a very sensitive means of diagnosing *M. pneumoniae* infection.

A recent large epidemiologic study from Taiwan provides strong evidence that *M. pneumoniae* infection is associated with the development of asthma (208). This study used data from the National Health Insurance Research Database of Taiwan to compare 1,591 patients with documented *M. pneumoniae* infection with 6,364 control individuals matched for age, sex, and incident year for the development of asthma. Patients with *M. pneumoniae* infection had a higher risk of developing asthma, and this risk was further augmented by comorbidities such as atopic disease. Although this is the strongest epidemiological study to date linking *M. pneumoniae* infection to the development of asthma, it suffers from the drawback that in Taiwan criteria for the diagnosis of *M. pneumoniae* infection, in addition to acute- and convalescent-phase IgG titers, are such that diagnosis can be made with a single positive IgM titer, which can be misleading. IgM antibody against *M. pneumoniae* is increased among childhood asthmatics (209)

and may persist for years (210). Regardless, because of the size of this study, it seems likely that *M. pneumoniae* infection has a true association with the development of chronic asthma in some individuals, particularly those with atopic disease.

There is little question that *M. pneumoniae* infection, either alone or in combination with respiratory viruses, is capable of inducing asthma exacerbations, but most studies suggest that this organism is responsible for a relatively modest proportion of asthma flares. Five PCR-based studies of acute asthma in hospitalized children revealed an incidence of *M. pneumoniae* ranging from 4.5 to 12.4% (211–215). An additional study based on analyses of acute- and convalescent-phase sera from adults estimated the incidence of *M. pneumoniae* at 18% in hospitalized asthmatics compared to 3% in controls (216). A recent study in adults with refractory asthma revealed a 52% prevalence, with 29 of 33 subjects positive by CARDS toxin antigen capture assay and only 10 of 33 positive by PCR (217) A subsequent study that employed a combination of the CARDS toxin antigen capture assay, PCR using the P1 adhesin and the CARDS toxin gene as targets, and serology found even higher percentages of *M. pneumoniae*-positive children with acute and refractory asthma (64 and 65%, respectively), although control subjects also exhibited surprisingly high 56% positivity (81). In this study, the CARDS toxin PCR was significantly more sensitive than the P1 PCR, but overall positive rates were 2.8% to 6.8% depending on the sampling site, similar to the range observed in the studies cited above. It remains to be seen whether the potentially ultrasensitive CARDS toxin antigen capture assay will be validated by other groups and find accepted use in detecting the presence of *M pneumoniae* in respiratory samples.

The next question deals with whether the prevalence of *M. pneumoniae* in patients with chronic asthma is higher than that in the general population and how a higher prevalence may relate to the persistence of asthmatic symptoms. The most convincing study is that by Martin et al. in which they enrolled 55 chronic stable adult asthmatics and 11 nonasthmatic controls and analyzed upper and airway samples for the presence of *M. pneumoniae* by culture and PCR (218). Twenty-three subjects were positive for *M. pneumoniae* by PCR (41.8%) and 7 were positive for *C. pneumoniae* (12.7%), with only 1 and 0 of the controls positive, respectively. No subjects were positive for *M. pneumoniae* antibody. This result indicates a very high prevalence of *M. pneumoniae* in chronic asthma, even in the absence of exacerbation, and also suggests that screening by serology for the presence of the organism in stable adult asthmatics may significantly underestimate its prevalence. As noted below, a multicenter study by Sutherland et al. designed to replicate these findings as well as the response of PCR-positive patients to macrolide therapy found only 12 of 92 patients positive by PCR in endobronchial biopsy specimens (13%), a number too low to provide sufficient power for the planned antibiotic treatment trial (219). Bébéar and colleagues found that only 13.6% of children with chronic asthma were positive for *M. pneumoniae* by PCR that and an even lower percentage (6.3%) of adults were (212). However, their samples were obtained using OP and NP swabs, whereas in the study by Martin et al., about 18% of the subjects were positive only from lower respiratory samples (218).

A final question is whether treatment with antibiotics affects the severity and frequency of exacerbations of asthmatics. If a significant proportion of chronic asthma is related to *M. pneumoniae* infection, then treatment with an antibiotic to which the organism is susceptible would be expected to produce improvement in symptoms and other measures of asthma severity. However, interpretation of any improvement seen in asthma symptoms in asthma patients following macrolide treatment is complicated by the fact that macrolides have well-documented anti-inflammatory properties. Kraft et al. treated their study subjects for 6 weeks with clarithromycin and obtained a clinically significant improvement in pulmonary functions only in the subgroup of subjects who were positive for the organism (220). As mentioned above Sutherland et al. (219) failed to find a statistically significant number of PCR-positive asthmatics and also failed to demonstrate benefit from macrolide therapy. However, all of their subjects were treated with inhaled corticosteroids, which may have blunted any response to the antibiotic. Stockholm et al. found that a 3-day course of azithromycin

significantly shortened the duration of wheezing episodes in children 1 to 3 years of age with asthma-like symptoms (221). Bacharier and colleagues reported the results of an extensive double-blind placebo-controlled trial testing the effects of treatment with azithromycin on progression to severe lower respiratory tract infection with wheezing (222). A total of 607 children with a history of wheezing with lower respiratory tract infections were enrolled. Their results demonstrated a clear protective benefit of azithromycin in preventing progression to severe lower respiratory infection, but treatment did not reduce the chances of a subsequent lower respiratory tract infection. Respiratory secretions were tested by PCR for the presence of wide group of respiratory viruses and cultured for various pathogenic bacteria but, they were not tested for *M. pneumoniae* or *C. pneumoniae*. The investigators were unable to come to a conclusion about the mechanism behind the protective effect of azithromycin. Clearly, more research is indicated to understand whether antimicrobial activity of macrolides, anti-inflammatory activity, or a combination is behind the protective effect of this class of medication in asthma and asthma-like diseases.

Animals, primarily rodents, have been used for decades in experimental studies of lung inflammation induced by *M. pneumoniae* and to test therapeutic drugs for efficacy. During early investigations of primary atypical pneumonia, Eaton et al. inoculated mice, guinea pigs, Syrian hamsters, and cotton rats and found that lung lesions were produced more reliably in the latter two types of rodents (223). With the development of inbred strains, better control of infectious agents in animal facilities, and, particularly, the increasing availability of transgenic and "knockout" mice, interest in using mice for infection studies with *M. pneumoniae* to study pathogenesis increased. In 1998, Wubbel et al. published a mouse model of *M. pneumoniae* infection, documenting histopathological changes in the lung and serological responses following short-term infection (224). Since that initial publication, numerous further studies in mice have been published, including long-term infection studies (225, 226) and characterization of the host immune response using mutant strains of mice (176, 227–229).

Despite obvious differences in anatomy and physiology and significant differences in immune responses, a number of murine models of asthma have been developed to study the pathophysiology of allergic airway hyperreactivity because of the same advantages mentioned above that recommend mice for infection studies (230–232). Using *M. pneumoniae* infection in mice, Martin and colleagues demonstrated that infection alone induces increased airway reactivity at 7, 14, and 21 days following infection and that the airway hyperreactivity correlates with histological inflammation (233). A number of subsequent studies have examined the effect of *M. pneumoniae* infection on allergic airway inflammation in mice and have documented the importance of STAT6 and IL-6 (234, 235) and surfactant protein A (SP-A) and SPLUNC1 (236) in modulating allergic airway inflammation and/or pathogen clearance. The results of these studies suggest that allergic airway inflammation downregulates the host response to *M. pneumoniae* and provides a plausible explanation for reports of increased prevalence of *M. pneumoniae* in chronic asthma as well as a potential role in the pathogenesis and perpetuation of this inflammatory respiratory disease.

Extrapulmonary Manifestations

M. pneumoniae is well recognized for producing a broad array of extrapulmonary manifestations that can affect almost every organ in the body. Even though many different extrapulmonary manifestations of *M. pneumoniae* infection have been characterized, they are not usually specific enough to confirm the diagnosis. Three different mechanisms have been put forward by Narita for extrapulmonary complications of *M. pneumoniae* infection. The first is a direct effect of the bacteria that are present at the site of inflammation mediated by cytokine release by the host. The second is an indirect effect, for example, through the production of autoimmunity or immune complexes. The third is a direct or indirect effect by the production of vasculitis or thrombosis as a result of cytokines and chemokines such as TNF-α and IL-8 or by immunomodulation through mediators such as complement and fibrin D-dimers (237).

The most noteworthy of extrapulmonary manifestations involve the central nervous system, and these can be present in about 6% of patients hospitalized with *M. pneumoniae* infection. Involvement can occur in the brain, spinal cord, meninges, and peripheral nerves. The primary brain manifestation is encephalitis, which occurs more commonly in children than in adults and may not have a respiratory component to the illness (180, 238–243). *M. pneumoniae* may be responsible for as many as 13% of encephalitis cases (244). Early-onset encephalitis is more likely to be PCR positive, indicating a direct effect of the organism, while late-onset encephalitis is usually PCR negative and associated with antiganglioside antibodies which may develop as a result of cross-reactivity with mycoplasmal glycolipids. *M. pneumoniae* antigen has been demonstrated in brain tissue by immunohistochemistry and by PCR in diseased areas of the brain in patients who died with fatal encephalitis, acute disseminated encephalomyelitis, or acute hemorrhagic leukoencephalitis, indicative of the severity that can occur with this infection (244–246). Stroke, striatal necrosis, and psychological disorders can occur following *M. pneumoniae* infection, most likely due to vascular injury (180). Bilateral striatal necrosis may involve local invasion by the organisms, as evidenced by positive PCR assays for *M. pneumoniae* DNA in a patient with this condition (247). Cerebellar dysfunction may also occur as a result of autoimmunity (180). Guillain-Barré paralysis is an occasional complication of *M. pneumoniae* infection involving antigalactocerebroside and antiganglioside antibodies, although the organisms have been isolated directly from cerebrospinal fluid (CSF) in patients with this condition (248–251). Transverse myelitis may occur as a result of early-onset direct invasion as well as a late-onset indirect-type mechanism (180). Aseptic meningitis is known to occur as a result of direct invasion, with accompanying elevation of IL-6 and IL-8. Peripheral nerve involvement may manifest as neuropathies and radiculopathies, including cranial nerve palsies. It is presumed that autoimmunity involving antiganglioside antibodies is responsible for neuropathies, but *M. pneumoniae* DNA has been reported in CSF from a patient with peripheral neuropathy (252).

A microbiological diagnosis of *M. pneumoniae* infection in many of the reports attempting to link such infections to central or peripheral nervous system sequelae is problematic because diagnosis invariably precipitates the use of intravenous immunoglobulin therapy, which prevents the determination of accurate convalescent-phase antibody titers. A recently reported series in children with encephalitis, including one fatality, exemplifies the problems in identifying *M. pneumoniae* as the trigger (248). All 7 children reported in the series were positive for IgG and IgM antibody against *M. pneumoniae* in serum, and one was positive in CSF. Only 2 were positive for *M. pneumoniae* DNA by PCR from throat swabs, and CSF was PCR negative in all cases. Only 3 of the 7 were reported to have antecedent respiratory symptoms, and both of the positive PCRs were obtained in this group. Because some neurological manifestations may occur without prominent respiratory symptoms and can be due to immunological mechanisms rather than direct invasion of the organisms, limiting attempts to diagnosis the condition to PCR testing of CSF or brain tissue may be insufficient. This is one instance in which demonstration of seroconversion may be very important for diagnosis.

Dermatological disorders, which include urticaria, anaphylactoid purpura, erythema multiforme, and SJS, are among the most common and potentially the most severe extrapulmonary complications of *M. pneumoniae* infection. Severe SJS produces tense bullae distributed over the skin, particularly the distal extremities, groin, and genitalia, with mucous membrane involvement in the eyes and oropharynx. In a retrospective review of 7 years of cases at the Mayo Clinic, 6 were described as *M. pneumoniae* related. The clinical course of these patients was milder than that in the drug-related cases. There are at least 2 cases in which the organism has been isolated from blisters, suggesting that the pathogenic mechanism involves direct infection of the organisms from bacteremic spread followed by skin damage due to cytokine production (237). There are also several reports of recurrent *M. pneumoniae*-associated SJS, and outbreaks with multiple cases have also been reported. Because of the distinctive appearance and

distribution of the blisters, it has been proposed that this disorder should be classified separately from drug-induced SJS (253). In contrast, urticaria, anaphylactoid purpura, and erythema multiforme associated with *M. pneumoniae* infections are most likely mediated through immunological reactions (254).

Another very common extrapulmonary manifestation associated with severe *M. pneumoniae* infection is the development of IgM autoantibodies to the I antigen on red blood cells. These autoantibodies are responsible for the phenomenon of "cold agglutinins." Autoantibodies to red blood cells may occasionally reach levels sufficient to trigger a brisk hemolytic anemia (255). This phenomenon may reach life-threatening proportions if the patient has an underlying hematological disorder such as sickle cell disease (256). Thrombotic thrombocytopenic purpura has also been described in association with *M. pneumoniae* infection (257). Disseminated intravascular coagulation may also occur in some persons who develop a hypercoagulable state as a consequence of complement activation or procoagulant induction, resulting in severe illness and sometimes fatal outcomes (254). Other cases of severe thrombocytopenia associated with *M. pneumoniae* infection have also been reported and have been considered to be a subset of immune thrombocytopenic purpura (258). In contrast, some patients with *M. pneumoniae* pneumonia may have thrombocytosis (259).

The role of *M. pneumoniae* in pericarditis, cardiac tamponade, myocarditis, myopericarditis, and endocarditis is small but well documented, and the effects range from mild or asymptomatic conditions to fatal illness (260). Illness appears to be related to direct detection of the organism by culture or PCR in the affected cardiac tissue and/or pericardial fluid in cases of pericarditis and endocarditis, but an autoimmune mechanism may also be operative in myocarditis (254). Many persons with cardiovascular manifestations do not have concurrent pneumonia (254).

Septic arthritis due to invasion of the joints by *M. pneumoniae* is a well-known complication in persons with antibody deficiency, but it may also occur in immunocompetent persons (254). Arthritis can also occur in association with pneumonia in immunocompetent persons, in which the mechanism may be of autoimmune origin (260). Another musculoskeletal manifestation that has been associated with *M. pneumoniae* is acute rhabdomyolysis, which has been reported numerous times, sometimes in association with neurological as well as other organ system manifestations (261, 262). It is possible that direct invasion, immunological, and/or vascular occlusive mechanisms could all be operative in mediating this complication (254).

A variety of nonspecific gastrointestinal symptoms commonly accompany mycoplasmal respiratory infections, but there are few data to validate a specific explanation for them. Hepatitis with severe liver dysfunction and pancreatitis has also been reported. Narita suggested that early-onset hepatic dysfunction is likely to be mediated by direct inflammatory reaction in the liver from disseminated *M. pneumoniae* infection, whereas later-onset hepatic dysfunction could be due to autoimmunity, vascular injury, or drug reactions (254).

Acute glomerulonephritis of various types, nephrotic syndrome, interstitial nephritis, and IgA nephropathy have occasionally been described in association with *M. pneumoniae* infection, presumably as a result of immune complex formation. However, the organism has also been detected by PCR from a kidney biopsy specimen of a child with membranoproliferative glomerulonephritis who also had elevated IgM (263) and by immunoperoxidase staining of kidney tissue of a patient with acute interstitial cystitis (264). Renal complications can be of great clinical importance since they can lead to renal failure (265, 266).

Other nonspecific manifestations can include conjunctivitis, iritis, and uveitis, which are presumed to have an immunological basis since the organism has not been detected in ocular tissues (254). Each of the aforementioned organ-specific extrapulmonary manifestations of *M. pneumoniae* infections can occur singly, or in some cases, multiple organ systems can be involved, leading to extremely severe and sometimes fatal illness. Due to the current understanding that many of the extrapulmonary complications have an immunological basis, measurement of the host immune re-

TABLE 1 Comparison of methods for detection of *M. pneumoniae* in clinical specimens

Category	Molecular assays	Culture	Serology
Specimen requirements	Specimen suitable for culture can be tested by molecular methods; organisms do not have to be viable; specimens can be frozen in an appropriate transport system until processed; formalin-fixed tissue can be tested by PCR	Specimens must not be allowed to dry out and must be maintained in appropriate transport media to preserve viability; refrigeration for short periods and freezing for transport are necessary for optimum detection	No special handling or storage other than refrigeration of serum is necessary
Equipment and facilities	Laboratories must have suitable facilities and trained personnel to perform molecular testing; instruments for real-time PCR and DNA extraction are expensive; loop-mediated isothermal amplification instruments are less costly	No special equipment is necessary other than incubators, microscopes, refrigerators and freezers for cultivation; species identification for clinical isolates requires additional procedures such as PCR	Single-test qualitative kits do not require any instrumentation, while automated assays may require spectrophotometers or fluorescence microscopes
Relative cost	Cost of reagents varies, depending on type of assay; costs and personnel time can be reduced if specimens can be processed in batches	Culture media are more expensive to purchase or prepare than media for other bacteria due to complex growth requirements	Costs of kits vary; some are designed for single use, while others are in multitest formats
Turnaround time	Real-time PCR can provide same-day results, including DNA extraction and amplification	Positive specimens may sometimes be detected in as few as 5 days, but incubation for up to 6 weeks is necessary to confirm negative results	Hands-on time may be as short as a few minutes for some qualitative or automated assays, but paired sera collected at least 14 days apart are needed for optimum performance
Analytical sensitivity	Most assays can detect <100 CFU/ml	May detect 100–1,000 viable organisms under optimum conditions	Serology measures host immune response and has no direct measurement of organism load; some infected patients never mount a detectable antibody response
Specificity	PCR assays with proper target selection and validation of amplification conditions can be very specific with no cross-reactivity	Culture is 100% specific when positive in symptomatic persons but requires another technique such as PCR to confirm species identity	Specificity varies, depending on the type of assay; complement fixation is nonspecific and cross-reactive; EIAs can also be nonspecific due to presence of high levels of background antibody in many persons if only a single measurement is taken
Commercial availability	Two products are available in US, several more in Europe; in-house PCRs are performed in reference labs	Commercially prepared media are sold in various countries	Qualitative and quantitative serology test kits are widely available commercially in a variety of formats

sponse through testing of paired sera for IgM and IgG in addition to PCR should always be attempted. Culture is rarely positive outside the respiratory tract, but it can be attempted if specimens are available. Treatment of extrapulmonary manifestations should include antibiotics, since the possibility of direct organism involvement can never be entirely excluded and reducing the bacterial load in the respiratory tract can potentially reduce further stimulation of the host immune system. Moreover, the anti-inflammatory effects of drugs such as macrolides may be beneficial in suppression of the immune response.

LABORATORY DETECTION

Detection of *M. pneumoniae* infections can be achieved using culture, serology, or molecular-based methods. Their relative advantages and disadvantages are summarized in Table 1. Some reasons that *M. pneumoniae* detection is not offered by very many diagnostic laboratories in the United States are that its slow growth and fastidious cultivation requirements make culture impractical, optimum serological testing requires acute- and convalescent-phase sera collected at least 2 weeks apart, and

molecular-based tests are expensive and still have limited availability. There is no FDA-cleared rapid point-of-care test for direct detection of *M. pneumoniae*. However, some qualitative serological tests that have a moderate complexity classification under the Clinical Laboratory Improvement Amendment can be performed in ambulatory care clinics, with results available in a few minutes. Such tests are rather commonly used, even though a single measurement of IgM and/or IgM/IgG is not particularly reliable, especially for adults (4, 267). A final reason for the lack of widespread diagnostic testing is that many clinicians have not expressed an urgent need to have microbiological confirmation for patient management purposes, and this opinion is reflected in clinical practice guidelines for treatment of community-acquired pneumonias that do not recommend pathogen-specific testing (268, 269).

Specimens for detection of *M. pneumoniae* respiratory tract infections by culture or molecular-based methods include NP or OP swabs, tracheal aspirates, lung tissue obtained by biopsy, pleural fluid, sputum, and BAL fluid. Extrapulmonary specimens include blood, CSF, pericardial fluid, skin lesions, or tissue from any organ. Several studies have evaluated various specimen types in order to determine which ones are most likely to yield a positive result for *M. pneumoniae*. The emphasis has been on PCR because culture is rarely used for diagnostic purposes. The general consensus of several studies is that sputum is the best specimen for *M. pneumoniae* detection by PCR and that it is superior to BAL fluid and/or NP or OP specimens (9, 270–272). However, Honda et al. (273) found a higher rate of detection from OP swabs than from BAL fluid or sputum. Reznikov et al. (274) found no significant difference in the detection of *M. pneumoniae* from NP aspirates versus OP swabs. Xu et al. found NP aspirates to be superior to BAL fluid (275). Loens et al. reported that numbers of *M. pneumoniae* in sputum range from 10^2 to 10^7 CFU/ml whereas OP swabs contain 10^2 to 10^3 CFU/ml, providing a plausible explanation for the superiority of sputum as a diagnostic specimen (270). From a practical standpoint, OP or NP specimens are the most common samples obtained for *M. pneumoniae* detection, since they are readily available and many patients, especially children, do not produce sufficient sputum for laboratory testing and are not so seriously ill as to warrant more invasive and expensive sampling methods. No matter which specimen type is collected, it is important to inoculate appropriate transport medium at the point of collection to avoid desiccation, preserve viability for culture, and maintain stability of DNA for molecular detection. Culture medium such as SP4 broth works well for transporting specimens for both culture and PCR, but most liquid commercially produced universal transport media are also acceptable, as long as they do not contain any antibiotics that would be inhibitory. Blood for PCR testing is collected in a tube containing acid citrate dextrose. For swab specimens, only Dacron or polyester swabs should be used, as calcium alginate and cotton swabs can be inhibitory. Refrigeration for short times or freezing at −80°C for longer periods of storage or transport is necessary for optimum detection by culture or molecular methods. Serum collected for antibody measurement does not require special handling other than refrigeration or freezing until processing. Further discussion of specimen collection, transport, storage, shipment, and culture procedures is provided in references 1, 276, and 277).

Culture

Culture is time-consuming and is seldom used to guide treatment of *M. pneumoniae* infections in an acute care setting, but it is sometimes used to obtain isolates for antimicrobial susceptibility testing and/or typing because it provides irrefutable evidence of infection with this organism when positive (100% specificity). Despite reports that culture is insensitive and of limited clinical utility, when performed in laboratories with experience, culture can yield a substantial number of positive tests. The UAB Diagnostic Mycoplasma Laboratory recently compared traditional culture using SP4 broth and agar with real-time PCR with the RepMp1 gene target in 821 OP swabs from patients with pneumonia and found that PCR detected 49/821 (6%), versus 40/821 (5%) by culture. It is not uncommon for growth in culture to take 3 to 4 weeks or longer.

Interestingly, a recent report suggested that inclusion of catalase in the culture medium can considerably increase the organism's rate of growth, possibly by destroying autoinhibitory hydrogen peroxide (278). However, it remains to be seen if this modification of established culture media will be validated by others and become widely adopted.

Various biochemical or immunological methods were once used to confirm species identification of mycoplasmas grown in broth and/or agar from respiratory tract cultures, since oral commensal mycoplasmas can sometimes cause diagnostic confusion with *M. pneumoniae*, but these have been mostly replaced by PCR performed on a subculture to determine which species is present. The current practice at the UAB Diagnostic Mycoplasma Laboratory is to report specimens with typical colonies grown on SP4 agar as either positive for *M. pneumoniae* or positive for *Mycoplasma* species but not *M. pneumoniae*, depending on PCR results. In some instances, a broth may produce a color change without growth of colonies. When the positive broth is subjected to PCR and *M. pneumoniae* DNA is detected, the culture is still reportable as positive for *M. pneumoniae*, confirmed by PCR.

M. pneumoniae culture is often performed in clinical antibiotic treatment trials of new antimicrobial agents for CABP in order to obtain isolates to be tested *in vitro* for antimicrobial susceptibilities and correlate with clinical outcomes. This is now much easier to accomplish since the publication of a guideline in 2011 by the Clinical and Laboratory Standards Institute (CLSI) that describes a standardized methodology for performing *in vitro* susceptibility tests on *M. pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma* spp. The document also includes appropriate quality control measures and MIC interpretive breakpoints for several drugs (279).

Serology

Host immune response. In order to understand the rationale for or against serological detection of infection, it is necessary to consider the immune response that occurs following respiratory infection with *M. pneumoniae* in an immunocompetent host. Following an initial infection which typically occurs sometime in childhood, the normal immune system responds by producing antibodies against protein and glycolipid antigens. These include antibodies reactive against the P1 adhesin protein and CARDS toxin. IgM can usually be detected within about 1 week after onset of clinical illness, followed by IgG about 2 weeks later. Antibody levels peak after 3 to 6 weeks, followed by a gradual decline over months to years (1). The presence of an elevated IgM level has been considered diagnostic of recent infection in children older than 6 months who are not likely to have experienced repeated mycoplasmal infections, but reinfections over time can lead to a blunting of the IgM response as typically occurs in adults. Even in young children, IgM can persist for up to several months (280). Use of serology for diagnosis is further complicated because of the variable length of time after onset of illness when a patient seeks medical care. Some individuals have a high background level of IgG such that measuring IgG in a single acute-phase serum specimen can lead to nonspecific results (281). In addition to specific antibodies against the pathogen itself, *M. pneumoniae* infections tend to elicit production of a variety of autoantibodies as well as mitogenic stimulation of T and B lymphocytes that can also lead to the autoimmune extrapulmonary complications discussed above. Persons with impaired antibody production from any cause may not mount any measurable immune response against *M. pneumoniae* and are also at greater risk for chronic, disseminated infection (4).

Shortcomings of commercial antibody assays. Several different test formats to measure antibody against *M. pneumoniae* have been developed since the 1980s. Many different types of antigens have been employed, including whole organisms, glycoproteins, proteins such as the CARDS toxin or P1 adhesin, and recombinant antigens, but there has been little effort to standardize them. There is no consensus on what constitutes the best test format or antigen type or even on the definition of what value should be considered a positive test result. Some quantitative enzyme-linked immunoassay (EIA) manufacturers provide interpretive guidelines for designation of a posi-

TABLE 2 Commercial serological test formats for *M. pneumoniae*[a]

Assay format	Antibodies measured	Equipment	Test description	Examples (references)
EIA	IgM, IgG (separately)	Spectrophotometer/EIA reader	Microtiter plate format with antigens adsorbed onto the polystyrene surface; dilutions of test serum are added to wells and incubated; antibodies bound to solid-phase antigen are visualized by using enzyme-labeled conjugates directed against the primary antibody and substrate are read in spectrophotometer; quantified amt of reactive conjugate is proportional to levels of antibody present; EIAs require very small serum volumes (<100 μl), are adaptable to testing large or small numbers of specimens, and can be made isotype specific; various different antigens are used depending on the manufacturer	ImmunoWELL *Mycoplasma* IgM and IgG Antibody (GenBio, San Diego, CA) (282, 475)
Membrane EIA	IgM alone or IgM and IgG together	None	Rapid, qualitative procedures designed for testing single serum specimens; a permeable membrane or filter paper is impregnated with antigen to which serum is added; this step is followed by addition of anti-human IgG or IgM enzyme conjugate; development of color after enzyme substrate is added constitutes a positive test	ImmunoCard (Meridian Bioscience, Inc., Cincinnati, OH (282, 476–479); *M. pneumoniae* IgG/IgM antibody test (Thermo Fisher, Lenexa, KS) (282, 478–481)
PA	IgG, IgM (separately or together)	None	Latex or gelatin carrier particles coated with antigen are incubated with test serum; if specific antibodies are present, the particles agglutinate, resulting in a visible reaction; PA products provide qualitative results that can be visualized on a card or quantitative data read in microtiter plate format	Serodia Myco II (Fujirebio Diagnostics, Inc., Malvern, PA) (482–485)
IFA	IgG or IgM (separately)	Fluorescence microscope	Antigen is fixed to glass slides; specific antibody is detected in dilutions of test serum after staining with anti-human IgM or IgG fluorochrome conjugate; IFA provides quantitative data but interpretation is very subjective	Mycoplasma antibody test system (Zeus Scientific, Inc., Branchburg, NJ) (476)

[a]Abbreviations: EIA, enzyme-linked immunoassay; PA, particle agglutination; IFA, indirect immunofluorescence.

tive result based on somewhat arbitrary values for IgM or IgG above a certain spectrophotometer reading cutoff tested on a single serum sample rather than designating a specific rise or fall in antibody titer in paired sera. Some assays are FDA cleared for use in the United States, whereas others are limited to use in other countries or in research settings. A summary of the four major commercialized *M. pneumoniae* assay formats along with some examples of these tests is provided in Table 2. The major types of commercial serological tests for *M. pneumoniae* include the microtiter plate EIA, the membrane EIA, indirect immunofluorescence (IFA), and particle agglutination (PA). In the United States, the EIA format has proven to be the most widely used and best studied (1). More details of these test formats and their comparative performances are available in an earlier review (1). Even though many of these commercial assays have been evaluated in published studies, for several reasons it is extremely difficult to accurately assess how well they perform. In some studies, commercial kits were compared against the less sensitive and less specific complement fixation test as the reference method, or kits were compared directly with one another and nothing else with no way to confirm whether or not *M. pneumoniae* infection was actually present. Tests were often compared using a single measurement obtained at variable times after onset of illness, and the definition of what is considered a positive test was not consistent.

Diagnostic sensitivity for serological diagnosis of acute *M. pneumoniae* infections depends on the timing of specimen collection as well as performance characteristics of the test used. The most accurate diagnostic yield of serological testing for *M. pneumoniae* is obtained when paired sera collected at least 2 weeks apart are tested simultaneously for both IgM and IgG and a 4-fold rise in titer can be documented (1).

This makes serology useful for epidemiological studies but not for patient management in an ambulatory care setting.

Talkington et al. (282) compared 8 commercial EIAs and found positive IgM in 14 to 45% of samples assayed. Positivity ranged from 39 to 88% when both acute- and convalescent-phase sera were analyzed. This study confirmed that these test kits had considerable variation in performance and ability to detect a positive result. These findings regarding poor performance of acute-phase IgM were confirmed by Miyashita et al. (192), who observed a 35% sensitivity for acute-phase IgM as measured by the Meridian ImmunoCard in adults with serologically and/or culture-proven pneumonia due to *M. pneumoniae*, and by Ozaki et al. (283), who found a sensitivity of only 32% for acute-phase IgM, rising to 89% when paired sera were analyzed for seroconversion. Liu et al. concluded that IgM has the highest sensitivity for detection of acute infection if it is tested 7 to 10 days after onset of illness (284). IgA can rise early in infection and return to normal sooner than either IgM or IgG (285, 286). While IgA is attractive as an alternative to IgM as a marker of acute infection, a study by Narita (287) found no advantage for testing IgA. Another recent study also demonstrated that a commercial IgA assay was less sensitive than IgM in hospitalized children and adolescents with radiologically confirmed pneumonia when paired sera were analyzed (288). Not only may the variable performance in serological tests noted in several studies may result in missing persons who are infected, but the nonspecific performance of some assays will also result in false positives. In addition to poor sensitivity, Miyashita et al. reported that the ImmunoCard showed positive reactivity in 61 of 200 (30%) healthy adults (192). Csango and coworkers found substantial numbers of positive IgM and IgA tests using 4 commercial serology kits in 102 healthy blood donors (289). Nir Paz and coworkers evaluated 8 commercial serology tests in 204 samples obtained from adult blood donors and children undergoing elective surgery and also found numerous samples positive for IgM and very poor interassay agreement (281).

Beersma and coworkers (267) published a comprehensive evaluation of 12 serology tests available in Europe, including EIAs, PAs, and complement fixation, using real-time PCR as a reference standard, and they also included PCR-negative control samples from subjects with and without respiratory tract symptoms. They found a wide range of results, with sensitivities for serological tests ranging from 35% to 77% and specificities ranging from 49% to 100%, again noting that single measurements in the acute phase were often negative in PCR-positive subjects. Similar results demonstrating poor specificity of positive IgM tests in children who were PCR positive for *M. pneumoniae* were reported by Chang et al. (290). Busson et al. (291) performed an evaluation of 10 serological assays used in Europe, including only one of the kits included in the study by Beersma et al. Similar to other studies, these investigators found variation in both sensitivity and specificity among the kits tested, with cross-reactivity in some instances with sera from patients with known cytomegalovirus or Epstein-Barr virus infections. Medjo et al. (292) reported the sensitivity and specificity of a single IgM measurement as 82% and 100%, respectively, compared to IgG serology and as 80% and 99%, respectively, compared to real-time PCR. The relatively high sensitivity of IgM serology in their study was likely due to the fact that sera were taken in the second week after disease onset, giving antibody time to develop, but this may not reflect what actually occurs in clinical practice.

Some investigators have indicated that recombinant proteins used as antigens would improve the specificity of serological assays for detection of *M. pneumoniae* (293–295). Dumke et al. (296) developed a line immunoblotting technique using several recombinant proteins or native purified proteins and determined that use of this technique to measure serum antibody in patients with and without *M. pneumoniae* infection showed high sensitivity and specificity compared to real-time PCR. Thus far, no products sold commercially in the United States utilize such features or recombinant proteins.

Until the availability of PCR dramatically changed and improved diagnostic testing options for *M. pneumoniae* infection, serology was the primary means for laboratory

diagnosis, even with its significant limitations. Presently, testing of acute-phase sera for antibody by qualitative tests continues to be commonly used for convenience purposes and because there is no other type of rapid test available in the United States, despite convincing evidence that such practice can be prone to false-negative as well as false-positive results.

Nonamplified Antigen Detection

The limitations of culture and serology for detection of *M. pneumoniae* were apparent by the early 1980s, when there were efforts to develop nonamplified antigen detection systems and DNA hybridization techniques in various formats, as discussed in an earlier review (1). The main problem with nonamplified antigen methods of organism detection in clinical specimens is that some natural infections caused by *M. pneumoniae* may produce an organism load that is about the same as the detection limit of antigen assays (approximately 10^3 CFU/ml), and thus they suffered from low analytical and clinical sensitivity. Antigen detection systems were largely abandoned by the 1990s in favor of nucleic acid amplification test (NAAT) methodology. However, there are some antigen detection systems still in use in Japan. A new commercial rapid antigen test based on L7/L12 ribosomal protein components of the 50S ribosome in a lateral-flow immunochromatographic format, known as Ribotest Mycoplasma (Asahi Kasei Pharma Co., Tokyo, Japan), has been used for testing OP swabs. Comparative evaluations of this product against real-time PCR showed that its sensitivity was approximately 60 to 70% (297, 298). Another product using colloidal gold in an immunochromatographic assay was 100% sensitive and 97.4% specific compared to real-time PCR (299). Although technology has advanced over the past several years, nonamplified antigen detection systems are not available in the United States.

Nucleic Acid Amplification

The first descriptions of molecular-based assays for direct detection of *M. pneumoniae* were published in the late 1980s, and since that time these techniques have become the diagnostic methods of choice for detection of *M. pneumoniae*. Several NAATs are now commercialized in both monoplex and multiplex formats. Molecular-based methods have superior analytical and clinical sensitivity in most instances over indirect methods of detection such as serology, as reviewed by Waites et al. (300). Although culture was long considered the gold standard for diagnosis of *M. pneumoniae* infection, the greater analytical sensitivity and shorter turnaround time of NAATs has led to their consideration as the "new gold standard," even though many of these assays have never been validated extensively against culture (83). The first PCR assay described for *M. pneumoniae* used the P1 cytadhesin gene as the target (301). Over the years, other gene targets have been described and various other NAAT formats have been developed. Other gene targets have included 16S rRNA, the 16S-23S rRNA spacer, the CARDS toxin gene, the ATPase operon, *dnaK*, *pdhA*, *tuf*, *parE*, *pdhA*, *ptsL*, and the noncoding repetitive element in *repMp1*. Assays targeting the P1 gene have been reported to be more sensitive than those targeted at the 16S rRNA by Ieven et al. (302), but the reverse result was found in a comparative study by Zhou et al. (303). A study by Peters et al. (217) showed that the CARDS toxin PCR detected *M. pneumoniae* in 10-fold more patients than a PCR using the P1 protein gene as the gene target. The UAB Diagnostic Mycoplasma Laboratory currently uses an internally validated *repMp1* real-time PCR assay with the Roche LightCycler system for clinical diagnostic purposes because its presence in multiple copies across the genome has the theoretical advantage of improving sensitivity for detection (300, 304). PCR platforms commonly used for detection of *M. pneumoniae* DNA in clinical specimens include the ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA), the iCycler iQ (Bio-Rad, Hercules, CA), and the LightCycler systems (Roche Diagnostics, Indianapolis, IN). Detection formats include agarose gel electrophoresis, SYBR green, TaqMan probes, hybridization probes, molecular beacons, scorpion probes, resequencing microarrays, capillary electrophoresis, and microchip electrophoresis (300). Conventional PCRs for *M. pneumoniae* have been

TABLE 3 Examples of commercial nucleic acid amplification tests for detection of *M. pneumoniae*[a]

Product	Technique, detection	Pathogen(s) detected	Manufacturer	Reference(s)
Loopamp *Mycoplasma pneumoniae* DNA amplification kit	LAMP,[c] turbidity	*M. pneumoniae*	Eiken Chemical, Tokyo, Japan	310, 313, 314
*illumi*gene Mycoplasma[b]	LAMP, turbidity	*M. pneumoniae*	Meridian BioScience, Inc., Cincinnati, OH, USA	307
FilmArray RP[b]	Multiplex PCR, microarray	*B. pertussis*, *M. pneumoniae*, *C. pneumoniae*, several respiratory viruses	bioMérieux/Biofire Diagnostics, Inc., Salt Lake City, UT, USA	315, 486
Chlamylege	Multiplex PCR, hybridization	*M. pneumoniae*, *C. pneumoniae*, *Legionella* spp.	Argene, Inc. Shirley, NY, USA	319
ArgeneChla/Myco pneumo assay	Multiplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*	bioMérieux/Argene Marcy l'Etoile, France	320
Mycoplasma/Chlamydophila pneumoniae real-time PCR kit	Duplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*	Diagenode, Liege, Belgium	317, 321
Simplex *M. pneumoniae*	Monoplex PCR, real time	*M. pneumoniae*	Focus Diagnostics, Cypress, CA, USA	317
Venor MP	Monoplex PCR, agarose gel and real time	*M. pneumoniae*	Minerva BioLabs, Berlin, Germany	317, 318, 330
Mycoplasma pn. Q-PCR Alert	Monoplex PCR, real time	*M. pneumoniae*	Nanogen Advanced Diagnostics, Buttigliera Alta, Italy	317, 330
Genaco Resplex I	Multiplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *H. influenzae*, *N. meningitidis*	Qiagen, Venlo, the Netherlands	330
RespiFinder 19	Multiplex PCR, capillary electrophoresis	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*, several viruses	Patho Finder, Maastrict, the Netherlands	330
RespiFinder SMART 22	Multiplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*, several viruses	Patho Finder, Maastrict, the Netherlands	320
Seeplex PneumoBacter ACE	Multiplex PCR, capillary electrophoresis	*S. pneumoniae*, *H. influenzae*, *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila*, *B. pertussis*	Seegene, Seoul, South Korea	320
GeneProof *M. pneumoniae*	Monoplex PCR, real time	*M. pneumoniae*	GeneProof, Brno, Czech Republic	321
BactoReal *Mycoplasma pneumoniae*	Monoplex PCR, real time	*M. pneumoniae*	Ingenetix GmbH, Vienna, Austria	321
M. pneumoniae LightMix kit	Monoplex PCR, real time	*M. pneumoniae*	TIB MolBiol, GmbH, Berlin, Germany	321
BD Probe Tec ET	Strand displacement, fluorescence	*M. pneumoniae*	BD Diagnostics, Sparks, MD, USA	330
NucliSENS Easy Q	Multiplex NASBA[d]	*M. pneumoniae*, *C. pneumoniae*	bioMérieux, Marcy l'Etoile, France	332, 333

[a]Examples are limited to commercial assays available in various countries for which publications in international journals were available. Some products evaluated in previous years may no longer be available or may now be sold by different companies.
[b]FDA cleared and available in the United States.
[c]LAMP, loop-mediated isothermal amplification.
[d]NASBA, nucleic acid sequence based amplification.

mostly replaced by real-time PCRs since the latter have several advantages, including quick turnaround times, various detection formats, lower likelihood of contamination, greater sensitivity and specificity, and ability to provide quantitative data, detect antimicrobial resistance genes, and analyze genetic relatedness of the organisms. Improved specificity is mainly due to the use of a third oligonucleotide probe that binds to the target sequence, thereby minimizing cross-reactivity and detection of undesired amplicons (300). A significant advantage of NAATs over serology is that only one specimen is needed, and the test may be positive much earlier in the course of infection. In addition to PCR, other NAAT formats include loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), and strand displacement assays (SDA). Several recent comprehensive reviews of PCR-based diagnostics for *M. pneumoniae* have been published, and readers are referred to them for more-detailed information on this topic (6, 83, 295, 300, 305).

Commercial nucleic acid-based diagnostic methods. Despite the recent profusion of molecular-based assays in Europe and the widely acknowledged need for such testing, development of this technology has progressed much more slowly in the United States. Table 3 provides examples of commercial nucleic acid amplification assays sold in various countries, including the assays available in the United States. The

table is limited to NAATs that have been included in published research studies. Much emphasis has been placed on multiplex tests, even though development of a sensitive and specific multiplex assay is more challenging because the numerous primer pairs can potentially lead to nonspecific amplifications and formation of primer-dimers, particularly if optimum PCR conditions and reagents differ among them (83). As of 2016, there are only two NAATs cleared by FDA for detection of *M. pneumoniae* in clinical specimens in the United States. These are the LAMP assay marketed as *illumi*gene Mycoplasma Direct and the FilmArray respiratory panel.

LAMP is a relatively new nucleic acid amplification alternative for direct detection of a variety of microorganisms in clinical specimens that is simpler and less expensive than PCR, making it suitable for laboratories that do not have facilities or experienced personnel for complex molecular-based testing. LAMP technology, first described in 2000 by Notomi et al. (306), utilizes oligonucleotide primers and a strand displacement DNA polymerase to amplify target DNA at a constant temperature. As target DNA is amplified in large amounts, pyrophosphate ions are produced as a by-product that combines with Mg^{2+}, yielding a precipitate in the reaction mixture that causes an increased turbidity which is detected as a change in absorbance. LAMP has been evaluated in several studies in various formats for detection of *M. pneumoniae* in OP swabs, including comparisons with serology, conventional, and real-time PCR, in Japan, China, and the United States (307–314), with generally favorable results in terms of diagnostic sensitivity. LAMP technology is now recommended as a first-line diagnostic method for detecting acute *M. pneumoniae* infections in Japan (310).

The *illumi*gene Mycoplasma detects *M. pneumoniae* as a single analyte under isothermal conditions from OP swabs, providing qualitative results in less than 1 h using primers directed against a 208-bp sequence of the *M. pneumoniae* genome found in the intracellular protease-like protein gene. Ratliff and coworkers (307) used the *illumi*gene Mycoplasma to correctly identify 36 *M. pneumoniae* reference strains and clinical isolates with a detection limit of 2,350 CFU/ml and no cross-reactivity. They also evaluated 214 archived respiratory specimens previously cultured for *M. pneumoniae* and reported a sensitivity of 100%, detecting 22/22 that were culture positive. Specificity was 190/192 (99%) when bidirectional sequencing of PCR products was used to resolve discrepancies. Although the *illumi*gene is approved for detection of *M. pneumoniae* from OP swabs, other respiratory specimens, including NP swabs, sputum, and BAL fluid, were included in this study. The *illumi*gene incubator/reader can process up to 10 specimens at the same time. There is now a newer version, the Meridian *illumi*gene Direct, that further simplifies the procedure, eliminating a DNA extraction step in favor of heating samples at 95°C for 10 min.

Investigators at the CDC developed a LAMP assay using the CARDS toxin gene as the target and determined that their assay had a detection limit of approximately 11 genome copies/reaction (311). In an attempt to produce a diagnostic test that could be used in a point-of-care setting, they tested NP and OP swab specimens with a prior DNA extraction or with only a heating step and used calcein to facilitate visual readout of results. LAMP detected 200/226 (89%) PCR-positive specimens using a validated assay targeting the same gene as the LAMP assay when there was prior DNA extraction. Use of LAMP directly without DNA extraction detected 32/39 (82%) PCR-positive specimens, with results available in approximately 70 min (311). As currently formulated, the LAMP reaction is potentially more specific than PCR due to the requirement for 4 primers having to recognize 6 sites on the target sequence. However, this requirement also means that LAMP assays require strict attention to optimization and validation procedures in order to avoid false-positive results (311). Unlike adaptations of real-time PCR, LAMP currently has no way to test specimens directly for macrolide resistance genes in *M. pneumoniae*. This LAMP assay has not been developed for use outside the CDC.

The FilmArray respiratory panel is a multiplexed automated PCR that integrates specimen processing, nucleic acid amplification, and detection into a single pouch. The assay includes DNA extraction, an initial reverse transcription, and multiplex PCR

followed by reactions for detection of 17 respiratory viruses and 3 bacteria in NP swab specimens. Bacterial species in the assay are *Bordetella pertussis*, *C. pneumoniae*, and *M. pneumoniae*. The assay requires less than 5 min of hand-on time, and results are available in about 1 h (315). Its primary use and published evaluations have focused on detection of respiratory viruses. One study reported that the FilmArray respiratory panel correctly detected 9/9 *M. pneumoniae* isolates in archived PCR-positive specimens (315). Additional studies must be performed with this product to understand fully how well it works in a clinical setting for organisms other than viruses. Relative disadvantages of the FilmArray respiratory panel in comparison to the single-analyte *illumi*gene Mycoplasma Direct are that the FilmArray instrument can handle only a single specimen at a time and the test has a much higher cost, considering that there are 20 pathogens included. However, if pathogens other than *M. pneumoniae* are of interest, the multiplex test is more cost-effective than if they had to be sought on an individual basis.

The CDC has utilized a commercial TaqMan Array Card (Thermo Fisher Scientific, Waltham, MA) that includes *M. pneumoniae* among several other pathogens for investigation of outbreaks of respiratory infections in the United States (305). They have also described a single-tube multiplex real-time PCR assay using 4 distinct hydrolysis probes that detect *M. pneumoniae*, *C. pneumoniae*, and *Legionella* spp. (316). The gene target for *M. pneumoniae* is MPN181, which encodes which encodes 50S ribosomal protein L18. This assay demonstrated sensitivity comparable to that of monoplex PCR using the same target.

Numerous monoplex and multiplex NAATs have been used in Europe for several years. Some of them have undergone comparative testing against one another and/or against other noncommercial NAATs. Several are CE certified. The most useful data come from studies that included comparisons of NAATs with serology and/or culture. It is not feasible to describe all of these NAATs, but it is pertinent to mention several that have been the subject of publications.

Touati et al. (317) evaluated five commercial PCR assays for detection of *M. pneumoniae* in respiratory tract specimens in comparison to an in-house real-time PCR assay. Kits from Nanogen Advanced Diagnostics, Focus Diagnostics, Diagenode SA, Cepheid, and Minerva Biolabs were included in the evaluation. All kits provided prompt and specific results, validated by the use of an internal control. Dumke and Jacobs (318) reported that the Venor Mp-QP and the artus LC PCR (Qiagen, research use only) were able to detect *M. pneumoniae* DNA at a concentration comparable to 1 CFU/μl when testing dilutions of type strains, as well as DNA extracted from respiratory tract specimens. They were also able to detect the different *M. pneumoniae* subtypes. The Chlamylege multiplex PCR assay used in conjunction with microplate hybridization with specific probes to detect *M. pneumoniae*, *C. pneumoniae*, and *Legionella* spp. was evaluated in 154 clinical specimens from patients with respiratory infections that included 9 patients with *M. pneumoniae* and in another evaluation of 220 endotracheal aspirates from children, among which there were 6 specimens positive for *M. pneumoniae* (319). Results were compared to detection by serology and/or in-house PCR assays. Chlamylege correctly identified all *M. pneumoniae*-positive and -negative specimens as well as two additional samples positive for *M. pneumoniae* in the second group. Pillet and coworkers (320) evaluated the technical performances of several commercial multiplex PCR kits sold in Europe for the diagnosis of respiratory infection. They compared the results against the ArgeneChla/Myco pneumo assay and found sensitivity and specificity of 70% and 100% for RespiFinder and 80 and 99% for Seeplex, respectively. Dumke and Jacobs (321) evaluated four commercial real-time PCR assays, the Diagenode *M. pneumoniae/C. pneumoniae* real-time PCR, GeneProof *M. pneumoniae*, Ingenetix BactoReal *Mycoplasma pneumoniae*, and LightMix kit *M. pneumoniae* assays. They compared the results to those of an in-house procedure using *repMp1* as the target, testing respiratory specimens from adults and children with symptoms of pneumonia, and found sensitivities ranging from 95 to 100%, with the Diagenode test detecting all 37 specimens that were positive by the *RepMp1* assay. All kits were able to detect at least 20 CFU/5 ml (52 fg DNA/5 ml) of sample.

NASBA is based on detection of RNA through a complex process in which double-stranded DNA is generated from RNA through reverse transcriptase, RNA polymerase (RNAP), and RNase H under isothermal conditions (305). This assay has been developed in both conventional and real-time formats and in monoplex and multiplex formats using 16S rRNA as the gene target. NASBA has been used commercially in Europe under the trade name NucliSENS for several years (322–324). NASBA can provide rapid results with sensitivity comparable to that of PCR at a detection threshold of 5 to 50 CFU. As expected, multiplex NASBA used to detect *C. pneumoniae* and *Legionella* species in addition to *M. pneumoniae* has somewhat lower sensitivity than monoplex NASBA (325). Even though NASBA is a plausible alternative to real-time PCR, thus far this method has not become widely used in diagnostic laboratories, although its lower cost and minimal equipment requirements make it a potential contender for development as a point-of-care test (305). Other techniques, including line blot assays and strand displacement assays, have been used alone and in combination with multiplex PCRs to detect *M. pneumoniae* and other respiratory pathogens (300, 319, 326–329). Some of these techniques, such as strand displacement, have been developed commercially in Europe (e.g., BD Probe Tec E) (330).

No matter which PCR format or platform is used, the type of specimen collection method, transport media and storage conditions, and method and efficiency of DNA extraction can affect whether the PCR assay itself will be able to confirm the presence or absence of *M. pneumoniae* DNA. Some of the commercial NAATs, such as the *illumi*gene Mycoplasma Direct and the FilmArray, do not require a separate DNA extraction step, as it is included in the overall assay procedure. Some laboratories may choose to use nonautomated methods such as lysis and proteinase K treatment for specimens such as body fluids other than blood and swabs in transport media. Potentially inhibitory specimens such as blood, tissue, lower respiratory tract secretions, and subcultures can be purified using the Qiagen DNA blood minikit (Qiagen) (300). For laboratories with a large specimen volume on which PCR testing needs to be done, consideration should be given to purchasing an automated or semiautomated DNA extraction system. There are now several alternatives available that utilize different technologies. The EasyMag extractor (bioMérieux) enabled superior amplification results when applied retrospectively to clinical specimens and compared to the Qiagen blood minikit and the NucliSENS miniMAG systems (331, 332). The NucliSENS EasyMag is an automated version of the NucliSENS miniMAG platform (325). Bésséde et al. (333) retrospectively tested respiratory specimens that were positive or negative for *M. pneumoniae* by real-time PCR. They extracted DNA using the NucliSENS EasyMag and then amplified the extracts by real-time NASBA using the NucliSENS EasyQ analyzer. The performance of the two systems was compared to results of PCR and MagNA Pure (Roche Diagnostics, Indianapolis, IN). They found the NucliSENS EasyMag/EasyQ combination to be equivalent to the MagNA Pure/in-house PCR system for *M. pneumoniae*. An evaluation of the MagNA Pure LC automated instrument (Roche Diagnostics, Indianapolis, IN), the InviMag Bacteria DNA minikit (Invitek, Berlin, Germany) using the KingFisher ml automated instrument (Thermo Scientific, Rochester, NY), the QIAamp DNA blood minikit (Qiagen, Valencia, CA), and the Invitrogen ChargeSwitch gDNA Mini Bacteria kit (Invitrogen, Carlsbad, CA) was performed at the CDC (334). They inoculated OP swabs obtained from 10 prescreened patients with serial dilutions of *M. pneumoniae* strain M129aq and tested 5 replicate swabs from each specimen. Real-time PCR was performed on the extracted specimens using the CARDS toxin gene target. They found that the two automated methods produced the lowest threshold cycle (C_T) values, and the lowest limit of detection was with the KingFisher method. The authors suggested that the automated methods offer the advantage of robotic precision to decrease human error and variability, resulting in superior DNA extraction performance and thereby justifying the significant cost for instrumentation.

Careful attention to quality control procedures for PCR is mandatory to limit false-positive and false-negative results. False-positive results from contamination can be a major

problem for conventional PCR but are less common with real-time PCR. Reasons for false-negative results can include the presence of PCR inhibitors in the specimen, suboptimal reagent preparation and reaction conditions, and inefficient extraction of target DNA. Inhibitory factors and suboptimal PCR conditions can be detected by adding a positive-control DNA after purification. However, this external control strategy cannot reveal inefficient DNA extraction. Use of an internal control added directly to the crude sample and processed for purification and amplification is the most accurate method to monitor the important steps of diagnostic PCR protocols (300).

Multicenter comparisons of various NAATs for *M. pneumoniae* detection (335, 336) reported significant variations in test performance among participating laboratories in Europe. Another interlaboratory comparison organized by Quality Control for Molecular Diagnostics (QCMD) (www.qcmd.org) (330) for *C. pneumoniae* and *M. pneumoniae* included several samples in BAL fluid or transport medium that were positive for one or the other organism in various concentrations or negative for both. Data from participating laboratories included testing by 5 conventional commercial PCRs, 10 conventional in-house PCRs, 4 real-time commercial PCRs, 46 real-time in-house PCRs, and 2 strand displacement amplification assays. Commercial PCRs included in the evaluation were the Minerva Biolabs Venor Mp PCR, Qiagen Genaco Resplex I, Seegene Seeplex PneumoBacter detection kit, Nanogen Mycoplasma pn Q-PCR Alert kit, and Qiagen artus *M. pneumoniae* PCR kit. Overall, the results showed that real-time commercial assays performed better than in-house assays, with all commercial assays correctly detecting all samples positive for 50 to 5,000 color-changing units (CCU)/ml of *M. pneumoniae*. Great variation was seen in assay performance at the lower concentrations. In addition to the QCMD program, an international standard for use with NAATs for detection of *M. pneumoniae* has been developed by the World Health Organization (337). Results of comparative studies clearly demonstrate the need for external proficiency testing to document accuracy of molecular testing for *M. pneumoniae* and the need for improvement of analytical sensitivity for some assays, as well as standardization of sample preparation, including DNA extraction, to achieve optimum results. Proficiency testing panels for NAATs for various pathogens, including *M. pneumoniae*, are also available through the College of American Pathology (CAP) (www.cap.org). The CAP does not provide any type of proficiency testing for detection of *M. pneumoniae* by culture, but the UAB Diagnostic Mycoplasma Laboratory has exchanged specimens with other laboratories at periodic intervals as a means for alternative proficiency testing. CAP provides a proficiency test for serological testing that includes IgM, IgG, and total antibodies against *M. pneumoniae*. Since molecular testing for *M. pneumoniae* is much less widespread in the United States than in Europe and there are many fewer assays available, there are no published interlaboratory evaluations or performance reports for proficiency tests from the United States.

Recommended Diagnostic Approach

Comparison of various NAATs, mainly PCR assays, with culture has yielded varied results. In view of the enhanced analytical sensitivity of the PCR assay over culture, a positive PCR result together with negative culture can be easily explained. However, in a situation of a negative PCR assay with a positive culture, the presence of inhibitors or some other technical problem with the PCR assay must be considered. PCR inhibition may be more likely to occur with NP aspirates than with OP swabs. Sometimes dilution of samples overcomes inhibition of PCR, but this is at the cost of diminished sensitivity because the DNA is diluted along with any inhibitors. Thus, the type of sample and its preparation can influence the ability to detect mycoplasma infection using a PCR assay.

It is not surprising that PCR results may not always correspond with serological results. For example, elderly adults with pneumonia might have age-related impairment in immunity, resulting in low antibody responses after *M. pneumoniae* infection. The same situation may occur with very young infants. A positive PCR test together with a negative serological test could also mean that the specimen was collected too soon in the course of the illness to allow sufficient time for antibody to develop. PCR

results may also become negative within days following antibiotic treatment, whereas serological results should remain positive for a longer time. It is not known with certainty whether there is a specific threshold quantity of *M. pneumoniae* in respiratory tract tissues that can differentiate colonization versus infection. Therefore, relying solely on 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, which sometimes occurs in children, or at a time when there is an epidemic ongoing in a given community with elevated exposure rates, and because of the propensity of this organism to cocirculate with other bacterial and viral pathogens.

A study performed in China analyzed data from children hospitalized with respiratory infections for *M. pneumoniae* using a commercial PA test for IgM and a quantitative real-time PCR (191) Concordance for the two methods for detection of *M. pneumoniae* infection was 90%, but 173 (7%) of children with a positive PCR result had no serological evidence of infection and only 72 (3%) IgM-positive children were PCR negative. Children who were PCR positive and IgM negative were primarily under 1 year of age, consistent with knowledge that infants may be unable to mount a vigorous humoral immune response to *M. pneumoniae*. Numerous other studies have compared real-time PCR with serology in adults and children, as reviewed by Ieven and Loens (83, 295). We agree with their conclusions that no single test will reliably detect all infections and that the most sensitive approach for early diagnosis of *M. pneumoniae* infection is a combination of IgM serology and real-time PCR, especially in children. This approach may be less optimal in adults. The additional expense of performing two tests has to be considered, along with very limited availability of NAATs in the United States.

Other New Diagnostic Methods

MALDI-TOF MS. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has become widespread as a rapid and accurate means for identification of Gram-positive and Gram-negative bacteria in clinical laboratories in many countries. This technique analyzes whole bacterial cells instead of nucleic acid. Although the initial cost for instrumentation is significant, the actual cost per test is negligible, and organism identification can be achieved within minutes.

Once an isolated colony is available from culture, a pulsed laser irradiates the sample, triggering ablation and desorption of the sample and matrix, which is then ionized and accelerated into a mass spectrometer to generate a unique spectrum that is compared with known spectra from a library of organisms. Pereyre and colleagues (338) used the Bruker Biotyper MALDI-TOF MS (Bruker Daltronics, Bremen, Germany) to generate spectra from reference strains of several human and animal *Mycoplasma* species and then applied this method to identify a collection of clinical isolates that had been characterized previously by phenotypic, antigenic, or molecular methods. MALDI-TOF MS correctly identified 96% of all isolates tested, which included 50 *M. pneumoniae* isolates, all of which were correctly identified and distinguished from *M. genitalium* and *Mycoplasma amphoriforme*. Moreover, all 50 isolates were correctly clustered into 2 groups, corresponding to the P1 subtypes. Xiao et al. then coupled this technology with ClinProTools software, and 7 biomarker peaks were selected to correctly identify the two subtypes from 38 *M. pneumoniae* isolates that had been positively identified by real-time PCR and classified as P1 subtype 1 or 2 by full-length sequencing of the P1 (MPN141) gene, and they found 100% sensitivity and specificity (339). MALDI-TOF MS is a viable means for rapid identification and subtyping of *M. pneumoniae* at low cost and high throughput, once it has been grown in culture, provided that a costly mass spectrometer is available. However, the time-consuming and insensitive nature of the prerequisite culture in order to perform MALDI-TOF MS potentially limits its usefulness for *M. pneumoniae*.

NA-SERS. A new and unique investigational technique for detection and typing of microbial pathogens is silver nanorod array-surface enhanced Raman spectroscopy (NA-SERS). The Raman signal enhancement permits the acquisition of unique SERS spectra within seconds without the need for sample amplification by growth or PCR

(340). Hennigan and coworkers applied this technology for detection and characterization of *M. pneumoniae* in OP swabs and simulated specimens (341). NA-SERS was able to distinguish strains M129, FH, and 11-3, which was derived from M129 with a nucleotide deletion resulting in loss of P1 adhesin and a reduced level of P65. NA-SERS also classified spectra for 10 specimens as positive or negative for *M. pneumoniae* with >97% accuracy at concentrations as low as 82 CFU per sample. Their further studies (342) demonstrated that NA-SERS provided a multivariate detection limit that was similar to that of quantitative PCR. A partial least-squares discriminatory analysis (PLS-DA) of the spectra correctly classified the two main subtypes of *M. pneumoniae* and the variants in 32 strains (343). Another evaluation of Raman spectra included 102 clinical isolates along with strains M-129 and FH to represent P1 subtypes 1 and 2, respectively (344). These researchers identified two major Raman clusters which together included 81% of the strains, but they did not correlate completely with P1 subtypes as determined previously by sequencing of the P1 gene (345). The different P1 subtypes were similarly distributed, and approximately 76% of the isolates were of subtype 1, approximately 20% of subtype 2, and approximately 5% of variant 2a. Only two of the strains (2%) could not be typed correctly. They also identified, for the first time, the presence of carotenoids in the Raman spectra of *M. pneumoniae*. Whether these new detection and strain typing methods will prove useful in epidemiological studies and characterization of virulence will require further prospective clinical studies. The major advantage of NA-SERS is its ability to detect and type *M. pneumoniae* in a single test. This nondestructive technique can be considered the next-generation method for mycoplasma detection and strain characterization.

NEW DEVELOPMENTS IN ANTIMICROBIAL CHEMOTHERAPY

Owing to the lack of a cell wall, *M. pneumoniae* is inherently resistant to beta-lactams, glycopeptides, and fosfomycin antimicrobials. Moreover, other drug classes, such as sulfonamides, trimethoprim, polymyxins, rifampin, nalidixic acid, and some oxazolidinones such as linezolid, are also inactive, leaving relatively few therapeutic alternatives for this organism. Historically, the main drug classes with efficacy against *M. pneumoniae* have included agents that act on the bacterial ribosome to inhibit protein synthesis, such as macrolides, ketolides, streptogramins, and tetracyclines, and agents that inhibit DNA replication, such as the fluoroquinolones (346). Macrolides and ketolides are the most potent agents against *M. pneumoniae*, with MICs that are typically ≤0.001 μg/ml (347). Azithromycin and clarithromycin have been used throughout the world to treat community-acquired bacterial respiratory infections because of their improved tolerability over erythromycin and, in the case of azithromycin, a much longer half-life that enables a 5-day course of treatment. Both doxycycline and tetracycline are uniformly active against *M. pneumoniae in vitro*, with doxycycline being the more potent drug with MICs usually ≤0.25 μg/ml (347). Tetracyclines should be considered if central nervous system involvement is suspected. Fluoroquinolone MICs are somewhat higher than those of macrolides, usually in the range of 0.06 to 0.125 μg/ml for agents such as moxifloxacin, but still well within the range to be considered susceptible (279). Fluoroquinolones have the advantage of being bactericidal, which can be important in eradication of infections in hosts with an impaired immune system and/or in unusual cases of systemic infection outside the respiratory tract (346). For many years, macrolides were the empirical treatments of choice for *M. pneumoniae* infections, particularly in children, for whom tetracyclines and fluoroquinolones were avoided because of potential toxicities, and little attention was given to the need for *in vitro* susceptibility testing to guide chemotherapy. Although resistance to tetracyclines and fluoroquinolones has never been documented to occur in *M. pneumoniae* under natural conditions, the situation has changed with regard to macrolides. This section will focus on the epidemiology, detection, and management of macrolide-resistant infections, as treatment of macrolide-susceptible *M. pneumoniae* infections has been discussed in detail in a previous review (1).

Macrolide Resistance

Macrolides and ketolides are primarily bacteriostatic agents that bind to specific nucleotides in domains II and/or V of 23S rRNA in the 50S bacterial ribosomal subunit, blocking protein synthesis by causing premature dissociation of peptidyl-tRNA from the ribosome (347). *In vitro* selection studies have demonstrated that point mutations in the peptidyl transferase loop of 23S rRNA as well as insertions or deletions in ribosomal proteins L4 and L22 can result in macrolide resistance in *M. pneumoniae* (348, 349). Reports from Japan and Israel showed that macrolide resistance can be documented after several days of exposure to erythromycin *in vivo* (350, 351). Naturally occurring macrolide resistance in *M. pneumoniae* is due to mutations in various positions in 23S rRNA (352). These have included C2611G, A2058G/C/T, and A2059G/C (*Escherichia coli* numbering system), corresponding to positions 2617, 2063, and 2064 in the *M. pneumoniae* numbering system. The transition mutation A2063G is the most common one reported. As the 23S rRNA gene is present in only one copy in the *M. pneumoniae* genome, a single mutational event can change the macrolide susceptibility phenotype from extremely susceptible to highly resistant, with concomitant resistance to streptogramin B, lincosamides, and ketolides (352). Whereas a mutation at position 2617 tends to produce lower levels of resistance, those at positions 2063 and 2064 lead to high-level macrolide resistance, with azithromycin MICs often exceeding 64 μg/ml (346). Because mycoplasmas have high mutation rates, such mutational events can rapidly accumulate in a population where selection is taking place.

Geographical data. The first reports of widespread macrolide-resistant *M. pneumoniae* (MRMP) appeared in Japan in the early 2000s, with subsequent spread through Asia and eventually to Europe and North America (346, 353). Prior to that time, surveillance studies from Japan (354, 355) and France (35) had shown no resistance or very minimal numbers of resistant isolates. Local outbreaks of MRMP have been reported (47, 356, 357), and numerous clinical studies of macrolide resistance from several countries worldwide have now been published, as summarized in Table 4. Over 90% of *M. pneumoniae* isolates are now resistant to macrolides in some regions of Japan and China. In Europe, prevalence is substantially lower than in Asia and varies from country to country, with recent reports ranging from very low levels of 1% in Slovenia (358) and 1.6% in Denmark (15) to 3.6% in Germany (359), 9.8% in France (360), 19% in the United Kingdom (23), 26% in Italy (361), and 30% in Israel (362). Interestingly, an evaluation of 114 *M. pneumoniae*-positive specimens collected between 1997 and 2008 and tested by a pyrosequencing assay in the Netherlands found no macrolide resistance (363). Various case reports indicate that macrolide resistance also occurs in other European countries, including Finland, Belgium, and Spain, even though no surveillance studies have been reported from these countries (364–366). In the United States, MRMP has been described as individual case reports, clusters of infections, including spread within families (47, 68, 367, 368), and one national surveillance study (62). Yamada et al. tested 49 *M. pneumoniae*-positive specimens collected from children in Missouri between 2007 and 2010 and found that 8.2% were macrolide resistant and contained the A2063G mutation (369). The CDC recently published results from 199 specimens obtained from case patients, small clusters, and outbreaks that occurred during 2006 to 2013, with an overall 10% rate of MRMP (47). They also performed macrolide resistance testing on *M. pneumoniae* isolates from children and adults hospitalized between January 2010 and June 2012. Among 202 PCR-positive specimens, 3.5% (7/202) were identified as macrolide resistant. Zheng et al. (62) reported that the prevalence of MRMP from several regions in the United States between 2012 and 2014 was 13.2%, similar to a study from Ontario, Canada, that reported macrolide resistance in 12.1% of adults and children between 2010 and 2011 (370).

Epidemiology and spread. *In vitro* exposure of *M. pneumoniae* to subinhibitory concentrations of azithromycin leads to the same mutations that occur in naturally occurring resistant organisms (346), suggesting that widespread macrolide usage has led to the global spread of macrolide resistance. Further evidence for this relationship

TABLE 4 Worldwide studies of macrolide-resistant *M. pneumoniae* infections

Country	Yr	Patient population	Resistance rate (%)	Comment(s)[a]	Reference
Japan	1985–1999	Not specified	0/381	This study demonstrated emergence of MRMP in Japan in 2000	355
	2000–2006	Not specified	15/85 (17.6)		
	2002–2004	Children	12/195 (6)	MRMP could be grouped into subtypes 1 and 2b	487
	2002–2006	Children	30/94 (31.9)	Efficacy of macrolide therapy as assessed by duration of fever and cough was 22.7% for MRMP isolates and 91.5% for susceptible ones	383
	2002–2006	Children	50/380 (13)	MRMP rate increased from 0% in 2002 to 30.6% in 2006	(391)
	2005–2009	Adults Children	2/32 (6) 18/27 (67)	A clear difference in MRMP rates between adult and pediatric patients was noted	(392)
	1983–1998	Not specified	0/296	Investigators concluded that macrolide resistance arose in Japan in 2000 and spread	354
	2000–2003	Children	13/76 (17)		
	2005–2010	Children	21/30 (70)	Macrolide therapy resulted in prolonged fever which resolved with minocycline	384
	2008–2012	Children	561/769 (73)	Gradual MRMP increase over time, varying according to geographic region	488
	2008–2011	Adults Adolescents Children	7/28 (25) 12/26 (46) 30/45 (66)	MRMP increased over time	394
	2009–2010	Children	39/47 (83)	Cluster of MRMP in 2 schools near one another suggests person-to-person transmission	489
	2010–2011	Children	58/65 (89.2)	Serum IFN-γ, IL-6, and IP-10 levels were higher in patients with MRMP	390
	2010–2012	Children	31/33 (93.9)	Most children with MRMP had received macrolides prior to hospitalization	490
	2009–2011	Children	124/190 (65.2)	Garenoxacin was the most potent agent tested against MRMP isolates	491
	2011	Children	176/202 (87.1)	Minocycline and doxycycline were effective treatments against MRMP	25
	2012–2013	Children	24/27 (89)	17/18 children with MRMP treated with macrolides did not respond, but they quickly improved when switched to minocycline or tosufloxacin	492
China	2005–2008	Children	44/53 (83)	Study conducted in Shanghai; MRMP increased from 17% in 2005 to 100% by 2007	493
	2003–2006	Children	46/50 (92)	Study conducted in Beijing; all children had prior treatment with macrolides	494
	2005–2009	Children	137/152 (90.1)	Study conducted in Shanghai; MLVA typing revealed 15 MRMP types	495
	2008–2009	Adults	46/67 (69)	Study conducted in Beijing; patients with MRMP required longer duration of treatment and longer time to resolution of fever	385
	2008–2009	Children	90/100 (90)	Study conducted in Shanghai; 93% of MRMP isolates were subtype 1 and 7% were subtype 2	496
	2008–2012	Children and adults	280/309 (90.6)	Study conducted in Beijing; MRMP rate was 98.1% in children under 14 years of age vs 83% among adolescents and adults	378
China and Australia	2008–2012	Children and adults	71/83 (85.5) in China; 1/30 (3.3) in Australia	Study conducted in Beijing and Sydney; there was no correlation between MLVA type and MRMP	451
China	2009–2010	Children	206/235 (87.7)	Study conducted in Hangzou; more complications occurred in MRMP infections	395
	2010–2011	Children	44/45 (97.8)	Study conducted in Beijing	497
	2010–2012	Adolescents and adults	114/136 (88.3)	Study conducted in Beijing; specific MLVA types were associated with MRMP and disease severity	27
	2011	Not specified	38/40 (95)	Study conducted in Beijing; based on MLVA analysis, MRMP isolates belonged to several different clones	380
	2011	Not specified	38/40 (95)	Study performed in Beijing; MLVA indicated that 2 separate clones were responsible for all MRMP	380

(Continued on next page)

TABLE 4 (Continued)

Country	Yr	Patient population	Resistance rate (%)	Comment(s)[a]	Reference
	2012–2014	Adults	71/71 (100)	Study conducted in Zhejiang. 92% of MRMP were subtype 1	393
	2011–2014	Adults and children	84/241 (34.9)	Study conducted in Hong Kong; MRMP rate increased each year and was 47.1% by 2014; specific MLVA type was associated with MRMP	357
	2010–2013	Children	34/48 (70.8)	Study conducted in Hong Kong	498
	2013	Children	128/130 (98.5)	Study conducted in Beijing; there was no correlation between MLVA type and MRMP	456
Taiwan	2011	Children	9/73 (12.3)	MRMP was associated with longer hospital stay	386
South Korea	2000–2011	Children	0/30 in 2000; 44/70 (62.9) in 2011	MRMP was not detected in 2000 but increased over time to 2011	499
	2010	Children	3/17 (17.64)	All 3 MRMP isolates were obtained from patients hospitalized in a primary care hospital	500
	2011	Adults Children	8/60 (13.3) 19/31 (61.3)	MRMP was significantly more common in children	388
	2011	Children	49/95 (51.6)	C-reactive protein elevation predicted lack of macrolide efficacy in MRMP infections	501
Denmark	2010–2011	Not specified	6/365 (1.6)	MRMP rate was very low despite heavy macrolide use during epidemics	15
Scotland	2010–2012	Adults and children	6/32 (19)	In 4 cases, MRMP arose during or following macrolide therapy	23
England and Wales	2014–2015	Adults	4/43 (9.3)	Three patients with MRMP required hospitalization	172
Germany	2003–2009	Adults	2/167 (1.2)	Two MRMP isolates were subtype 1 and 3 were subtype 2	502
	1991–2009	Adults	3/99 (3)		
	2009–2012	Adults and children	3/84 (3.6)	Specimens obtained from different parts of Germany	359
France	1994–2006	Not specified	2/155 (1.3)	Both MRMP isolates were from children and isolated in 1999	35
	2005–2007	Not specified	5/51 (9.8)	PCR and melting curve analysis were used to detect 23S rRNA mutations	430
Italy	2010	Children	11/43 (26)	No association between MRMP and subtype was detected	371
Slovenia	2006–2014	Adults and children	7/783 (1)	Subtype 2 predominated in 2010, while subtype 1 predominated in 2014	358
Israel	2010	Adults and children	9/30 (30)	*In vivo* development of resistance during treatment was documented; treatment with doxycycline and later ciprofloxacin was necessary to cure one patient	362
USA and Europe	1995–1999	Not specified	2/41 (4.8)	Multicountry surveillance study; one MRMP isolate was from USA and the other was from Finland	364
USA	2006–2007	Not specified	3/11 (27)	Outbreak in Rhode Island evaluated by PCR for mutations; 0/53 isolates obtained prior to 1998 were MRMP	368
	2007–2010	Adults and children	4/49 (8.2)	Study conducted in Missouri	369
	2006–2013	Adults and children	10	MRMP was detected in 5 of 10 (50%) outbreaks	47
	2010–2012	Adults and children	7/202 (3.5)	5 of 7 patients with MRMP had recently received macrolide therapy	72
	2012–2014	Adults and children	12/91 (13.2)	Study conducted in 6 states across the USA	62
Canada	2010–2011	Adults and children	11/91 (12.1)	Study conducted in Ontario; 3 MRMP isolates were subtype 1 and 2 were subtype 2	370

[a]MRMP, macrolide-resistant *M. pneumoniae*. Some studies detected macrolide resistance by broth microdilution MICs, while others detected rRNA mutations by various molecular-based methods such as real-time PCR and sequencing.

comes from studies in which development of macrolide resistance was documented during a course of treatment by comparing strains isolated before and after initiation of macrolide therapy and documenting the presence of new mutations in the latter (23, 361, 366, 371–375). Molecular genotyping has been used to prove the persistence of an identical strain with a new mutation that arose during therapy (366, 373, 374).

Several investigations have utilized various molecular-based typing systems to aid in understanding how MRMP infections are spread and the types of organisms that are responsible. Methods used have included simple P1 subtyping, restriction fragment length polymorphisms (RFLP) of the P1 adhesin gene, and MLVA. Investigations in North America, Europe, and Asia (47, 376–380) found no clear association between P1 subtypes or a particular MLVA type and macrolide resistance, indicating a polyclonal origin of macrolide resistance, consistent with the emergence of resistance *de novo* during treatment rather than person-to-person spread of a single clone. Macrolide resistance tends to occur in whichever P1 subtype happens to be circulating in the community at the time of an outbreak (380). However, a recent study from Hong Kong demonstrated that during an epidemic outbreak in which MRMP in hospitalized persons increased substantially, the major MLVA types did not change, suggesting that there was increased transmission of existing MLVA types rather than introduction of new types in the community (357). In particular, the increase in macrolide-resistant organisms was predominantly a result of increasing resistance in MLVA type 4-5-7-2, a common type that occurs worldwide. Between 2011 and 2014, macrolide resistance in this MLVA type increased from 25% to 100%, while other MLVA types remained predominantly macrolide-susceptible (357). Qu et al. (27) also reported a significant correlation between MLVA type 4-5-7-2 and macrolide resistance and that this MLVA type was also associated with more severe disease. As mentioned earlier, Chironna et al. have also described clonal spread of MLVA type 4-5-7-2 that was macrolide resistant among 7 members of the same family (50).

Clinical significance. The clinical relevance of MRMP has been debated because whether resistant strains can cause more severe or prolonged disease has not been completely clarified and some patients may benefit from administration of macrolides even in the presence of resistance (354, 381). Prospective, randomized, double-blinded, controlled studies would be needed in order to compare clinical outcomes in persons with macrolide-resistant infections treated with a macrolide such as azithromycin versus another agent such as a fluoroquinolone. However, such studies have not been performed and are unlikely to be conducted in the future for ethical reasons because of growing evidence that these drug-resistant infections may not respond clinically to macrolides. Several studies have reported that individuals infected with MRMP who receive macrolide treatment can experience a longer febrile period and extended antibiotic therapy compared with those infected with macrolide-susceptible strains (382–390). Matsubara et al. (383) reported that the clinical efficacy rate of macrolide therapy was 91.5%, versus 22.7% in macrolide-susceptible versus MRMP infections. In another study, PCR was used to track numbers of *M. pneumoniae* organisms in NP samples from patients with macrolide-resistant versus macrolide-susceptible infections who received a macrolide. The numbers of organisms were significantly greater in the group with macrolide-resistant infections (384).

The presence of MRMP does not change the clinical or radiographic presentation of pneumonia, so it is impossible to guess in advance who may be harboring resistant organisms (354, 382, 383, 385, 386, 391). Thus far, most macrolide-resistant infections have been reported in children, because most studies have focused on this population and *M. pneumoniae* infections tend to be especially common in the pediatric age groups, but macrolide-resistant infections can also occur in adolescents and adults (385, 392–394). It is likely that many adults acquire infection from children.

Generally speaking, acquisition of genetic information mediating antimicrobial resistance does not make a bacterium more virulent. However, it can certainly make treatment more difficult and result in more complications if an ineffective antimicrobial is used. Zhou et al. (395) reported that hospitalized children who were treated with

azithromycin and turned out to have MRMP not only had longer duration of fever and hospitalization than those infected with macrolide-susceptible organisms but also experienced more serious radiological findings, significantly more extrapulmonary complications (29.6% versus 10.3%), and more serious pneumonias. These authors suggested that ineffectively treated MRMP pneumonia led to a stronger host response with secretion of more cytokine-mediated inflammation, particularly IL-8 and IL-18, which may be related to severity of illness in children (396). However, another study found no difference in IL-8 and IL-18 in children with MRMP infection but did find higher levels of IL-10, interferon gamma (IFN-γ), and interferon gamma-induced protein 10 (IP-10) (390). They speculated that milder disease in their patients or a difference in blood sampling times could explain the apparent discrepancy with other studies. Life-threatening pneumonia and meningoencephalitis due to MRMP have also been reported (397, 398).

Need for Alternative Treatments

Even though there is some disagreement regarding the value of antimicrobial treatment for mycoplasmal pneumonia in children (399, 400), many clinicians feel that in the absence of resistance, macrolides are useful, perhaps for both their antimicrobial effect and their anti-inflammatory properties mediated through cytokine inhibition (387, 401). Overt macrolide treatment failures have been reported, highlighting a need for alternative antibiotic treatment options for infections caused by MRMP (68, 361, 367, 402–405). Various case reports have shown that alternative agents such as quinolones (ciprofloxacin or levofloxacin), tetracyclines (doxycycline or minocycline), or the glycylcycline tigecycline (402) may result in clinical improvement after macrolide treatment failure in children (68, 361, 362, 387, 391, 404, 406). Atkinson et al. (404) reported a severe case of SJS in a child who was shown to have MRMP and who failed azithromycin therapy but responded to levofloxacin. According to an Italian study, seven of eight patients with MRMP infection (87.5%) had persistence of symptoms that led to clarithromycin being replaced by levofloxacin, which was followed by the prompt resolution of fever and cough (387). Okada et al. reported that minocycline and doxycycline were each significantly more effective than tosufloxacin in achieving defervescence within 24 h and in decreasing numbers of *M. pneumoniae* DNA copies 3 days after initiation in an outbreak involving Japanese children who had macrolide-resistant infections (25). Prior administration of macrolides was significantly associated with occurrence of macrolide resistance in that study. Similar findings demonstrating superiority of minocycline over tosufloxacin have been reported by Kawai et al. (406). Matsubara and colleagues reported that 10 of 22 (45.5%) children with macrolide-resistant infections required substitution of levofloxacin or minocycline to resolve the infection (383). Similar findings were described in a study by Morozumi et al. (391), in which 19 of 53 (35.8%) patients with macrolide-resistant infections had macrolide treatment changed to levofloxacin or minocycline, versus only 4/58 (6.9%) of children with susceptible isolates. Other quinolones such as garenoxacin, which is active *in vitro* and *in vivo* against *M. pneumoniae*, are sometimes used in Japan (407, 408). Although there are no data concerning the clinical efficacy of garenoxacin against MRMP, there is no reason to believe it should not be effective, as there is no cross-resistance of quinolones with macrolides. Normally, drugs in the fluoroquinolone and tetracycline classes would not be used in children, but until new classes of drugs that are effective against MRMP become available, there may be no other realistic choices. Streptogramins such as quinupristin-dalfopristin and pristinamycin are available in some countries. They retain activity against macrolide-resistant organisms, but they have not been utilized thus far for this purpose in any published reports of which we are aware (409).

Guidelines for treatment of community-acquired respiratory pneumonia in children have been published by various professional organizations, such as the British Thoracic Society (410), Pediatric Infectious Disease Society/Infectious Disease Society of America (269), and Japanese Society of Pediatric Pulmonology/Japanese Society for Pediatric Infectious Diseases (411). The 2011 Japanese guidelines rec-

ommend use of tosufloxacin instead of a macrolide when a MRMP infection is suspected, in consideration of potentially greater toxicity of tetracyclines in children. The other guidelines address laboratory diagnosis of *M. pneumoniae* but do not mention MRMP, considering the lack of data and presumed infrequent occurrence in North America and Europe as of 2011, when the latest versions were published. The Pediatric Infectious Disease Society/Infectious Disease Society of America (269) recommends azithromycin as preferred treatment with erythromycin and levofloxacin as alternatives, while the British Thoracic Society document mentions only macrolides. Given the common use of macrolides to treat pediatric respiratory infections, judicious use of antimicrobial drugs should be emphasized. In some instances, mild respiratory infections may not need antibiotics at all, in the opinion of Swedish investigators (17). Okada, et al. reported that macrolides account for 30% of all oral antibacterial drugs prescribed in Japan and concluded that the increase in macrolide-resistant bacteria during the past several years in that country was closely related to selective pressure resulting from widespread macrolide use (25). Regular surveillance for resistance worldwide would help monitor the trend. In Asia, where resistance rates are extremely high, clinicians should consider using an alternative to macrolides as initial treatment of suspected or confirmed *M. pneumoniae* infection. However, in Europe and North America, where resistance is still much less common, macrolides may still be considered the first-line treatment, but with careful follow up and consideration for change to another drug class if the clinical response is unsatisfactory (412). Even though macrolide resistance is the only type of naturally occurring acquired antimicrobial resistance in *M. pneumoniae*, tetracycline and fluoroquinolone resistances are known to occur as a result of selective pressure in urogenital mycoplasmas, and resistance to these agents has been produced experimentally by *in vitro* selection in *M. pneumoniae* with target mutations identified in the mutants (346, 413).

In addition to antimicrobials, plasmapheresis and intravenous immunoglobulin therapy have also been shown to be beneficial in some cases of central nervous system disease (414). Corticosteroids such as solumedrol given intravenously may also be effective in ameliorating symptoms of *M. pneumoniae* infection, particularly neurological manifestations and perhaps the dermatological lesions associated with SJS (415). Corticosteroids have received considerable attention and have shown promising results in treatment of refractory pneumonia due to *M. pneumoniae*, particularly when combined with appropriate antimicrobials (416). Luo et al. reported that children with refractory *M. pneumoniae* infections who received prednisolone combined with azithromycin for 5 days had clinical and radiological improvement sooner than children who received only azithromycin, but they did not test for macrolide resistance (417). Combination treatment of a child with progressive, severe systemic MRMP infection with high-dose methylprednisone pulse therapy, intravenous immunoglobulin, and moxifloxacin led to a full recovery after no response occurred with azithromycin and methylprednisone at standard dosage. Six additional patients who received a combination of glucocorticoids and ciprofloxacin for refractory *M. pneumoniae* infections have also been described (418). The rapid response to corticosteroids in children with severe pneumonias illustrates the importance of a hyperactive immune reaction in the host that may overshadow the pathological effects of *M. pneumoniae* on the respiratory tract in some cases. Systematic studies are needed to determine the true benefit of combinations of antimicrobial agents and immunomodulators in treatment of severe mycoplasmal infections, both macrolide susceptible and resistant, as well as the optimal dose and timing of pulse corticosteroid treatment (419).

New Investigational Antimicrobial Agents

Over the past several years, there has been interest in development of new agents for treatment of CABP, stimulated to a great extent by increased prevalence of multidrug-resistant *S. pneumoniae*. Since *M. pneumoniae* and other atypical pathogens such as *Legionella* spp. and *C. pneumoniae* are also causes of CABP, drugs suitable for empirical use need to be active against all of these organisms. Fortunately, agents that

TABLE 5 MICs of new antimicrobial agents tested against macrolide-resistant *Mycoplasma pneumoniae*[a]

Antimicrobial	Class	No. of isolates tested	MIC range (μg/ml)	Reference
Lefamulin	Pleuromutilin	42	≤0.001–0.008	424
Solithromycin	Ketolide	6	0.25–0.5	424
Nafithromycin	Ketolide	2	16	420
Omadacycline	Aminomethylcycline	10	0.125–0.25	427
Zoliflodacin	Spiropyrimidinetrione	3	0.5–1	425

[a]Azithromycin MICs, 2 to >32 μg/ml.

are active against macrolide-resistant pneumococci are usually active against MRMP. Table 5 summarizes the *in vitro* activity of some new antimicrobials against MRMP.

Ketolides are semisynthetic derivatives of erythromycin. One promising agent in this class is the investigational agent solithromycin (Cempra Pharmaceuticals), the most potent antimicrobial agent ever tested against *M. pneumoniae* (MIC_{90} = 0.000125 μg/ml) (347). The extremely low MICs enable it to retain some activity against organisms with 23S rRNA mutations, even though their MICs are several dilutions higher. The highest reported solithromycin MICs measured against macrolide-resistant *M. pneumoniae* in an *in vitro* study were 0.5 μg/ml (347). Solithromycin is currently undergoing clinical trials for CABP and has performed in a comparable manner to moxifloxacin (89.2% versus 90.5% clinical efficacy) in treatment of 79 adults with *M. pneumoniae* pneumonia, which included one macrolide-resistant strain (58). Even though solithromycin MICs against MRMP are relatively low, additional patients must be treated before strong conclusions can be made regarding its clinical efficacy in the presence of 23S rRNA mutations. As larger numbers of patients are treated with solithromycin, there is hope that problems that the earlier ketolide telithromycin had with hepatotoxicity will not occur. Another ketolide undergoing clinical development for treatment for CABP is nafithromycin (WCK4873; Wockhardt, Ltd.). Although this drug was very active against 18 macrolide-susceptible *M. pneumoniae*, with all MICs being ≤0.0001 μg/ml, MICs for 2 strains known to be macrolide resistant were elevated (16 μg/ml), thus limiting its utility to treatment of macrolide-susceptible *M. pneumoniae* infections (420). Another novel class of macrolides known as the acylides, which are derivatives of clarithromycin, had low MICs (0.015 to 0.5 μg/ml) against 7 macrolide-susceptible ATCC strains of *M. pneumoniae* (421). Although no MRMP strains were tested, the 8 acylides tested had MICs ranging from 0.03 to 8 μg/ml against 12 high-level telithromycin-resistant *ermB*-positive *S. pneumoniae* strains selected by serial passages in subinhibitory telithromycin, suggesting that there may be some activity in the setting of macrolide resistance. It should be noted, however, that the mechanism of macrolide resistance in *M. pneumoniae* is mediated by mutations in 23S rRNA rather than *ermB*. A novel bridged bicyclic macrolide (bicyclolide), S-013420, was tested against 26 macrolide-susceptible *M. pneumoniae* strains and 5 MRMP strains (422). Despite potent activity against the former, with an MIC_{90} of 0.001 μg/ml, MICs against MRMP were much higher (8 to 16 μg/ml).

Pleuromutilins inhibit bacterial growth by binding to the peptidyl transferase center of the 50S ribosomal subunit, blocking protein synthesis, and have been used to treat mycoplasmal respiratory infections in swine and poultry. Lefamulin (BC-3781) (Nabriva Therapeutics) is a new semisynthetic pleuromutilin available in intravenous and oral formulations with potent activity against a variety of Gram-positive and Gram-negative bacteria, including multidrug-resistant strains, and is currently in clinical development for treatment of CABP. Sader et al. reported potent activity of lefamulin (MIC_{90} = 0.006 μg/ml) against 50 *M. pneumoniae* isolates from Germany, where macrolide resistance rates are low, but these organisms were not actually tested against macrolides for comparison (423). Lefamulin was subsequently tested against a collection of macrolide-susceptible and MRMP strains in comparison to azithromycin, erythromycin, tetracycline, and moxifloxacin. Lefamulin was highly active against all strains tested, with MICs of ≤0.008 μg/ml, comparable to azithromycin for macrolide-susceptible strains. The

lefamulin MIC_{90} (0.002 μg/ml) for macrolide-resistant strains was the lowest among all drugs tested. Like the fluoroquinolones, lefamulin was also bactericidal against *M. pneumoniae* (424).

Zoliflodacin is an investigational spiropyrimidinetrione DNA gyrase/topoisomerase inhibitor (Entasis Therapeutics) in clinical development for sexually transmitted infections. It has potent inhibitory and bactericidal activity against *M. pneumoniae*, with MICs similar to those of levofloxacin and doxycycline. Its activity is unaffected by resistance to other drug classes, including fluoroquinolones and macrolides (425). Even though zoliflodacin is not currently being developed for CABP, its broad spectrum of activity *in vitro* against respiratory as well as urogenital pathogens makes it a potential drug for further study. Another class of antimicrobial agents, the isothiazoloquinolones, act in a manner similar to that for the fluoroquinolones. One such agent, ACH-702, is in preclinical development by Achillon Pharmaceuticals. In one report that demonstrated that it had good *in vitro* activity against *M. pneumoniae* (MIC_{90} = 0.015 μg/ml) (426), no mention of macrolide-resistant organisms was made.

Omadacycline (Paratek Pharmaceuticals) is a new aminomethylcycline currently in clinical development for use against acute skin and skin structure infections, CABP, and urinary tract infections. Although the binding site is similar to that of tetracycline, a significant advantage of this agent is that it retains activity against microorganisms with the two main tetracycline resistance mechanisms, efflux and ribosomal protection. This agent was active *in vitro* against 10 MRMP strains, with MICs comparable to those of doxycycline (427).

A somewhat unusual approach to identification of new therapies for *M. pneumoniae* has been the investigation of various anticancer and antiviral nucleoside and nucleobase analogs that will selectively kill bacteria and not harm the host. Drugs in these categories might logically be active against *M. pneumoniae* because it depends on an exogenous supply of precursors for nucleotide biosynthesis, as it is unable to synthesize purine and pyrimidine bases *de novo*. A recent study (428) found that 7 such analogs inhibited *M. pneumoniae* growth at clinically achievable plasma concentrations, suggesting that enzymes involved in nucleotide biosynthesis are potential future targets for drug development.

Balish and Distelhorst (429) emphasized the importance of developing narrow-spectrum agents to treat *M. pneumoniae* infections in order to avoid the collateral effects on the host microbiota that can occur with broad-spectrum agents. Some potential targets of theoretical importance mentioned include the CARDS toxin, glycerol-3-phosphate, various metabolic pathway inhibitors, inhibitors of the attachment organelle assembly or function, and, as noted previously (428), inhibitors of nucleoside/nucleotide synthesis. Although the concept of developing a therapeutic agent with a very narrow-spectrum target specific to *M. pneumoniae* is completely logical from a scientific point of view in order to tailor therapy, this approach may not presently be practical in most ambulatory care settings, as the use of such an agent would require advance knowledge at the time treatment is initiated that an infection is due to *M. pneumoniae* alone and not to another pathogen or a mixture of *M. pneumoniae* and other pathogens, as is known to occur in many cases. Therefore, such agents could come into play primarily when point-of-care diagnosis of *M. pneumoniae* infection has become available.

Rapid Detection of Macrolide Resistance

A very important strategic intervention for control of MRMP is rapid testing for 23S rRNA mutations that confer macrolide resistance, directly in clinical specimens or in cultured isolates, in order to guide antimicrobial therapy. In addition to time-consuming and labor-intensive Sanger sequencing of 23S rRNA, various rapid methods have been developed, including real-time PCR-HRM, PCR-RFLP, and pyrosequencing assays to detect MRMP (46, 354, 367, 368, 430–433). The UAB Diagnostic Mycoplasma Laboratory performs real-time PCR for detection of *M. pneumoniae* in clinical specimens followed with a reflexive test for macrolide resistance in all PCR-positive samples. This technique enables detection of all 3 point mutations in the 23S

rRNA gene known to be associated with macrolide resistance in *M. pneumoniae*, based on different melting points for the mutated nucleotide base pairs compared to those of the wild type (367).

Sequencing must still be performed to determine whether the mutation occurs at position 2063 or 2064, but this is not necessary for clinical diagnosis. The CDC has employed their HRM method to identify macrolide resistance in outbreak settings in the United States (47). Other direct molecular methods for detection of macrolide resistance that have been described are a Cycleave PCR method (433), a combination of nested PCR, single-strand conformation polymorphisms (SSCPs), and capillary electrophoresis (434), single-nucleotide polymorphism (SNP) PCR (435), Simple Probe PCR (436), and allele-specific PCR amplification (437), each of which is described in more depth in the original publications.

MOLECULAR TYPING

Considering the overall low sequence diversity and the relatively high number of repetitive elements (RepMPs) throughout the genome (comprising approximately 8% of the total genome), efforts to distinguish strains have focused mainly on the sequence variations in the P1 operon (MPN140 to MPN142), where the sequence identity drops (111), as well as the variations in the copy number of tandem repeats. Several typing methods have been developed based on these elements, including P1 typing and MLVA (376). With advances in the whole-genome sequencing, more regions in the genome that have high discriminatory power have been identified. A multilocus sequence typing (MLST) method and an SNP genotyping method were developed with the aid of whole-genome sequencing analysis (111, 438). Whole-genome sequencing may eventually displace the traditional single- or multiple-locus genotyping of *M. pneumoniae* and will be used in routine clinical and outbreak investigations. Advances in other techniques, such as NA-SERS and MALDI-TOF MS, have also been used for strain typing of *M. pneumoniae* (338, 344), as described in previous sections. A comparative summary of typing methods used for *M. pneumoniae* is provided in Table 6. Since the value of strain typing in studying epidemiology and outbreaks of *M. pneumoniae* infections and in tracking spread of MRMP has been described in previous sections, further commentary is limited mainly to descriptions and comparisons of the various methods.

P1 Typing

P1 gene sequencing, performed by various methods, was the most commonly used genotyping method until newer methods were developed (35, 36, 38, 345, 439, 440). Early techniques used RFLP or pulsed-field gel electrophoresis (PFGE) (441). PFGE requires growing large volumes of the organisms in order to obtain sufficient amounts of genomic DNA, and its discriminating power is somewhat low compared to those of other methods (442). The more recent methods have been mainly PCR based. Several post-PCR procedures to detect the sequence variations have been developed. All methods allow detection of the two major subtypes as well as some variants. RFLP uses several enzymes to digest the amplicons and group them based on their band patterns (440, 441, 443). Denaturing gradient gel electrophoresis (DGGE), which can detect single base differences, can also be employed to distinguish P1 PCR products (38). Using this method, a type 2 variant, V2d, was identified (38). PCR-HRM provided a rapid one-step method for typing *M. pneumoniae* isolates (41). This method differentiates the 2 P1 subtypes based on the difference of melting temperature of a 1,900-bp amplicon carrying multiple SNPs without sequencing or hybridization procedures. Three variants were identified in a study using this method (41). To type *M. pneumoniae* directly in clinical specimens, a culture-independent amplification and sequencing method was developed by Dumke et al., and variant V2b was identified by this method (345). Random amplification of polymorphic DNA (RAPD) analysis has also been used to classify *M. pneumoniae* strains according to P1 subtypes, with results similar to those obtained by PCR-RFLP (441, 444). NASBA has been used to amplify RNA under isother-

TABLE 6 Summary of typing methods for *Mycoplasma pneumoniae*[a]

Category and typing scheme	Technique	Types	Advantage(s)	Disadvantage(s)	Reference(s)
Single-gene/locus typing		Subtypes 1 and 2 plus variants	Separates clinical isolates into 2 major stable subtypes	Not able to track strains	
P1	RFLP				440, 441, 443
	DGGE				38
	Real-time PCR coupled with HRM analysis		Fast		42
	PCR and sequencing		Culture independent		345
16S rRNA gene	NASBA				445
16S-23S rRNA gene spacer region	PCR and sequencing				440
SNPs in MPN528a and P1	Pyrosequencing				431
MPN459 and MPNA5864	Duplex real-time PCR		Fast, culture independent		46
MLVA			More discriminative than P1 typing	No correlation with P1 types	376
Five-locus scheme	Multiplex PCR and capillary electrophoresis/sequencing	>26 MLVA types	Strain tracking	Locus 1 is not stable, too discriminative	451, 452
Four-locus scheme	Multiplex PCR and capillary electrophoresis/sequencing	Fewer than major MLVA types	Stable locus, correlation with macrolide-resistant phenotype	Less discriminative than 5-locus scheme	66, 453, 454
MLST			More discriminatory than four-locus MLVA typing and P1 typing methods		
Eight genes/locus	Snapshot minisequencing	9 SNP types	Correlation with P1 types, fast, culture independent	No correlation with MLVA types	438
Eight housekeeping genes	PCR and sequencing	12 sequence types	Correlation with P1 types and MLVA types, culture independent	Laborious and expensive sequencing	458
Whole bacterial cells					
MALDI-TOF MS	MALDI-TOF MS	Subtypes 1 and 2 plus variants	Quickly identifies and types in one step	Culture dependent, cost of mass spectrometer	338, 339
NA-SERS	NA-SERS	Subtypes 1 and 2 plus variants	Detects and types in one step, easy sample prepn		343

[a]Abbreviations: RFLP, restriction fragment length polymorphism; DGGE, denaturing gradient gel electrophoresis; HRM, high-resolution melt; NASBA, nucleic acid sequence-based amplification; SNP, single-nucleotide polymorphism; MLVA, multilocus variable-number tandem-repeat analysis; MLST, multilocus sequence typing; MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; NA-SERS, nanorod array-surface-enhanced Raman spectroscopy.

mal conditions, followed by hybridization using 16S rRNA to assess differences between the two P1 subtypes (445). Another DNA amplification method for distinguishing the P1 subtypes is the amplified fragment length polymorphism (AFLP) technique.

Although the sequence variation of the RepMp elements in the P1 gene was the initial basis on which to classify the two main *M. pneumoniae* subtypes, other sequence differences between subtypes 1 and 2 are observed throughout the genome, including all RepMp elements and many other genes/regions (32, 39, 111). A total of 8 variants of RepMp4 and 10 variants of RepMp2/3 elements exist in the genome of strain M129 (113), and intragenomic homologous DNA recombination events occur among these loci and within other repetitive elements such as RepMp1 (32, 35, 39). With the identification of more P1 subtype variants that are generated by homologous recombination, P1 typing itself appears to be ambiguous to classify *M. pneumoniae*. However, according to extended genome sequencing and whole genomic sequence analysis of clinical isolates collected from various times and geographic regions, the two subtypes of *M. pneumoniae* appear to be evolutionarily stable lineages (111, 446, 447). One subtype does not switch to another, even when homologous recombination occurs at the P1 region. Thus, characterizing clinical strains into two main subtypes, not restricted to the P1 genotypes, should still bear some biological meaning. Studies have shown that subtype 1 and subtype 2 strains form different biofilms *in vitro* (99) and that subtype 2 strains may have higher expression of CARDS toxin (446). More importantly, the correlation between subtypes and disease characteristics or antimicrobial resistance has not been confirmed since the P1 typing scheme was established. Subtype-specific combinations of the repetitive elements in the P1 and OFR6 genes have been proven not to be essential for the adherence of *M. pneumoniae* to the host (448). A recent report indicated that clinical severity of *M. pneumoniae* infection is associated with bacterial load but not with the *M. pneumoniae* subtypes in 45 patients in Sweden (48). Experiments involving 140 specimens from Germany using sera of acute-phase patients with a known *M. pneumoniae* type in the respiratory tract resulted in no correlation of IgA and IgG antibodies to subtype- and variant-specific regions of the P1 gene with the genotype of the *M. pneumoniae* strain causing the actual infection (449). Because disease correlation is a common question any typing methods must face, more studies need to be done to test whether there is a relationship between P1 subtypes and virulence.

Single SNP/Gene Typing Methods

The difference between the two main subtypes of *M. pneumoniae* is not restricted to the P1 gene, and other loci (mainly SNPs) and various methods have been employed to differentiate the two subtypes. The 16S rRNA, 23S rRNA gene, and 16S16S-23S rRNA gene spacer regions are the earlier targets. A NASBA assay was designed to detect differences between the subtypes using two type-specific probes (445). Sequencing of the 750-bp fragment of the entire 16S-23S rRNA gene spacer region and partial 23S rRNA gene revealed an extra adenosine in subtype 2 strains that was absent from the subtype 1 strains, which has also been used for distinguishing them (440). Spuesens et al. used pyrosequencing to discriminate the two subtypes based on an SNP in MPN528a and another SNP in the conserved region of the P1-coding gene (431). Zhao et al. developed a duplex real-time PCR assay targeting two subtype-specific genes, MPN459 for subtype 1 and MPNA5864 for subtype 2, to type *M. pneumoniae* directly from clinical specimens (46). This real-time method is fast, sensitive, and culture independent. Using it, the authors detected a subtype shift from type 1 to type 2 that occurred in 2013 in Beijing.

Multilocus Variable-Number Tandem-Repeat Analysis

Multilocus variable-number tandem-repeat analysis (MLVA) was developed in 2009 by Degrange et al. and soon became an additional classification level for *M. pneumoniae* (376). The initial development selected five variable-number tandem-repeat (VNTR) loci (Mpn1 and Mpn13 to -16) that are polymorphic and stable after brief broth

passages. The five-locus typing scheme revealed 26 VNTR types from 265 clinical strains that the authors designated A to Z, based on five-digit codes representing the number of repeats at each locus.

The MLVA technique greatly expands the typing scheme for *M. pneumoniae* beyond what is offered by the methods that can identify only the two major subtypes and variants. A particularly attractive feature of this typing method is that it can be adapted to direct testing of clinical specimens without requirements for growing the bacterial isolates (450), thereby greatly expanding its application in surveillance and epidemiology of *M. pneumoniae* infections. This method has been used to track the clonal spread of a specific strain during an outbreak (49). Xue et al. (451) used the five-locus MLVA scheme, P1-RFLP, and detection of macrolide resistance mutations to compare 83 specimens from Beijing, China, and 30 from Sydney, Australia. They identified 18 MLVA types, including 8 that were previously unknown, and most belonged to P1 subtype 1. There was greater diversity among specimens from Beijing and much more macrolide resistance (85.5%% versus 3.3%) than in those from Sydney. Benitez et al. (452) also used the five-locus MLVA scheme to type 154 clinical isolates obtained over a 50-year period from North America, Europe, and Asia, identifying clonal spread of specific strains during outbreaks. However, these investigators and subsequent reports indicated that the Mpn1 locus, which is localized in the variable *hsdS* gene, is unstable (452, 453). Therefore, several researchers have proposed to exclude this locus and form a four-locus MLVA scheme (66, 453, 454). The discriminatory power of the modified MLVA scheme was reduced and resulted in fewer predominant MLVA types. The three most common MLVA types in the past 10 years are 4-5-7-2, 3-5-6-2, and 3-6-6-2 according to the analysis of data from North America, Asia, and Europe (47, 453, 455). The four-locus scheme maintained the correlation between the MLVA type 4-5-7-2 and macrolide resistance (455). The biological basis for this correlation is unknown. However, in another study (456), they used the four-locus MLVA scheme to type and analyze *M. pneumoniae* clinical specimens from Beijing, China, and found no correlation between MLVA type and macrolide resistance, even though MLVA type 4-5-7-2 was the most common one detected. To improve the discriminative power of the current four-locus scheme, more effort is needed to find new loci and modify the MLVA scheme. This could be carried out with the aid of whole-genome sequence analysis. Depending on the purpose of the typing, a highly stable scheme is preferred for long-term or global epidemiological studies, whereas a highly discriminative scheme is good for short-term epidemiological studies (457).

Multilocus Sequence Typing

An early attempt to develop such a method for typing *M. pneumoniae* using housekeeping genes and structural genes failed (447). With the analysis of the whole-genome sequences of more clinical isolates, a new multilocus sequence typing (MLST) scheme was developed based on the polymorphisms of eight housekeeping genes (*ppa*, *pgm*, *gyrB*, *gmk*, *glyA*, *atpA*, *arcC*, and *adk*) (458). Twelve sequence types (STs) were identified in 57 clinical isolates, and two distinct genetic clusters were formed, representing the two main subtypes of the genomes. This scheme is more discriminatory than the four-locus MLVA typing and P1 typing methods. Correlations between STs and P1 types and between STs and MLVA types were observed. This method is PCR based and does not require culturing the organism. The disadvantage, however, is the need for a large amount of laborious and expensive sequencing. This MLST scheme for strain typing is supported by a public web-based database (http://pubmlst.org/mpneumoniae).

Another multilocus SNP typing method, using SNaPshot technology, overcomes the sequencing disadvantage (438). Similarly, eight genes (*gyrA*, *gmk*, *glpK*, *rpoB*, *rplB*, the P1 gene, MPN582, and MPN442) were selected according to the extensive analysis of the whole-genome sequences of the clinical strains. The SNaPshot minisequencing technology is based on the single-base extension of a specially designed minisequencing primer that anneals one base upstream of the SNP using a fluorochrome-labeled dideoxynucleotide (ddNTP). The SNPs can thus be detected by fluorescence and size without

sequencing. The discriminatory power of this method is also higher than that of the four-locus MLVA scheme. A strong correlation between the SNP types and P1 types was observed, whereas the SNP type does not predict the MLVA type. This method is very fast, with a global turnaround time of 7 h starting from the DNA extracts. It can also be adapted to type clinical specimens without the need for culture.

KNOWLEDGE GAINED FROM COMPARATIVE GENOMICS

The application of whole-genome sequencing and other "-omics" opened a new door to understand this tiny organism. Since the first *M. pneumoniae* genome sequence was published (113), there have been 58 genome sequences deposited in the National Center for Biotechnology Information (NCBI). Among them, 57 have been sequenced by next-generation sequencing (NGS) technology, and 55 were posted after 2014. The current bottleneck in genome sequencing is not obtaining the sequence but the analysis, understanding, and application of the information gained from the sequences. The same situation also applies to other "-omics," i.e., transcriptomics and proteomics. Thus, close collaborations between mycoplasmologists and bioinformaticists in the "big data" era should lead to broader and deeper understanding of *M. pneumoniae* and its interaction with its host. An online database, MyMpn (http://mympn.crg.eu), which hosts the omic-scale data sets generated by experimental and computational analysis has been developed to promote the understanding the biology of *M. pneumoniae* on a global scale (459).

Comparative Genome Analyses

The genome sequence of strain M129 (ATCC 29342) was the first finished *M. pneumoniae* genome, obtained using the cosmid library and Sanger sequencing technology (113). It was reported in 1996 and reannotated in 2000, showing 816,394 bp, 730 genes, and an average GC content of 40% (113, 460). All subsequent genome sequences were obtained by NGS technologies. In 2015, two independent studies on comparative genome analysis of *M. pneumoniae* were reported. Xiao et al. sequenced 15 strains that were obtained from 3 continents over 6 decades and completely assembled them into single circular contigs (111). Lluch-Senar et al. sequenced 23 clinical isolates, mainly from Europe, but did not complete the assembly of the whole genome (446). Both groups of investigators found an overall high degree of sequence similarity (>99%) among the strains, although the two main subgroups of type 1 and type 2 can be clearly differentiated by the SNPs and indels. The higher similarity (>99.9%) within the strains of the same subgroup suggests that the subtype 1 and subtype 2 groups are clonal and recently diverged (111). The *M. pneumoniae* genome looks extraordinarily stable over time and geographic distance, without evidence of recent horizontal gene transfer. There are 182 genes that are conserved in all genomes without any variations, whereas P1 and ORF6 presented the greatest variations among the strains, suggesting the existence of positive selection pressure (111). Two large subtype-specific genomic structural variations were also identified. By examination of SNPs versus nonsynonymous mutation rates in the OFRs, several putative virulence factors were revealed, including recombination machinery and a hydrogen peroxide production system (446). Gene-specific analysis indicated that both coding and promoter regions of the CARDS toxin-coding gene are conserved, suggesting little selective pressure on this gene (MPN372) and the essentiality of its function. The fact that no nonsynonymous mutations are found in both copies of the *arcA* genes (MPN304 and MPN560) in the arginine deiminase pathway in all sequenced genomes suggests that the genes are not diverging and may be functional with unidentified activities not associated with the arginine dihydrolase metabolic pathway, which is inactive in *M. pneumoniae* (461). The identification of multiple copies of *hsdS* genes (encoding S subunit of type I restriction enzyme) with variable tandem-repeat numbers in the target recognition domain across the genome is striking and may indicate an epigenetic mechanism for gene regulation.

Application of Knowledge Gained from Whole-Genome Sequence Analysis

Two research groups have recently used the whole-genome sequencing information to develop two new MLST schemes, as discussed in Molecular Typing above (438, 458). These two studies are the first examples of the NGS-aided *M. pneumoniae* research. Li et al. reported whole-genome sequencing of two MRMP isolates with different responses to azithromycin and identified SNP differences in their P1 genes (462). The significance of this finding is unclear at present. Shahbaaz et al. performed an *in silico* search for uncharacterized adhesin and virulence protein genes in the genome of strain 309 and predicted 83 virulent hypothetical proteins (463). To improve the MLVA typing scheme, Zhang et al. reanalyzed the published NGS data on *M. pneumoniae* genomes and characterized inter- and intrastrain variability of VNTRs (464). There were 13 VNTRs identified, displaying different levels of inter- and intrastrain copy number variations. Several new MLVA schemes were proposed for different purposes of strain typing.

Other "-Omics" Studies

Despite having a genome that is reduced in size, *M. pneumoniae* possesses a surprisingly dynamic and complex regulation system for information flow from genome to RNA to protein. Several regulation layers and mechanisms, such as genome methylation, transcriptional regulation, noncoding RNAs (ncRNAs), translational regulation, and posttranscriptional modifications, have been identified (465–468). Lluch-Senar et al. performed single-molecule real-time (SMRT) sequencing to determine the methylomes of *M. pneumoniae* (465). Two new methylation motifs were identified: a widespread m6A methylation motif (5′-CTAT-3′) and a complex type I m6A sequence motif (5′-GAN7TAY-3′/3′-CTN7ATR-5′). The distribution of methylation sites across the genome suggests a potential role for methylation in regulating the cell cycle and gene expression. Transcriptome analysis of *M. pneumoniae* has been advancing with the improvement of techniques over the past 2 decades. Güell et al. identified the exon- and intron-like structure within operons in *M. pneumoniae* and suggested that complex gene regulation mechanisms resembling that of eukaryotes exist in *M. pneumoniae* (467). Frequent antisense transcripts were also identified, and a subsequent analysis indicated that most of them are the consequence of transcriptional noise (469). Junier et al. performed a hierarchical genomic analysis of transcriptome sequencing (RNA-Seq) data across 115 conditions and revealed that basal coordination of transcription in *M. pneumoniae* is driven by pervasive transcription (470). They identified three qualitatively distinct levels of coexpression and found that the degree of coexpression between codirectional genes and operons is tightly related to the capacity to be transcribed into the same mRNA. The pervasive transcription is repressed by DNA-bound RNAPs, strong intrinsic terminators, and large intergenic distances. The proteomic studies have been able to experimentally identify about 90% of predicted proteins thus far (468). Kühner et al. employed tandem affinity purification-mass spectrometry to analyze the protein complexes in *M. pneumoniae* and identified 62 homomultimeric and 116 heteromultimeric soluble protein complexes (468). A subsequent study using two-dimensional gel electrophoresis and mass spectrometry further detected 63 phosphorylated proteins in *M. pneumoniae* and identified 16 phosphorylation sites (102). Other posttranslational modifications have been described systematically. Van Noort et al. combined genetics and high-resolution quantitative mass spectrometry to measure the global effect of kinase and phosphatase deletions on proteome abundance, protein phosphorylation, and lysine acetylation of proteins in *M. pneumoniae* and revealed cross talk between phosphorylation and lysine acetylation (471). Large-scale multiomics data are presently accumulating rapidly, and many secrets of this "simple" organism are being revealed.

CONCLUSIONS AND FUTURE NEEDS

Over the past several years, knowledge concerning the mechanisms by which *M. pneumoniae* causes disease, epidemiology of infection, clinical spectrum of illness, and appreciation for its role as a common and significant pathogen of CABP has dramatically increased. Many new data have come from novel techniques for genome sequenc-

ing and molecular methods for strain typing. It is now appreciated that *M. pneumoniae* can cause respiratory infection in persons of all ages and can account for a substantial portion of CABP, especially during epidemic periods. Although most *M. pneumoniae* infections are fairly mild, severe and even fatal pneumonia, spread in communities, among persons in close contact, and within family groups, is well described. Autoimmune and inflammatory sequelae are common complications involving multiple organs. With the development and recent commercialization in the United States of molecular-based tests that can detect *M. pneumoniae* in clinical specimens with same-day turnaround, diagnostic laboratories can now offer improved microbiological diagnosis. However, there are two obstacles remaining that must be overcome if such diagnostic testing is to become more widely used by clinicians. First, there needs to be a true point-of care test for rapid detection of *M. pneumoniae* for use in an ambulatory care setting that incorporates direct detection of the organism or its DNA, rather than an indirect measure of antibody response. Such a test would eliminate some of the current barriers to real-time PCR testing such as the expensive equipment required, along with molecular diagnostics laboratory facilities and personnel trained in complex molecular-based testing. A commercial test that can also detect the presence of macrolide resistance gene mutations would also be very beneficial, especially if such resistance continues to increase in the United States as it has in Asia. Until this is achieved, many clinicians who want to confirm a microbiological diagnosis of *M. pneumoniae* infection are likely to continue to rely on rapid qualitative serology tests that have only fair diagnostic sensitivity when based on a single acute-phase specimen. The second obstacle to increased diagnostic testing for *M. pneumoniae* is the lack of definitive recommendations for performing microbiological testing from influential professional organizations such as the Infectious Disease Society of America. We suggest that a reconsideration of whether such testing should be recommended is reasonable in the next guideline update now that there are molecular-based tests available for *M. pneumoniae* detection, quantitative data concerning the frequency of macrolide resistance, and numerous well-described outbreaks that have involved persons with severe and even fatal infections due to this organism. Since macrolide resistance is now well documented in the United States and can be associated with serious infections and treatment failures, a national surveillance system to document the occurrence and disease burden of *M. pneumoniae* and, most importantly, the quantitation and geographic distribution of macrolide resistance are important goals. Antimicrobials that are not affected by macrolide resistance and that can be used safely in children as well as adults for treatment of respiratory infections caused by *M. pneumoniae* as well as other pathogens of CABP are urgently needed.

Discussion of control strategies for any microbial disease should include mention of a vaccine to prevent infection with and/or illness due to the etiological organism. Despite the potential benefit of an effective vaccine and considerable research that was performed over time, little effort has taken place in recent years to develop one for *M. pneumoniae*. A systematic review and meta-analysis of protective effects of and adverse reactions to *M. pneumoniae* inactivated vaccines published by Linchevski et al. in 2009 concluded that vaccines may have some benefit, particularly for high-risk closed populations and the elderly (472). The authors did not specifically address the emergence of MRMP, but it seems that this development is another good reason that vaccine research should be restarted.

REFERENCES

1. Waites KB, Talkington DF. 2004. *Mycoplasma pneumoniae* and its role as a human pathogen. Clin Microbiol Rev 17:697–728. https://doi.org/10.1128/CMR.17.4.697-728.2004.
2. Atkinson TP, Balish MF, Waites KB. 2008. Epidemiology, clinical manifestations, pathogenesis and laboratory detection of *Mycoplasma pneumoniae* infections. FEMS Microbiol Rev 32:956–973. https://doi.org/10.1111/j.1574-6976.2008.00129.x.
3. Waites KB, Balish MF, Atkinson TP. 2008. New insights into the pathogenesis and detection of *Mycoplasma pneumoniae* infections. Future Microbiol 3:635–648. https://doi.org/10.2217/17460913.3.6.635.
4. Atkinson TP, Waites KB. 2014. *Mycoplasma pneumoniae* infections in childhood. Pediatr Infect Dis J 33:92–94. https://doi.org/10.1097/INF.0000000000000171.
5. Jacobs E, Ehrhardt I, Dumke R. 2015. New insights in the outbreak

pattern of *Mycoplasma pneumoniae*. Int J Med Microbiol 305:705–708. https://doi.org/10.1016/j.ijmm.2015.08.021.
6. Loens K, Goossens H, Ieven M. 2010. Acute respiratory infection due to *Mycoplasma pneumoniae:* current status of diagnostic methods. Eur J Clin Microbiol Infect Dis 29:1055–1069. https://doi.org/10.1007/s10096-010-0975-2.
7. Marston BJ, Plouffe JF, File TM, Jr, Hackman BA, Salstrom SJ, Lipman HB, Kolczak MS, Breiman RF. 1997. Incidence of community-acquired pneumonia requiring hospitalization. Results of a population-based active surveillance study in Ohio. The Community-Based Pneumonia Incidence Study Group. Arch Intern Med 157:1709–1718.
8. Winchell JM. 2013. *Mycoplasma pneumoniae*—a national public health perspective. Curr Pediatr Rev 9:324–333. https://doi.org/10.2174/15733963113099990009.
9. Dorigo-Zetsma JW, Verkooyen RP, van Helden HP, van der Nat H, van den Bosch JM. 2001. Molecular detection of *Mycoplasma pneumoniae* in adults with community-acquired pneumonia requiring hospitalization. J Clin Microbiol 39:1184–1186. https://doi.org/10.1128/JCM.39.3.1184-1186.2001.
10. Miyashita N, Ouchi K, Kawasaki K, Oda K, Kawai Y, Shimizu H, Kobashi Y, Oka M. 2008. *Mycoplasma pneumoniae* pneumonia in the elderly. Med Sci Monit 14:CR387–C391.
11. Gadsby NJ, Reynolds AJ, McMenamin J, Gunson RN, McDonagh S, Molyneaux PJ, Yirrell DL, Templeton KE. 2012. Increased reports of *Mycoplasma pneumoniae* from laboratories in Scotland in 2010 and 2011—impact of the epidemic in infants. Euro Surveill 17(10):pii=20110. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20110.
12. Onozuka D, Hashizume M, Hagihara A. 2009. Impact of weather factors on *Mycoplasma pneumoniae* pneumonia. Thorax 64:507–511. https://doi.org/10.1136/thx.2008.111237.
13. Onozuka D, Chaves LF. 2014. Climate variability and nonstationary dynamics of *Mycoplasma pneumoniae* pneumonia in Japan. PLoS One 9:e95447. https://doi.org/10.1371/journal.pone.0095447.
14. Lenglet A, Herrador Z, Magiorakos AP, Leitmeyer K, Coulombier D, European Working Group on Mycoplasma pneumoniae Surveillance. 2012. Surveillance status and recent data for *Mycoplasma pneumoniae* infections in the European Union and European Economic Area, January 2012. Euro Surveill 17(5):pii=20075. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20075.
15. Uldum SA, Bangsborg JM, Gahrn-Hansen B, Ljung R, Molvadgaard M, Fons Petersen R, Wiid Svarrer C. 2012. Epidemic of *Mycoplasma pneumoniae* infection in Denmark, 2010 and 2011. Euro Surveill 17(5):pii=20073. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20073.
16. Blystad H, Anestad G, Vestrheim DF, Madsen S, Ronning K. 2012. Increased incidence of *Mycoplasma pneumoniae* infection in Norway 2011. Euro Surveill 17(5):pii=20074. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20074.
17. Linde A, Ternhag A, Torner A, Claesson B. 2012. Antibiotic prescriptions and laboratory-confirmed cases of *Mycoplasma pneumoniae* during the epidemic in Sweden in 2011. Euro Surveill 17(6):pii=20082. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20082.
18. Chalker V, Stocki T, Mentasti M, Fleming D, Harrison T. 2011. Increased incidence of *Mycoplasma pneumoniae* infection in England and Wales in 2010: multilocus variable number tandem repeat analysis typing and macrolide susceptibility. Euro Surveill 16(19):pii=19865. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19865.
19. Eibach D, Casalegno JS, Escuret V, Billaud G, Mekki Y, Frobert E, Bouscambert-Duchamp M, Lina B, Morfin F. 2012. Increased detection of *Mycoplasma pneumoniae* infection in children, Lyon, France, 2010 to 2011. Euro Surveill 17(8):pii=20094. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20094.
20. Polkowska A, Harjunpaa A, Toikkanen S, Lappalainen M, Vuento R, Vuorinen T, Kauppinen J, Flinck H, Lyytikainen O. 2012. Increased incidence of *Mycoplasma pneumoniae* infection in Finland, 2010–2011. Euro Surveill 17(5):pii=20072. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20072.
21. Reinton N, Manley L, Tjade T, Moghaddam A. 2013. Respiratory tract infections during the 2011 *Mycoplasma pneumoniae* epidemic. Eur J Clin Microbiol Infect Dis 32:835–840. https://doi.org/10.1007/s10096-013-1818-8.
22. Brown RJ, Nguipdop-Djomo P, Zhao H, Stanford E, Spiller OB, Chalker VJ. 2016. *Mycoplasma pneumoniae* epidemiology in England and Wales: a national perspective. Front Microbiol 7:157. https://doi.org/10.3389/fmicb.2016.00157.
23. Ferguson GD, Gadsby NJ, Henderson SS, Hardie A, Kalima P, Morris AC, Hill AT, Cunningham S, Templeton KE. 2013. Clinical outcomes and macrolide resistance in *Mycoplasma pneumoniae* infection in Scotland, UK. J Med Microbiol 62:1876–1882. https://doi.org/10.1099/jmm.0.066191-0.
24. Pereyre S, Charron A, Hidalgo-Grass C, Touati A, Moses AE, Nir-Paz R, Bebear C. 2012. The spread of *Mycoplasma pneumoniae* is polyclonal in both an endemic setting in France and in an epidemic setting in Israel. PLoS One 7:e38585. https://doi.org/10.1371/journal.pone.0038585.
25. Okada T, Morozumi M, Tajima T, Hasegawa M, Sakata H, Ohnari S, Chiba N, Iwata S, Ubukata K. 2012. Rapid effectiveness of minocycline or doxycycline against macrolide-resistant *Mycoplasma pneumoniae* infection in a 2011 outbreak among Japanese children. Clin Infect Dis 55:1642–1649. https://doi.org/10.1093/cid/cis784.
26. Kim EK, Youn YS, Rhim JW, Shin MS, Kang JH, Lee KY. 2015. Epidemiological comparison of three *Mycoplasma pneumoniae* pneumonia epidemics in a single hospital over 10 years. Korean J Pediatr 58:172–177. https://doi.org/10.3345/kjp.2015.58.5.172.
27. Qu J, Yu X, Liu Y, Yin Y, Gu L, Cao B, Wang C. 2013. Specific multilocus variable-number tandem-repeat analysis genotypes of *Mycoplasma pneumoniae* are associated with diseases severity and macrolide susceptibility. PLoS One 8:e82174. https://doi.org/10.1371/journal.pone.0082174.
28. Nir-Paz R, Abutbul A, Moses AE, Block C, Hidalgo-Grass C. 2012. Ongoing epidemic of *Mycoplasma pneumoniae* infection in Jerusalem, Israel, 2010 to 2012. Euro Surveill 17(8):pii=20095. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20095.
29. Martinez MA, Ruiz M, Zunino E, Luchsinger V, Aguirre R, Avendano LF. 2010. Identification of P1 types and variants of *Mycoplasma pneumoniae* during an epidemic in Chile. J Med Microbiol 59:925–929. https://doi.org/10.1099/jmm.0.018333-0.
30. Jiang W, Yan Y, Ji W, Wang Y, Chen Z. 2014. Clinical significance of different bacterial load of *Mycoplasma pneumoniae* in patients with Mycoplasma pneumoniae pneumonia. Braz J Infect Dis 18:124–128. https://doi.org/10.1016/j.bjid.2013.06.004.
31. Chalker V, Stocki T, Litt D, Bermingham A, Watson J, Fleming D, Harrison T. 2012. Increased detection of *Mycoplasma pneumoniae* infection in children in England and Wales, October 2011 to January 2012. Euro Surveill 17(6):pii=20081. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20081.
32. Spuesens EB, Oduber M, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C. 2009. Sequence variations in RepMP2/3 and RepMP4 elements reveal intragenomic homologous DNA recombination events in *Mycoplasma pneumoniae*. Microbiology 155:2182–2196. https://doi.org/10.1099/mic.0.028506-0.
33. Rocha EP, Blanchard A. 2002. Genomic repeats, genome plasticity and the dynamics of Mycoplasma evolution. Nucleic Acids Res 30:2031–2042. https://doi.org/10.1093/nar/30.9.2031.
34. Dorigo-Zetsma JW, Wilbrink B, Dankert J, Zaat SA. 2001. *Mycoplasma pneumoniae* P1 type 1- and type 2-specific sequences within the P1 cytadhesin gene of individual strains. Infect Immun 69:5612–5618. https://doi.org/10.1128/IAI.69.9.5612-5618.2001.
35. Pereyre S, Charron A, Renaudin H, Bebear C, Bebear CM. 2007. First report of macrolide-resistant strains and description of a novel nucleotide sequence variation in the P1 adhesin gene in *Mycoplasma pneumoniae* clinical strains isolated in France over 12 years. J Clin Microbiol 45:3534–3539. https://doi.org/10.1128/JCM.01345-07.
36. Kenri T, Taniguchi R, Sasaki Y, Okazaki N, Narita M, Izumikawa K, Umetsu M, Sasaki T. 1999. Identification of a new variable sequence in the P1 cytadhesin gene of *Mycoplasma pneumoniae:* evidence for the generation of antigenic variation by DNA recombination between repetitive sequences. Infect Immun 67:4557–4562.
37. Ruland K, Wenzel R, Herrmann R. 1990. Analysis of three different repeated DNA elements present in the P1 operon of *Mycoplasma pneumoniae*: size, number and distribution on the genome. Nucleic Acids Res 18:6311–6317. https://doi.org/10.1093/nar/18.21.6311.
38. Xiao J, Liu Y, Wang M, Jiang C, You X, Zhu C. 2014. Detection of *Mycoplasma pneumoniae* P1 subtype variations by denaturing gradient gel electrophoresis. Diagn Microbiol Infect Dis 78:24–28. https://doi.org/10.1016/j.diagmicrobio.2013.08.008.
39. Musatovova O, Kannan TR, Baseman JB. 2012. *Mycoplasma pneumoniae* large DNA repetitive elements RepMP1 show type specific organization

among strains. PLoS One 7:e47625. https://doi.org/10.1371/journal.pone.0047625.
40. Kenri T, Ohya H, Horino A, Shibayama K. 2012. Identification of *Mycoplasma pneumoniae* type 2b variant strains in Japan. J Med Microbiol 61:1633–1635. https://doi.org/10.1099/jmm.0.046441-0.
41. Schwartz SB, Mitchell SL, Thurman KA, Wolff BJ, Winchell JM. 2009. Identification of P1 variants of *Mycoplasma pneumoniae* by use of high-resolution melt analysis. J Clin Microbiol 47:4117–4120. https://doi.org/10.1128/JCM.01696-09.
42. Schwartz SB, Thurman KA, Mitchell SL, Wolff BJ, Winchell JM. 2009. Genotyping of *Mycoplasma pneumoniae* isolates using real-time PCR and high-resolution melt analysis. Clin Microbiol Infect 15:756–762. https://doi.org/10.1111/j.1469-0691.2009.02814.x.
43. Dumke R, Catrein I, Herrmann R, Jacobs E. 2004. Preference, adaptation and survival of *Mycoplasma pneumoniae* subtypes in an animal model. Int J Med Microbiol 294:149–155. https://doi.org/10.1016/j.ijmm.2004.06.020.
44. Lind K, Benzon MW, Jensen JS, Clyde WA, Jr. 1997. A seroepidemiological study of *Mycoplasma pneumoniae* infections in Denmark over the 50-year period 1946-1995. Eur J Epidemiol 13:581–586. https://doi.org/10.1023/A:1007353121693.
45. Kenri T, Okazaki N, Yamazaki T, Narita M, Izumikawa K, Matsuoka M, Suzuki S, Horino A, Sasaki T. 2008. Genotyping analysis of *Mycoplasma pneumoniae* clinical strains in Japan between 1995 and 2005: type shift phenomenon of *M. pneumoniae* clinical strains. J Med Microbiol 57:469–475. https://doi.org/10.1099/jmm.0.47634-0.
46. Zhao F, Liu L, Tao X, He L, Meng F, Zhang J. 2015. Culture-independent detection and genotyping of *Mycoplasma pneumoniae* in clinical specimens from Beijing, China. PLoS One 10:e0141702. https://doi.org/10.1371/journal.pone.0141702.
47. Diaz MH, Benitez AJ, Winchell JM. 2015. Investigations of *Mycoplasma pneumoniae* Infections in the United States: trends in molecular typing and macrolide resistance from 2006 to 2013. J Clin Microbiol 53:124–130. https://doi.org/10.1128/JCM.02597-14.
48. Nilsson AC, Bjorkman P, Welinder-Olsson C, Widell A, Persson K. 2010. Clinical severity of *Mycoplasma pneumoniae* (MP) infection is associated with bacterial load in oropharyngeal secretions but not with MP genotype. BMC Infect Dis 10:39. https://doi.org/10.1186/1471-2334-10-39.
49. Pereyre S, Renaudin H, Charron A, Bebear C. 2012. Clonal spread of *Mycoplasma pneumoniae* in primary school, Bordeaux, France. Emerg Infect Dis 18:343–345. https://doi.org/10.3201/eid1802.111379.
50. Chironna M, Loconsole D, De Robertis AL, Morea A, Scalini E, Quarto M, Tafuri S, Germinario C, Manzionna M. 2016. Clonal spread of a unique strain of macrolide-resistant *Mycoplasma pneumoniae* within a single family in Italy. Medicine (Baltimore, MD) 95:e3160. https://doi.org/10.1097/MD.0000000000003160.
51. Song JH, Oh WS, Kang CI, Chung DR, Peck KR, Ko KS, Yeom JS, Kim CK, Kim SW, Chang HH, Kim YS, Jung SI, Tong Z, Wang Q, Huang SG, Liu JW, Lalitha MK, Tan BH, Van PH, Carlos CC, So T, Asian Network for Surveillance of Resistant Pathogens Study Group. 2008. Epidemiology and clinical outcomes of community-acquired pneumonia in adult patients in Asian countries: a prospective study by the Asian network for surveillance of resistant pathogens. Int J Antimicrob Agents 31:107–114. https://doi.org/10.1016/j.ijantimicag.2007.09.014.
52. Lui G, Ip M, Lee N, Rainer TH, Man SY, Cockram CS, Antonio GE, Ng MH, Chan MH, Chau SS, Mak P, Chan PK, Ahuja AT, Sung JJ, Hui DS. 2009. Role of 'atypical pathogens' among adult hospitalized patients with community-acquired pneumonia. Respirology 14:1098–1105. https://doi.org/10.1111/j.1440-1843.2009.01637.x.
53. Deng J, Zheng Y, Zhao R, Wright PF, Stratton CW, Tang YW. 2009. Culture versus polymerase chain reaction for the etiologic diagnosis of community-acquired pneumonia in antibiotic-pretreated pediatric patients. Pediatr Infect Dis J 28:53–55. https://doi.org/10.1097/INF.0b013e3181817bd2.
54. Samransamruajkit R, Jitchaiwat S, Wachirapaes W, Deerojanawong J, Sritippayawan S, Prapphal N. 2008. Prevalence of *Mycoplasma* and *Chlamydia* pneumonia in severe community-acquired pneumonia among hospitalized children in Thailand. Jpn J Infect Dis 61:36–39.
55. Cao B, Ren LL, Zhao F, Gonzalez R, Song SF, Bai L, Yin YD, Zhang YY, Liu YM, Guo P, Zhang JZ, Wang JW, Wang C. 2010. Viral and *Mycoplasma pneumoniae* community-acquired pneumonia and novel clinical outcome evaluation in ambulatory adult patients in China. Eur J Clin Microbiol Infect Dis 29:1443–1448. https://doi.org/10.1007/s10096-010-1003-2.
56. von Baum H, Welte T, Marre R, Suttorp N, Luck C, Ewig S. 2009. *Mycoplasma pneumoniae* pneumonia revisited within the German Competence Network for Community-Acquired Pneumonia (CAPNETZ). BMC Infect Dis 9:62. https://doi.org/10.1186/1471-2334-9-62.
57. Dumke R, Schnee C, Pletz MW, Rupp J, Jacobs E, Sachse K, Rohde G, Capnetz Study G. 2015. *Mycoplasma pneumoniae* and *Chlamydia* spp. infection in community-acquired pneumonia, Germany, 2011-2012. Emerg Infect Dis 21:426–434. https://doi.org/10.3201/eid2103.140927.
58. Oldach D, Metev H, Mykietiuk A, Tanaseanu C, Das A, Jamieson B, Clark K, Sheets A, Keedy K, Waites KB, Fernandes P. 2015. Characterization of *Mycoplasma pneumoniae* infection and outcomes in the SOLITAIRE-oral, global phase 3 clinical trial for solithromycin, abstr 893. Abstr Infect Dis Soc Am, San Diego, CA.
59. Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM, Reed C, Grijalva CG, Anderson EJ, Courtney DM, Chappell JD, Qi C, Hart EM, Carroll F, Trabue C, Donnelly HK, Williams DJ, Zhu Y, Arnold SR, Ampofo K, Waterer GW, Levine M, Lindstrom S, Winchell JM, Katz JM, Erdman D, Schneider E, Hicks LA, McCullers JA, Pavia AT, Edwards KM, Finelli L, CDC EPIC Study Team. 2015. Community-acquired pneumonia requiring hospitalization among U.S. adults. N Engl J Med 373:415–427. https://doi.org/10.1056/NEJMoa1500245.
60. Jain S, Williams DJ, Arnold SR, Ampofo K, Bramley AM, Reed C, Stockmann C, Anderson EJ, Grijalva CG, Self WH, Zhu Y, Patel A, Hymas W, Chappell JD, Kaufman RA, Kan JH, Dansie D, Lenny N, Hillyard DR, Haynes LM, Levine M, Lindstrom S, Winchell JM, Katz JM, Erdman D, Schneider E, Hicks LA, Wunderink RG, Edwards KM, Pavia AT, McCullers JA, Finelli L, CDC EPIC Study Team. 2015. Community-acquired pneumonia requiring hospitalization among U.S. children. N Engl J Med 372:835–845. https://doi.org/10.1056/NEJMoa1405870.
61. Diaz MH, Cross KE, Benitez AJ, Hicks LA, Kutty P, Bramley AM, Chappell JD, Hymas W, Patel A, Qi C, Williams DJ, Arnold SR, Ampofo K, Self WH, Grijalva CG, Anderson EJ, McCullers JA, Pavia AT, Wunderink RG, Edwards KM, Jain S, Winchell JM. 2016. Identification of bacterial and viral codetections with *Mycoplasma pneumoniae* using the TaqMan array card in patients hospitalized with community-acquired pneumonia. Open Forum Infect Dis 3:ofw071. https://doi.org/10.1093/ofid/ofw071.
62. Zheng X, Lee S, Selvarangan R, Qin X, Tang YW, Stiles J, Hong T, Todd K, Ratliff AE, Crabb DM, Xiao L, Atkinson TP, Waites KB. 2015. Macrolide-resistant *Mycoplasma pneumoniae*, United States. Emerg Infect Dis 21:1470–1472. https://doi.org/10.3201/eid2108.150273.
63. Chiu CY, Chen CJ, Wong KS, Tsai MH, Chiu CH, Huang YC. 2015. Impact of bacterial and viral coinfection on mycoplasmal pneumonia in childhood community-acquired pneumonia. J Microbiol Immunol Infect 48:51–56. https://doi.org/10.1016/j.jmii.2013.06.006.
64. Mandell LA. 2015. Community-acquired pneumonia: an overview. Postgrad Med 127:607–615. https://doi.org/10.1080/00325481.2015.1074030.
65. Hastings DL, Harrington KJ, Kutty PK, Rayman RJ, Spindola D, Diaz MH, Thurman KA, Winchell JM, Safranek TJ, Centers for Disease Control and Prevention. 2015. *Mycoplasma pneumoniae* outbreak in a long-term care facility—Nebraska, 2014. MMWR Morb Mortal Wkly Rep 64:296–299.
66. Waller JL, Diaz MH, Petrone BL, Benitez AJ, Wolff BJ, Edison L, Tobin-D'Angelo M, Moore A, Martyn A, Dishman H, Drenzek CL, Turner K, Hicks LA, Winchell JM. 2014. Detection and characterization of *Mycoplasma pneumoniae* during an outbreak of respiratory illness at a university. J Clin Microbiol 52:849–853. https://doi.org/10.1128/JCM.02810-13.
67. Olson D, Watkins LK, Demirjian A, Lin X, Robinson CC, Pretty K, Benitez AJ, Winchell JM, Diaz MH, Miller LA, Foo TA, Mason MD, Lauper UL, Kupfer O, Kennedy J, Glode MP, Kutty PK, Dominguez SR. 2015. Outbreak of *Mycoplasma pneumoniae*-associated Stevens-Johnson syndrome. Pediatrics 136:e386–e394. https://doi.org/10.1542/peds.2015-0278.
68. Tsai V, Pritzker BB, Diaz MH, Winchell JM, Hicks LA, Petrone B, Benitez A, Wolff BJ, Soyemi KL. 2013. Cluster of macrolide-resistant *Mycoplasma pneumoniae* infections in Illinois in 2012. J Clin Microbiol 51:3889–3892. https://doi.org/10.1128/JCM.01613-13.
69. Rhea SK, Cox SW, Moore ZS, Mays ER, Benitez AJ, Diaz MH, Winchell JM, Centers for Disease Control and Prevention. 2014. Notes from the field: atypical pneumonia in three members of an extended family—South Carolina and North Carolina, July-August 2013. MMWR Morb Mortal Wkly Rep 63:734–735.
70. Walter ND, Grant GB, Bandy U, Alexander NE, Winchell JM, Jordan HT,

Sejvar JJ, Hicks LA, Gifford DR, Alexander NT, Thurman KA, Schwartz SB, Dennehy PH, Khetsuriani N, Fields BS, Dillon MT, Erdman DD, Whitney CG, Moore MR. 2008. Community outbreak of *Mycoplasma pneumoniae* infection: school-based cluster of neurologic disease associated with household transmission of respiratory illness. J Infect Dis 198: 1365–1374. https://doi.org/10.1086/592281.

71. Ralston D, Cochran B. 1979. A college epidemic of *Mycoplasma pneumoniae*. J Am Coll Health Assoc 27: 264:266.
72. Diaz MH, Benitez AJ, Cross KE, Hicks LA, Kutty P, Bramley AM, Chappell JD, Hymas W, Patel A, Qi C, Williams DJ, Arnold SR, Ampofo K, Self WH, Grijalva CG, Anderson EJ, McCullers JA, Pavia AT, Wunderink RG, Edwards KM, Jain S, Winchell JM. 2015. Molecular detection and characterization of *Mycoplasma pneumoniae* among patients hospitalized with community-acquired pneumonia in the United States. Open Forum Infect Dis 2:ofv106. https://doi.org/10.1093/ofid/ofv106.
73. Sliman JA, Metzgar D, Asseff DC, Coon RG, Faix DJ, Lizewski S. 2009. Outbreak of acute respiratory disease caused by *Mycoplasma pneumoniae* on board a deployed U.S. navy ship. J Clin Microbiol 47: 4121–4123. https://doi.org/10.1128/JCM.01926-09.
74. Esposito S, Blasi F, Bosis S, Droghetti R, Faelli N, Lastrico A, Principi N. 2004. Aetiology of acute pharyngitis: the role of atypical bacteria. J Med Microbiol 53:645–651. https://doi.org/10.1099/jmm.0.05487-0.
75. Esposito S, Bosis S, Begliatti E, Droghetti R, Tremolati E, Tagliabue C, Bellasio M, Blasi F, Principi N. 2006. Acute tonsillopharyngitis associated with atypical bacterial infection in children: natural history and impact of macrolide therapy. Clin Infect Dis 43:206–209. https://doi.org/10.1086/505120.
76. Centor RM, Atkinson TP, Ratliff AE, Xiao L, Crabb DM, Estrada CA, Faircloth MB, Oestreich L, Hatchett J, Khalife W, Waites KB. 2015. The clinical presentation of *Fusobacterium*-positive and streptococcal-positive pharyngitis in a university health clinic: a cross-sectional study. Ann Intern Med 162:241–247. https://doi.org/10.7326/M14-1305.
77. Piacentini GL, Peroni DG, Blasi F, Pescollderungg L, Goller P, Gallmetzer L, Drago L, Bodini A, Boner AL. 2010. Atypical bacteria in adenoids and tonsils of children requiring adenotonsillectomy. Acta Otolaryngol 130: 620–625. https://doi.org/10.3109/00016480903359921.
78. Waites KB, Atkinson TP. 2009. The role of Mycoplasma in upper respiratory infections. Curr Infect Dis Rep 11:198–206. https://doi.org/10.1007/s11908-009-0030-6.
79. Pandak N, Pajic-Penavic I, Zidovec-Lepej S, Planinic A, Troselj-Vukic B, Peric L. 2014. *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* were not identified in sinus mucosa of patients with chronic rhinosinusitis. Eur Arch Otorhinolaryngol 271:1553–1555. https://doi.org/10.1007/s00405-013-2745-0.
80. Spuesens EB, Fraaij PL, Visser EG, Hoogenboezem T, Hop WC, van Adrichem LN, Weber F, Moll HA, Broekman B, Berger MY, van Rijsoort-Vos T, van Belkum A, Schutten M, Pas SD, Osterhaus AD, Hartwig NG, Vink C, van Rossum AM. 2013. Carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of symptomatic and asymptomatic children: an observational study. PLoS Med 10:e1001444. https://doi.org/10.1371/journal.pmed.1001444.
81. Wood PR, Hill VL, Burks ML, Peters JI, Singh H, Kannan TR, Vale S, Cagle MP, Principe MF, Baseman JB, Brooks EG. 2013. *Mycoplasma pneumoniae* in children with acute and refractory asthma. Ann Allergy Asthma Immunol 110:328–334.e1. https://doi.org/10.1016/j.anai.2013.01.022.
82. Palma SC, Martinez TM, Salinas SM, Rojas GP. 2005. Asymptomatic pharyngeal carriage of *Mycoplasma pneumoniae* in Chilean children. Rev Chilena Infectol 22:247–250. https://doi.org/10.4067/S0716-10182005000300005.
83. Loens K, Ieven M. 2016. *Mycoplasma pneumoniae:* current knowledge on nucleic acid amplification techniques and serological diagnostics. Front Microbiol 7:448. https://doi.org/10.3389/fmicb.2016.00448.
84. Simecka JW. 2013. What have we learned from animal models of *Mycoplasma pneumoniae* disease: virulence mechanisms and host responses. Curr Pediatr Rev 9:314–323. https://doi.org/10.2174/15733963090413122311065 5.
85. Chu HW, Breed R, Rino JG, Harbeck RJ, Sills MR, Martin RJ. 2006. Repeated respiratory *Mycoplasma pneumoniae* infections in mice: effect of host genetic background. Microbes Infect 8:1764–1772. https://doi.org/10.1016/j.micinf.2006.02.014.
86. Yancey AL, Watson HL, Cartner SC, Simecka JW. 2001. Gender is a major factor in determining the severity of mycoplasma respiratory disease in mice. Infect Immun 69:2865–2871. https://doi.org/10.1128/IAI.69.5.2865-2871.2001.
87. Guo L, Liu F, Lu MP, Zheng Q, Chen ZM. 2015. Increased T cell activation in BALF from children with *Mycoplasma pneumoniae* pneumonia. Pediatr Pulmonol 50:814–819. https://doi.org/10.1002/ppul.23095.
88. Krause DC, Leith DK, Wilson RM, Baseman JB. 1982. Identification of *Mycoplasma pneumoniae* proteins associated with hemadsorption and virulence. Infect Immun 35:809–817.
89. Loveless RW, Feizi T. 1989. Sialo-oligosaccharide receptors for *Mycoplasma pneumoniae* and related oligosaccharides of poly-N-acetyllactosamine series are polarized at the cilia and apical-microvillar domains of the ciliated cells in human bronchial epithelium. Infect Immun 57:1285–1289.
90. Krivan HC, Olson LD, Barile MF, Ginsburg V, Roberts DD. 1989. Adhesion of *Mycoplasma pneumoniae* to sulfated glycolipids and inhibition by dextran sulfate. J Biol Chem 264:9283–9288.
91. Bredt W. 1968. Motility and multiplication of *Mycoplasma pneumoniae*. A phase contrast study. Pathol Microbiol (Basel) 32:321–326.
92. Hasselbring BM, Page CA, Sheppard ES, Krause DC. 2006. Transposon mutagenesis identifies genes associated with *Mycoplasma pneumoniae* gliding motility. J Bacteriol 188:6335–6345. https://doi.org/10.1128/JB.00698-06.
93. Prince OA, Krunkosky TM, Krause DC. 2014. In vitro spatial and temporal analysis of *Mycoplasma pneumoniae* colonization of human airway epithelium. Infect Immun 82:579–586. https://doi.org/10.1128/IAI.01036-13.
94. Miyata M. 2010. Unique centipede mechanism of Mycoplasma gliding. Annu Rev Microbiol 64:519–537. https://doi.org/10.1146/annurev.micro.112408.134116.
95. Szczepanek SM, Majumder S, Sheppard ES, Liao X, Rood D, Tulman ER, Wyand S, Krause DC, Silbart LK, Geary SJ. 2012. Vaccination of BALB/c mice with an avirulent *Mycoplasma pneumoniae* P30 mutant results in disease exacerbation upon challenge with a virulent strain. Infect Immun 80:1007–1014. https://doi.org/10.1128/IAI.06078-11.
96. Krunkosky TM, Jordan JL, Chambers E, Krause DC. 2007. *Mycoplasma pneumoniae* host-pathogen studies in an air-liquid culture of differentiated human airway epithelial cells. Microb Pathog 42:98–103. https://doi.org/10.1016/j.micpath.2006.11.003.
97. Hao Y, Kuang Z, Jing J, Miao J, Mei LY, Lee RJ, Kim S, Choe S, Krause DC, Lau GW. 2014. *Mycoplasma pneumoniae* modulates STAT3-STAT6/EGFR-FOXA2 signaling to induce overexpression of airway mucins. Infect Immun 82:5246–5255. https://doi.org/10.1128/IAI.01989-14.
98. Kornspan JD, Tarshis M, Rottem S. 2011. Adhesion and biofilm formation of *Mycoplasma pneumoniae* on an abiotic surface. Arch Microbiol 193:833–836. https://doi.org/10.1007/s00203-011-0749-y.
99. Simmons WL, Daubenspeck JM, Osborne JD, Balish MF, Waites KB, Dybvig K. 2013. Type 1 and type 2 strains of *Mycoplasma pneumoniae* form different biofilms. Microbiology 159:737–747. https://doi.org/10.1099/mic.0.064782-0.
100. Balish MF. 2014. *Mycoplasma pneumoniae*, an underutilized model for bacterial cell biology. J Bacteriol 196:3675–3682. https://doi.org/10.1128/JB.01865-14.
101. Seto S, Layh-Schmitt G, Kenri T, Miyata M. 2001. Visualization of the attachment organelle and cytadherence proteins of *Mycoplasma pneumoniae* by immunofluorescence microscopy. J Bacteriol 183: 1621–1630. https://doi.org/10.1128/JB.183.5.1621-1630.2001.
102. Schmidl SR, Gronau K, Pietack N, Hecker M, Becher D, Stulke J. 2010. The phosphoproteome of the minimal bacterium *Mycoplasma pneumoniae:* analysis of the complete known Ser/Thr kinome suggests the existence of novel kinases. Mol Cell Proteomics 9:1228–1242. https://doi.org/10.1074/mcp.M900267-MCP200.
103. Daubenspeck JM, Liu R, Dybvig K. 2016. Rhamnose links moonlighting proteins to membrane phospholipid in mycoplasmas. PLoS One 11: e0162505. https://doi.org/10.1371/journal.pone.0162505.
104. Krause DC, Baseman JB. 1983. Inhibition of *Mycoplasma pneumoniae* hemadsorption and adherence to respiratory epithelium by antibodies to a membrane protein. Infect Immun 39:1180–1186.
105. Baseman JB, Cole RM, Krause DC, Leith DK. 1982. Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. J Bacteriol 151:1514–1522.
106. Nakane D, Adan-Kubo J, Kenri T, Miyata M. 2011. Isolation and characterization of P1 adhesin, a leg protein of the gliding bacterium *Mycoplasma pneumoniae*. J Bacteriol 193:715–722. https://doi.org/10.1128/JB.00796-10.
107. Sperker B, Hu P, Herrmann R. 1991. Identification of gene products of the P1 operon of *Mycoplasma pneumoniae*. Mol Microbiol 5:299–306. https://doi.org/10.1111/j.1365-2958.1991.tb02110.x.

108. Layh-Schmitt G, Herrmann R. 1992. Localization and biochemical characterization of the ORF6 gene product of the *Mycoplasma pneumoniae* P1 operon. Infect Immun 60:2906–2913.
109. Waldo RH, III, Jordan JL, Krause DC. 2005. Identification and complementation of a mutation associated with loss of *Mycoplasma pneumoniae* virulence-specific proteins B and C. J Bacteriol 187:747–751. https://doi.org/10.1128/JB.187.2.747-751.2005.
110. Ruland K, Himmelreich R, Herrmann R. 1994. Sequence divergence in the ORF6 gene of *Mycoplasma pneumoniae*. J Bacteriol 176:5202–5209. https://doi.org/10.1128/jb.176.17.5202-5209.1994.
111. Xiao L, Ptacek T, Osborne JD, Crabb DM, Simmons WL, Lefkowitz EJ, Waites KB, Atkinson TP, Dybvig K. 2015. Comparative genome analysis of *Mycoplasma pneumoniae*. BMC Genomics 16:610. https://doi.org/10.1186/s12864-015-1801-0.
112. Spuesens EB, van de Kreeke N, Estevao S, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C. 2011. Variation in a surface-exposed region of the *Mycoplasma pneumoniae* P40 protein as a consequence of homologous DNA recombination between RepMP5 elements. Microbiology 157:473–483. https://doi.org/10.1099/mic.0.045591-0.
113. Himmelreich R, Hilbert H, Plagens H, Pirkl E, Li BC, Herrmann R. 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. Nucleic Acids Res 24:4420–4449. https://doi.org/10.1093/nar/24.22.4420.
114. Baseman JB, Morrison-Plummer J, Drouillard D, Puleo-Scheppke B, Tryon VV, Holt SC. 1987. Identification of a 32-kilodalton protein of *Mycoplasma pneumoniae* associated with hemadsorption. Isr J Med Sci 23:474–479.
115. Dallo SF, Lazzell AL, Chavoya A, Reddy SP, Baseman JB. 1996. Biofunctional domains of the *Mycoplasma pneumoniae* P30 adhesin. Infect Immun 64:2595–2601.
116. Chang HY, Jordan JL, Krause DC. 2011. Domain analysis of protein P30 in *Mycoplasma pneumoniae* cytadherence and gliding motility. J Bacteriol 193:1726–1733. https://doi.org/10.1128/JB.01228-10.
117. Chang HY, Prince OA, Sheppard ES, Krause DC. 2011. Processing is required for a fully functional protein P30 in *Mycoplasma pneumoniae* gliding and cytadherence. J Bacteriol 193:5841–5846. https://doi.org/10.1128/JB.00104-11.
118. Nakane D, Kenri T, Matsuo L, Miyata M. 2015. Systematic structural analyses of attachment organelle in *Mycoplasma pneumoniae*. PLoS Pathog 11:e1005299. https://doi.org/10.1371/journal.ppat.1005299.
119. Jordan JL, Berry KM, Balish MF, Krause DC. 2001. Stability and subcellular localization of cytadherence-associated protein P65 in *Mycoplasma pneumoniae*. J Bacteriol 183:7387–7391. https://doi.org/10.1128/JB.183.24.7387-7891.2001.
120. Hasselbring BM, Sheppard ES, Krause DC. 2012. P65 truncation impacts P30 dynamics during *Mycoplasma pneumoniae* gliding. J Bacteriol 194:3000–3007. https://doi.org/10.1128/JB.00091-12.
121. Cloward JM, Krause DC. 2009. *Mycoplasma pneumoniae* J-domain protein required for terminal organelle function. Mol Microbiol 71:1296–1307. https://doi.org/10.1111/j.1365-2958.2009.06602.x.
122. Kenri T, Seto S, Horino A, Sasaki Y, Sasaki T, Miyata M. 2004. Use of fluorescent-protein tagging to determine the subcellular localization of *Mycoplasma pneumoniae* proteins encoded by the cytadherence regulatory locus. J Bacteriol 186:6944–6955. https://doi.org/10.1128/JB.186.20.6944-6955.2004.
123. Jordan JL, Chang HY, Balish MF, Holt LS, Bose SR, Hasselbring BM, Waldo RH, III, Krunkosky TM, Krause DC. 2007. Protein P200 is dispensable for *Mycoplasma pneumoniae* hemadsorption but not gliding motility or colonization of differentiated bronchial epithelium. Infect Immun 75:518–522. https://doi.org/10.1128/IAI.01344-06.
124. Hasselbring BM, Krause DC. 2007. Cytoskeletal protein P41 is required to anchor the terminal organelle of the wall-less prokaryote *Mycoplasma pneumoniae*. Mol Microbiol 63:44–53. https://doi.org/10.1111/j.1365-2958.2006.05507.x.
125. Cloward JM, Krause DC. 2011. Loss of co-chaperone TopJ impacts adhesin P1 presentation and terminal organelle maturation in *Mycoplasma pneumoniae*. Mol Microbiol 81:528–539. https://doi.org/10.1111/j.1365-2958.2011.07712.x.
126. Cloward JM, Krause DC. 2010. Functional domain analysis of the *Mycoplasma pneumoniae* co-chaperone TopJ. Mol Microbiol 77:158–169. https://doi.org/10.1111/j.1365-2958.2010.07196.x.
127. Hasselbring BM, Krause DC. 2007. Proteins P24 and P41 function in the regulation of terminal-organelle development and gliding motility in *Mycoplasma pneumoniae*. J Bacteriol 189:7442–7449. https://doi.org/10.1128/JB.00867-07.
128. Hatchel JM, Balish MF. 2008. Attachment organelle ultrastructure correlates with phylogeny, not gliding motility properties, in *Mycoplasma pneumoniae* relatives. Microbiology 154:286–295. https://doi.org/10.1099/mic.0.2007/012765-0.
129. Hatchel JM, Balish RS, Duley ML, Balish MF. 2006. Ultrastructure and gliding motility of *Mycoplasma amphoriforme*, a possible human respiratory pathogen. Microbiology 152:2181–2189. https://doi.org/10.1099/mic.0.28905-0.
130. Yasutomi M, Okazaki S, Hata I, Tanizawa A, Tamamura S, Kawakita M, Ohshima Y. 11 December 2014. Cytokine profiles in *Mycoplasma pneumoniae* infection-associated hemophagocytic lymphohistiocytosis. J Microbiol Immunol Infect https://doi.org/10.1016/j.jmii.2014.11.015.
131. Martinelli L, Garcia-Morales L, Querol E, Pinol J, Fita I, Calisto BM. 2016. Structure-guided mutations in the terminal organelle protein MG491 cause major motility and morphologic alterations on *Mycoplasma genitalium*. PLoS Pathog 12:e1005533. https://doi.org/10.1371/journal.ppat.1005533.
132. Calisto BM, Broto A, Martinelli L, Querol E, Pinol J, Fita I. 2012. The EAGR box structure: a motif involved in mycoplasma motility. Mol Microbiol 86:382–393. https://doi.org/10.1111/j.1365-2958.2012.08200.x.
133. Balish MF, Hahn TW, Popham PL, Krause DC. 2001. Stability of *Mycoplasma pneumoniae* cytadherence-accessory protein HMW1 correlates with its association with the triton shell. J Bacteriol 183:3680–3688. https://doi.org/10.1128/JB.183.12.3680-3688.2001.
134. Henderson GP, Jensen GJ. 2006. Three-dimensional structure of *Mycoplasma pneumoniae*'s attachment organelle and a model for its role in gliding motility. Mol Microbiol 60:376–385. https://doi.org/10.1111/j.1365-2958.2006.05113.x.
135. Seybert A, Herrmann R, Frangakis AS. 2006. Structural analysis of *Mycoplasma pneumoniae* by cryo-electron tomography. J Struct Biol 156:342–354. https://doi.org/10.1016/j.jsb.2006.04.010.
136. Garcia-Morales L, Gonzalez-Gonzalez L, Querol E, Pinol J. 2016. A minimized motile machinery for *Mycoplasma genitalium*. Mol Microbiol 100:125–138. https://doi.org/10.1111/mmi.13305.
137. Jeffery CJ. 2014. An introduction to protein moonlighting. Biochem Soc Trans 42:1679–1683. https://doi.org/10.1042/BST20140226.
138. Dallo SF, Kannan TR, Blaylock MW, Baseman JB. 2002. Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in *Mycoplasma pneumoniae*. Mol Microbiol 46:1041–1051. https://doi.org/10.1046/j.1365-2958.2002.03207.x.
139. Balasubramanian S, Kannan TR, Baseman JB. 2008. The surface-exposed carboxyl region of *Mycoplasma pneumoniae* elongation factor Tu interacts with fibronectin. Infect Immun 76:3116–3123. https://doi.org/10.1128/IAI.00173-08.
140. Thomas C, Jacobs E, Dumke R. 2013. Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*. Microbiology 159:352–365. https://doi.org/10.1099/mic.0.061184-0.
141. Grundel A, Friedrich K, Pfeiffer M, Jacobs E, Dumke R. 2015. Subunits of the pyruvate dehydrogenase cluster of *Mycoplasma pneumoniae* are surface-displayed proteins that bind and activate human plasminogen. PLoS One 10:e0126600. https://doi.org/10.1371/journal.pone.0126600.
142. Grundel A, Pfeiffer M, Jacobs E, Dumke R. 2015. Network of surface-displayed glycolytic enzymes in *Mycoplasma pneumoniae* and their interactions with human plasminogen. Infect Immun 84:666–676. https://doi.org/10.1128/IAI.01071-15.
143. Dumke R, Hausner M, Jacobs E. 2011. Role of *Mycoplasma pneumoniae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mediating interactions with the human extracellular matrix. Microbiology 157:2328–2338. https://doi.org/10.1099/mic.0.048298-0.
144. Kannan TR, Provenzano D, Wright JR, Baseman JB. 2005. Identification and characterization of human surfactant protein A binding protein of *Mycoplasma pneumoniae*. Infect Immun 73:2828–2834. https://doi.org/10.1128/IAI.73.5.2828-2834.2005.
145. Kannan TR, Baseman JB. 2006. ADP-ribosylating and vacuolating cytotoxin of *Mycoplasma pneumoniae* represents unique virulence determinant among bacterial pathogens. Proc Natl Acad Sci U S A 103:6724–6729. https://doi.org/10.1073/pnas.0510644103.
146. Becker A, Kannan TR, Taylor AB, Pakhomova ON, Zhang Y, Somarajan SR, Galaleldeen A, Holloway SP, Baseman JB, Hart PJ. 2015. Structure of CARDS toxin, a unique ADP-ribosylating and vacuolating cytotoxin

from *Mycoplasma pneumoniae*. Proc Natl Acad Sci U S A 112:5165–5170. https://doi.org/10.1073/pnas.1420308112.
147. Hardy RD, Coalson JJ, Peters J, Chaparro A, Techasaensiri C, Cantwell AM, Kannan TR, Baseman JB, Dube PH. 2009. Analysis of pulmonary inflammation and function in the mouse and baboon after exposure to *Mycoplasma pneumoniae* CARDS toxin. PLoS One 4:e7562. https://doi.org/10.1371/journal.pone.0007562.
148. Medina JL, Coalson JJ, Brooks EG, Winter VT, Chaparro A, Principe MF, Kannan TR, Baseman JB, Dube PH. 2012. *Mycoplasma pneumoniae* CARDS toxin induces pulmonary eosinophilic and lymphocytic inflammation. Am J Respir Cell Mol Biol 46:815–822. https://doi.org/10.1165/rcmb.2011-0135OC.
149. Medina JL, Coalson JJ, Brooks EG, Le Saux CJ, Winter VT, Chaparro A, Principe MF, Solis L, Kannan TR, Baseman JB, Dube PH. 2014. *Mycoplasma pneumoniae* CARDS toxin exacerbates ovalbumin-induced asthma-like inflammation in BALB/c mice. PLoS One 9:e102613. https://doi.org/10.1371/journal.pone.0102613.
150. Techasaensiri C, Tagliabue C, Cagle M, Iranpour P, Katz K, Kannan TR, Coalson JJ, Baseman JB, Hardy RD. 2010. Variation in colonization, ADP-ribosylating and vacuolating cytotoxin, and pulmonary disease severity among Mycoplasma pneumoniae strains. Am J Respir Crit Care Med 182:797–804. https://doi.org/10.1164/rccm.201001-0080OC.
151. Johnson C, Kannan TR, Baseman JB. 2011. Cellular vacuoles induced by *Mycoplasma pneumoniae* CARDS toxin originate from Rab9-associated compartments. PLoS One 6:e22877. https://doi.org/10.1371/journal.pone.0022877.
152. Bose S, Segovia JA, Somarajan SR, Chang TH, Kannan TR, Baseman JB. 2014. ADP-ribosylation of NLRP3 by *Mycoplasma pneumoniae* CARDS toxin regulates inflammasome activity. mBio 5:e02186-14. https://doi.org/10.1128/mBio.02186-14.
153. Im H, Ammit AJ. 2014. The NLRP3 inflammasome: role in airway inflammation. Clin Exp Allergy 44:160–172. https://doi.org/10.1111/cea.12206.
154. Krishnan M, Kannan TR, Baseman JB. 2013. *Mycoplasma pneumoniae* CARDS toxin is internalized via clathrin-mediated endocytosis. PLoS One 8:e62706. https://doi.org/10.1371/journal.pone.0062706.
155. Kannan TR, Musatovova O, Balasubramanian S, Cagle M, Jordan JL, Krunkosky TM, Davis A, Hardy RD, Baseman JB. 2010. *Mycoplasma pneumoniae* community acquired respiratory distress syndrome toxin expression reveals growth phase and infection-dependent regulation. Mol Microbiol 76:1127–1141. https://doi.org/10.1111/j.1365-2958.2010.07092.x.
156. Somarajan SR, Al-Asadi F, Ramasamy K, Pandranki L, Baseman JB, Kannan TR. 2014. Annexin A2 mediates *Mycoplasma pneumoniae* community-acquired respiratory distress syndrome toxin binding to eukaryotic cells. mBio 5:e01497-14. https://doi.org/10.1128/mBio.01497-14.
157. Vilei EM, Frey J. 2001. Genetic and biochemical characterization of glycerol uptake in *Mycoplasma mycoides* subsp. mycoides SC: its impact on H(2)O(2) production and virulence. Clin Diagn Lab Immunol 8:85–92.
158. Pilo P, Vilei EM, Peterhans E, Bonvin-Klotz L, Stoffel MH, Dobbelaere D, Frey J. 2005. A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. mycoides small colony. J Bacteriol 187:6824–6831. https://doi.org/10.1128/JB.187.19.6824-6831.2005.
159. Hames C, Halbedel S, Hoppert M, Frey J, Stulke J. 2009. Glycerol metabolism is important for cytotoxicity of *Mycoplasma pneumoniae*. J Bacteriol 191:747–753. https://doi.org/10.1128/JB.01103-08.
160. Miles RJ, Taylor RR, Varsani H. 1991. Oxygen uptake and H_2O_2 production by fermentative *Mycoplasma* spp. J Med Microbiol 34:219–223. https://doi.org/10.1099/00222615-34-4-219.
161. Halbedel S, Hames C, Stulke J. 2007. Regulation of carbon metabolism in the mollicutes and its relation to virulence. J Mol Microbiol Biotechnol 12:147–154. https://doi.org/10.1159/000096470.
162. Grosshennig S, Schmidl SR, Schmeisky G, Busse J, Stulke J. 2013. Implication of glycerol and phospholipid transporters in *Mycoplasma pneumoniae* growth and virulence. Infect Immun 81:896–904. https://doi.org/10.1128/IAI.01212-12.
163. Maenpuen S, Watthaisong P, Supon P, Sucharitakul J, Parsonage D, Karplus PA, Claiborne A, Chaiyen P. 2015. Kinetic mechanism of L-alpha-glycerophosphate oxidase from *Mycoplasma pneumoniae*. FEBS J 282:3043–3059. https://doi.org/10.1111/febs.13247.
164. Kannan TR, Baseman JB. 2000. Hemolytic and hemoxidative activities in *Mycoplasma penetrans*. Infect Immun 68:6419–6422. https://doi.org/10.1128/IAI.68.11.6419-6422.2000.
165. Grosshennig S, Ischebeck T, Gibhardt J, Busse J, Feussner I, Stulke J. 2016. Hydrogen sulfide is a novel potential virulence factor of *Mycoplasma pneumoniae*: characterization of the unusual cysteine desulfurase/desulfhydrase HapE. Mol Microbiol 100:42–54. https://doi.org/10.1111/mmi.13300.
166. Dhandayuthapani S, Blaylock MW, Bebear CM, Rasmussen WG, Baseman JB. 2001. Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in *Mycoplasma genitalium*. J Bacteriol 183:5645–5650. https://doi.org/10.1128/JB.183.19.5645-5650.2001.
167. Saikolappan S, Sasindran SJ, Yu HD, Baseman JB, Dhandayuthapani S. 2009. The *Mycoplasma genitalium* MG_454 gene product resists killing by organic hydroperoxides. J Bacteriol 191:6675–6682. https://doi.org/10.1128/JB.01066-08.
168. Zhang W, Baseman JB. 2014. Functional characterization of osmotically inducible protein C (MG_427) from *Mycoplasma genitalium*. J Bacteriol 196:1012–1019. https://doi.org/10.1128/JB.00954-13.
169. Pritchard RE, Prassinos AJ, Osborne JD, Raviv Z, Balish MF. 2014. Reduction of hydrogen peroxide accumulation and toxicity by a catalase from *Mycoplasma iowae*. PLoS One 9:e105188. https://doi.org/10.1371/journal.pone.0105188.
170. Pritchard RE, Balish MF. 2015. *Mycoplasma iowae:* relationships among oxygen, virulence, and protection from oxidative stress. Vet Res 46:36. https://doi.org/10.1186/s13567-015-0170-7.
171. Somarajan SR, Kannan TR, Baseman JB. 2010. *Mycoplasma pneumoniae* Mpn133 is a cytotoxic nuclease with a glutamic acid-, lysine- and serine-rich region essential for binding and internalization but not enzymatic activity. Cell Microbiol 12:1821–1831. https://doi.org/10.1111/j.1462-5822.2010.01513.x.
172. Yamamoto T, Kida Y, Sakamoto Y, Kuwano K. 2016. Mpn491, a secreted nuclease of *Mycoplasma pneumoniae*, plays a critical role in evading killing by neutrophil extracellular traps. Cell Microbiol https://doi.org/10.1111/cmi.12666.
173. Taylor-Robinson D, Webster AD, Furr PM, Asherson GL. 1980. Prolonged persistence of *Mycoplasma pneumoniae* in a patient with hypogammaglobulinaemia. J Infect 2:171–175. https://doi.org/10.1016/S0163-4453(80)91284-0.
174. Roifman CM, Rao CP, Lederman HM, Lavi S, Quinn P, Gelfand EW. 1986. Increased susceptibility to *Mycoplasma* infection in patients with hypogammaglobulinemia. Am J Med 80:590–594. https://doi.org/10.1016/0002-9343(86)90812-0.
175. Johnston CL, Webster AD, Taylor-Robinson D, Rapaport G, Hughes GR. 1983. Primary late-onset hypogammaglobulinaemia associated with inflammatory polyarthritis and septic arthritis due to *Mycoplasma pneumoniae*. Ann Rheum Dis 42:108–110.
176. Lai JF, Zindl CL, Duffy LB, Atkinson TP, Jung YW, van Rooijen N, Waites KB, Krause DC, Chaplin DD. 2010. Critical role of macrophages and their activation via MyD88-NFkappaB signaling in lung innate immunity to *Mycoplasma pneumoniae*. PLoS One 5:e14417. https://doi.org/10.1371/journal.pone.0014417.
177. Wu Q, Jiang D, Minor MN, Martin RJ, Chu HW. 2011. In vivo function of airway epithelial TLR2 in host defense against bacterial infection. Am J Physiol Lung Cell Mol Physiol 300:L579–L586. https://doi.org/10.1152/ajplung.00336.2010.
178. Shimizu T, Kimura Y, Kida Y, Kuwano K, Tachibana M, Hashino M, Watarai M. 2014. Cytadherence of Mycoplasma pneumoniae induces inflammatory responses through autophagy and toll-like receptor 4. Infect Immun 82:3076–3086. https://doi.org/10.1128/IAI.01961-14.
179. Narita M, Tanaka H, Abe S, Yamada S, Kubota M, Togashi T. 2000. Close association between pulmonary disease manifestation in *Mycoplasma pneumoniae* infection and enhanced local production of interleukin-18 in the lung, independent of gamma interferon. Clin Diagn Lab Immunol 7:909–914.
180. Narita M. 2009. Pathogenesis of neurologic manifestations of *Mycoplasma pneumoniae* infection. Pediatr Neurol 41:159–166. https://doi.org/10.1016/j.pediatrneurol.2009.04.012.
181. Tani K, Shimizu T, Kida Y, Kuwano K. 2011. *Mycoplasma pneumoniae* infection induces a neutrophil-derived antimicrobial peptide, cathelin-related antimicrobial peptide. Microbiol Immunol 55:582–588. https://doi.org/10.1111/j.1348-0421.2011.00353.x.
182. Di YP. 2011. Functional roles of SPLUNC1 in the innate immune response against Gram-negative bacteria. Biochem Soc Trans 39:1051–1055. https://doi.org/10.1042/BST0391051.
183. Chu HW, Thaikoottathil J, Rino JG, Zhang G, Wu Q, Moss T, Refaeli Y, Bowler R, Wenzel SE, Chen Z, Zdunek J, Breed R, Young R, Allaire E, Martin RJ. 2007. Function and regulation of SPLUNC1 protein in Myco-

plasma infection and allergic inflammation. J Immunol 179:3995–4002. https://doi.org/10.4049/jimmunol.179.6.3995.
184. Gally F, Di YP, Smith SK, Minor MN, Liu Y, Bratton DL, Frasch SC, Michels NM, Case SR, Chu HW. 2011. SPLUNC1 promotes lung innate defense against *Mycoplasma pneumoniae* infection in mice. Am J Pathol 178:2159–2167. https://doi.org/10.1016/j.ajpath.2011.01.026.
185. Thaikoottathil J, Chu HW. 2011. MAPK/AP-1 activation mediates TLR2 agonist-induced SPLUNC1 expression in human lung epithelial cells. Mol Immunol 49:415–422. https://doi.org/10.1016/j.molimm.2011.08.005.
186. Gross CA, Bowler RP, Green RM, Weinberger AR, Schnell C, Chu HW. 2010. beta2-agonists promote host defense against bacterial infection in primary human bronchial epithelial cells. BMC Pulm Med 10:30. https://doi.org/10.1186/1471-2466-10-30.
187. Gally F, Minor MN, Smith SK, Case SR, Chu HW. 2012. Heat shock factor 1 protects against lung mycoplasma pneumoniae infection in mice. J Innate Immun 4:59–68. https://doi.org/10.1159/000333089.
188. Jiang D, Nelson ML, Gally F, Smith S, Wu Q, Minor M, Case S, Thaikoottathil J, Chu HW. 2012. Airway epithelial NF-kappaB activation promotes *Mycoplasma pneumoniae* clearance in mice. PLoS One 7:e52969. https://doi.org/10.1371/journal.pone.0052969.
189. Jiang W, Qian L, Liang H, Tian M, Liu F, Zhao D. 2014. Relationships between the varied ciliated respiratory epithelium abnormalities and severity of Mycoplasma pneumoniae pneumonia. Scand J Infect Dis 46:486–492. https://doi.org/10.3109/00365548.2014.885658.
190. Smith CB, Chanock RM, Friedewald WT, Alford RH. 1967. *Mycoplasma pneumoniae* infections in volunteers. Ann N Y Acad Sci 143:471–483. https://doi.org/10.1111/j.1749-6632.1967.tb27691.x.
191. He XY, Wang XB, Zhang R, Yuan ZJ, Tan JJ, Peng B, Huang Y, Liu EM, Fu Z, Bao LM, Zou L. 2013. Investigation of *Mycoplasma pneumoniae* infection in pediatric population from 12,025 cases with respiratory infection. Diagn Microbiol Infect Dis 75:22–27. https://doi.org/10.1016/j.diagmicrobio.2012.08.027.
192. Miyashita N, Kawai Y, Yamaguchi T, Ouchi K, Oka M, Atypical Pathogen Study Group. 2011. Clinical potential of diagnostic methods for the rapid diagnosis of *Mycoplasma pneumoniae* pneumonia in adults. Eur J Clin Microbiol Infect Dis 30:439–446. https://doi.org/10.1007/s10096-010-1107-8.
193. Foy HM. 1993. Infections caused by *Mycoplasma pneumoniae* and possible carrier state in different populations of patients. Clin Infect Dis 17(Suppl 1):S37–S46. https://doi.org/10.1093/clinids/17.Supplement_1.S37.
194. Kannan TR, Hardy RD, Coalson JJ, Cavuoti DC, Siegel JD, Cagle M, Musatovova O, Herrera C, Baseman JB. 2012. Fatal outcomes in family transmission of *Mycoplasma pneumoniae*. Clin Infect Dis 54:225–231. https://doi.org/10.1093/cid/cir769.
195. Li YN, Liu L, Qiao HM, Cheng H, Cheng HJ. 2014. Post-infectious bronchiolitis obliterans in children: a review of 42 cases. BMC Pediatr 14:238. https://doi.org/10.1186/1471-2431-14-238.
196. Izumikawa K, Izumikawa K, Takazono T, Kosai K, Morinaga Y, Nakamura S, Kurihara S, Imamura Y, Miyazaki T, Tsukamoto M, Yanagihara K, Hara K, Kohno S. 2014. Clinical features, risk factors and treatment of fulminant *Mycoplasma pneumoniae* pneumonia: a review of the Japanese literature. J Infect Chemother 20:181–185. https://doi.org/10.1016/j.jiac.2013.09.009.
197. Daxboeck F, Eisl B, Burghuber C, Memarsadeghi M, Assadian O, Stanek G. 2007. Fatal *Mycoplasma pneumoniae* pneumonia in a previously healthy 18-year-old girl. Wien Klin Wochenschr 119:379–384. https://doi.org/10.1007/s00508-007-0810-7.
198. Dorigo-Zetsma JW, Zaat SA, Vriesema AJ, Dankert J. 1999. Demonstration by a nested PCR for *Mycoplasma pneumoniae* that *M. pneumoniae* load in the throat is higher in patients hospitalised for *M. pneumoniae* infection than in non-hospitalised subjects. J Med Microbiol 48:1115–1122. https://doi.org/10.1099/00222615-48-12-1115.
199. Wang M, Wang Y, Yan Y, Zhu C, Huang L, Shao X, Xu J, Zhu H, Sun X, Ji W, Chen Z. 2014. Clinical and laboratory profiles of refractory *Mycoplasma pneumoniae* pneumonia in children. Int J Infect Dis 29:18–23. https://doi.org/10.1016/j.ijid.2014.07.020.
200. Park SJ, Pai KS, Kim AR, Lee JH, Shin JI, Lee SY. 2012. Fulminant and fatal multiple organ failure in a 12-year-old boy with *Mycoplasma pneumoniae* infection. Allergy Asthma Immunol Res 4:55–57. https://doi.org/10.4168/aair.2012.4.1.55.
201. Muir MT, Cohn SM, Louden C, Kannan TR, Baseman JB. 2011. Novel toxin assays implicate *Mycoplasma pneumoniae* in prolonged ventilator course and hypoxemia. Chest 139:305–310. https://doi.org/10.1378/chest.10-1222.
202. Miyashita N, Kawai Y, Inamura N, Tanaka T, Akaike H, Teranishi H, Wakabayashi T, Nakano T, Ouchi K, Okimoto N. 2015. Setting a standard for the initiation of steroid therapy in refractory or severe *Mycoplasma pneumoniae* pneumonia in adolescents and adults. J Infect Chemother 21:153–160. https://doi.org/10.1016/j.jiac.2014.10.008.
203. Miyashita N, Akaike H, Teranishi H, Nakano T, Ouchi K, Okimoto N. 2014. Chest computed tomography for the diagnosis of *Mycoplasma pneumoniae* infection. Respirology 19:144–145. https://doi.org/10.1111/resp.12218.
204. Rappaport SM. 2016. Genetic factors are not the major causes of chronic diseases. PLoS One 11:e0154387. https://doi.org/10.1371/journal.pone.0154387.
205. Bonnelykke K, Ober C. 2016. Leveraging gene-environment interactions and endotypes for asthma gene discovery. J Allergy Clin Immunol 137:667–679. https://doi.org/10.1016/j.jaci.2016.01.006.
206. Jackson DJ, Gern JE, Lemanske RF, Jr. 2016. The contributions of allergic sensitization and respiratory pathogens to asthma inception. J Allergy Clin Immunol 137:659–665. (Quiz, 666.) https://doi.org/10.1016/j.jaci.2016.01.002.
207. Larsen JM, Brix S, Thysen AH, Birch S, Rasmussen MA, Bisgaard H. 2014. Children with asthma by school age display aberrant immune responses to pathogenic airway bacteria as infants. J Allergy Clin Immunol 133:1008–1013. https://doi.org/10.1016/j.jaci.2014.01.010.
208. Yeh JJ, Wang YC, Hsu WH, Kao CH. 2016. Incident asthma and *Mycoplasma pneumoniae:* a nationwide cohort study. J Allergy Clin Immunol 137:1017–1023.e6. https://doi.org/10.1016/j.jaci.2015.09.032.
209. Smith-Norowitz TA, Silverberg JI, Kusonruksa M, Weaver D, Ginsburg D, Norowitz KB, Durkin HG, Hammerschlag MR, Bluth MH, Kohlhoff SA. 2013. Asthmatic children have increased specific anti-mycoplasma pneumoniae IgM but not IgG or IgE values independent of history of respiratory tract infection. Pediatr Infect Dis J https://doi.org/10.1097/INF.0b013e3182862ea8.
210. Atkinson TP, Duffy LB, Pendley D, Dai Y, Cassell GH. 2009. Deficient immune response to *Mycoplasma pneumoniae* in childhood asthma. Allergy Asthma Proc 30:158–165. https://doi.org/10.2500/aap.2009.30.3207.
211. Duenas Meza E, Jaramillo CA, Correa E, Torres-Duque CA, Garcia C, Gonzalez M, Rojas D, Hernandez A, Paez AM, Delgado MDP. 2016. Virus and Mycoplasma pneumoniae prevalence in a selected pediatric population with acute asthma exacerbation. J Asthma 53:253–260. https://doi.org/10.3109/02770903.2015.1075548.
212. Bebear C, Raherison C, Nacka F, de Barbeyrac B, Pereyre S, Renaudin H, Girodet PO, Marquant F, Desjardins S, Chene G, Fayon M. 2014. Comparison of *Mycoplasma pneumoniae* infections in asthmatic children versus asthmatic adults. Pediatr Infect Dis J 33:e71–e75. https://doi.org/10.1097/INF.0000000000000063.
213. Qasem JA, Al-Khalaf BN, Qasem AA, Ghulam AH, Bidass G. 2013. Application of three uniplex polymerase chain reaction assays for the detection of atypical bacteria in asthmatic patients in Kuwait. J Infect Public Health 6:134–141. https://doi.org/10.1016/j.jiph.2012.12.002.
214. Maffey AF, Barrero PR, Venialgo C, Fernandez F, Fuse VA, Saia M, Villalba A, Fermepin MR, Teper AM, Mistchenko AS. 2010. Viruses and atypical bacteria associated with asthma exacerbations in hospitalized children. Pediatr Pulmonol 45:619–625. https://doi.org/10.1002/ppul.21236.
215. Varshney AK, Chaudhry R, Saharan S, Kabra SK, Dhawan B, Dar L, Malhotra P. 2009. Association of Mycoplasma pneumoniae and asthma among Indian children. FEMS Immunol Med Microbiol 56:25–31. https://doi.org/10.1111/j.1574-695X.2009.00543.x.
216. Lieberman D, Printz S, Ben-Yaakov M, Lazarovich Z, Ohana B, Friedman MG, Dvoskin B, Leinonen M, Boldur I. 2003. Atypical pathogen infection in adults with acute exacerbation of bronchial asthma. Am J Respir Crit Care Med 167:406–410. https://doi.org/10.1164/rccm.200209-996OC.
217. Peters JSH, Brooks EG, Diaz J, Kannan TR, Coalson JJ, Baseman JG, Cagle M, Baseman JB. 2011. Persistence of community-acquired respiratory distress syndrome toxin-producing Mycoplasma pneumoniae in refractory asthma. Chest 140:401–407. https://doi.org/10.1378/chest.11-0221.
218. Martin RJ, Kraft M, Chu HW, Berns EA, Cassell GH. 2001. A link between chronic asthma and chronic infection. J Allergy Clin Immunol 107:595–601. https://doi.org/10.1067/mai.2001.113563.
219. Sutherland ER, King TS, Icitovic N, Ameredes BT, Bleecker E, Boushey HA, Calhoun WJ, Castro M, Cherniack RM, Chinchilli VM, Craig TJ, Denlinger L, DiMango EA, Fahy JV, Israel E, Jarjour N, Kraft M, Lazarus

SC, Lemanske RF, Jr, Peters SP, Ramsdell J, Sorkness CA, Szefler SJ, Walter MJ, Wasserman SI, Wechsler ME, Chu HW, Martin RJ. 2010. A trial of clarithromycin for the treatment of suboptimally controlled asthma. J Allergy Clin Immunol 126:747–753. https://doi.org/10.1016/j.jaci.2010.07.024.
220. Kraft M, Cassell GH, Pak J, Martin RJ. 2002. Mycoplasma pneumoniae and Chlamydia pneumoniae in asthma: effect of clarithromycin. Chest 121:1782–1788. https://doi.org/10.1378/chest.121.6.1782.
221. Stokholm J, Chawes BL, Vissing NH, Bjarnadottir E, Pedersen TM, Vinding RK, Schoos AM, Wolsk HM, Thorsteinsdottir S, Hallas HW, Arianto L, Schjorring S, Krogfelt KA, Fischer TK, Pipper CB, Bonnelykke K, Bisgaard H. 2016. Azithromycin for episodes with asthma-like symptoms in young children aged 1-3 years: a randomised, double-blind, placebo-controlled trial. Lancet Respir Med 4:19–26. https://doi.org/10.1016/S2213-2600(15)00500-7.
222. Bacharier LB, Guilbert TW, Mauger DT, Boehmer S, Beigelman A, Fitzpatrick AM, Jackson DJ, Baxi SN, Benson M, Burnham CA, Cabana M, Castro M, Chmiel JF, Covar R, Daines M, Gaffin JM, Gentile DA, Holguin F, Israel E, Kelly HW, Lazarus SC, Lemanske RF, Jr, Ly N, Meade K, Morgan W, Moy J, Olin T, Peters SP, Phipatanakul W, Pongracic JA, Raissy HH, Ross K, Sheehan WJ, Sorkness C, Szefler SJ, Teague WG, Thyne S, Martinez FD. 2015. Early administration of azithromycin and prevention of severe lower respiratory tract illnesses in preschool children with a history of such illnesses: a randomized clinical trial. JAMA 314:2034–2044. https://doi.org/10.1001/jama.2015.13896.
223. Eaton MD, Meikejohn G, Van Herick W. 1944. Studies on the etiology of primary atypical pneumonia: a filterable agent transmissible to cotton rats, hamsters, and chick embryos. J Exp Med 79:649–667. https://doi.org/10.1084/jem.79.6.649.
224. Wubbel L, Jafri HS, Olsen K, Shelton S, Barton Rogers B, Gambill G, Patel P, Keyser E, Cassell G, McCracken GH. 1998. Mycoplasma pneumoniae pneumonia in a mouse model. J Infect Dis 178:1526–1529. https://doi.org/10.1086/314439.
225. Hardy RD, Jafri HS, Olsen K, Hatfield J, Iglehart J, Rogers BB, Patel P, Cassell G, McCracken GH, Ramilo O. 2002. Mycoplasma pneumoniae induces chronic respiratory infection, airway hyperreactivity, and pulmonary inflammation: a murine model of infection-associated chronic reactive airway disease. Infect Immun 70:649–654. https://doi.org/10.1128/IAI.70.2.649-654.2002.
226. Hayakawa M, Taguchi H, Kamiya S, Fujioka Y, Watanabe H, Kawai S, Kobayashi H. 2002. Animal model of Mycoplasma pneumoniae infection using germfree mice. Clin Diagn Lab Immunol 9:669–676.
227. Michels NM, Chu HW, LaFasto SC, Case SR, Minor MN, Martin RJ. 2010. Mast cells protect against airway Mycoplasma pneumoniae under allergic conditions. Clin Exp Allergy 40:1406–1413. https://doi.org/10.1111/j.1365-2222.2010.03488.x.
228. Ledford JG, Goto H, Potts EN, Degan S, Chu HW, Voelker DR, Sunday ME, Cianciolo GJ, Foster WM, Kraft M, Wright JR. 2009. SP-A preserves airway homeostasis during Mycoplasma pneumoniae infection in mice. J Immunol 182:7818–7827. https://doi.org/10.4049/jimmunol.0900452.
229. Salvatore CM, Fonseca-Aten M, Katz-Gaynor K, Gomez AM, Mejias A, Somers C, Chavez-Bueno S, McCracken GH, Hardy RD. 2007. Respiratory tract infection with Mycoplasma pneumoniae in interleukin-12 knockout mice results in improved bacterial clearance and reduced pulmonary inflammation. Infect Immun 75:236–242. https://doi.org/10.1128/IAI.01249-06.
230. Martin RA, Hodgkins SR, Dixon AE, Poynter ME. 2014. Aligning mouse models of asthma to human endotypes of disease. Respirology 19:823–833. https://doi.org/10.1111/resp.12315.
231. Chapman DG, Tully JE, Nolin JD, Janssen-Heininger YM, Irvin CG. 2014. Animal models of allergic airways disease: where are we and where to next? J Cell Biochem 115:2055–2064. https://doi.org/10.1002/jcb.24881.
232. Kumar RK, Herbert C, Foster PS. 2016. Mouse models of acute exacerbations of allergic asthma. Respirology 21:842–849. https://doi.org/10.1111/resp.12760.
233. Martin RJ, Chu HW, Honour JM, Harbeck RJ. 2001. Airway inflammation and bronchial hyperresponsiveness after *Mycoplasma pneumoniae* infection in a murine model. Am J Respir Cell Mol Biol 24:577–582. https://doi.org/10.1165/ajrcmb.24.5.4315.
234. Wu Q, Martin RJ, Lafasto S, Efaw BJ, Rino JG, Harbeck RJ, Chu HW. 2008. Toll-like receptor 2 down-regulation in established mouse allergic lungs contributes to decreased mycoplasma clearance. Am J Respir Crit Care Med 177:720–729. https://doi.org/10.1164/rccm.200709-1387OC.
235. Wu Q, Martin RJ, Rino JG, Breed R, Torres RM, Chu HW. 2007. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory Mycoplasma pneumoniae infection. Microbes Infect 9:78–86. https://doi.org/10.1016/j.micinf.2006.10.012.
236. Ledford JG, Mukherjee S, Kislan MM, Nugent JL, Hollingsworth JW, Wright JR. 2012. Surfactant protein-A suppresses eosinophil-mediated killing of Mycoplasma pneumoniae in allergic lungs. PLoS One 7:e32436. https://doi.org/10.1371/journal.pone.0032436.
237. Narita M. 2016. Classification of extrapulmonary manifestations due to *Mycoplasma pneumoniae* infection on the basis of possible pathogenesis. Front Microbiol 7:23. https://doi.org/10.3389/fmicb.2016.00023.
238. Kammer J, Ziesing S, Davila LA, Bultmann E, Illsinger S, Das AM, Haffner D, Hartmann H. 2016. Neurological manifestations of Mycoplasma pneumoniae infection in hospitalized children and their long-term follow-up. Neuropediatrics 14:14.
239. Meyer Sauteur PM, Moeller A, Relly C, Berger C, Plecko B, Nadal D. 2016. Swiss national prospective surveillance of paediatric Mycoplasma pneumoniae-associated encephalitis. Swiss Med Wkly 146:w14222. https://doi.org/10.4414/smw.2016.14222.
240. Pillai SC, Hacohen Y, Tantsis E, Prelog K, Merheb V, Kesson A, Barnes E, Gill D, Webster R, Menezes M, Ardern-Holmes S, Gupta S, Procopis P, Troedson C, Antony J, Ouvrier RA, Polfrit Y, Davies NW, Waters P, Lang B, Lim MJ, Brilot F, Vincent A, Dale RC. 2015. Infectious and autoantibody-associated encephalitis: clinical features and long-term outcome. Pediatrics https://doi.org/10.1542/peds.2014-2702.
241. Al-Zaidy SA, MacGregor D, Mahant S, Richardson SE, Bitnun A. 2015. Neurological complications of PCR-proven M. pneumoniae infections in children: prodromal illness duration may reflect pathogenetic mechanism. Clin Infect Dis 61:1092–1098. https://doi.org/10.1093/cid/civ473.
242. Yimenicioglu S, Yakut A, Ekici A, Bora Carman K, Cagri Dinleyici E. 2014. Mycoplasma pneumoniae infection with neurologic complications. Iran J Pediatr 24:647–651.
243. Lin JJ, Lin KL, Chiu CH, Hsia SH, Wang HS, Chou IJ, Lin YT. 2014. Antineuronal antibodies and infectious pathogens in severe acute pediatric encephalitis. J Child Neurol 29:11–16. https://doi.org/10.1177/0883073812461944.
244. Stamm B, Moschopulos M, Hungerbuehler H, Guarner J, Genrich GL, Zaki SR. 2008. Neuroinvasion by *Mycoplasma pneumoniae* in acute disseminated encephalomyelitis. Emerg Infect Dis 14:641–643. https://doi.org/10.3201/eid1404.061366.
245. Powers JM, Johnson MD. 2012. Mycoplasmal panencephalitis: a neuropathologic documentation. Acta Neuropathol 124:143–148. https://doi.org/10.1007/s00401-012-0960-1.
246. Magun R, Verschoor CP, Bowdish DM, Provias J. 2016. *Mycoplasma pneumoniae*, a trigger for Weston Hurst syndrome. Neurol Neuroimmunol Neuroinflamm 3:e187. https://doi.org/10.1212/NXI.0000000000000187.
247. Saitoh S, Wada T, Narita M, Kohsaka S, Mizukami S, Togashi T, Kajii N. 1993. *Mycoplasma pneumoniae* infection may cause striatal lesions leading to acute neurologic dysfunction. Neurology 43:2150–2151. https://doi.org/10.1212/WNL.43.10.2150.
248. Meyer Sauteur PM, Roodbol J, Hackenberg A, de Wit MY, Vink C, Berger C, Jacobs E, van Rossum AM, Jacobs BC. 2015. Severe childhood Guillain-Barre syndrome associated with Mycoplasma pneumoniae infection: a case series. J Peripher Nerv Syst 26:12122.
249. Samukawa M, Hamada Y, Kuwahara M, Takada K, Hirano M, Mitsui Y, Sonoo M, Kusunoki S. 2014. Clinical features in Guillain-Barre syndrome with anti-Gal-C antibody. J Neurol Sci 337:55–60. https://doi.org/10.1016/j.jns.2013.11.016.
250. Wakerley BR, Yuki N. 2013. Infectious and noninfectious triggers in Guillain-Barre syndrome. Expert Rev Clin Immunol 9:627–639. https://doi.org/10.1586/1744666X.2013.811119.
251. Sharma MB, Chaudhry R, Tabassum I, Ahmed NH, Sahu JK, Dhawan B, Kalra V. 2011. The presence of *Mycoplasma pneumoniae* infection and GM1 ganglioside antibodies in Guillain-Barre syndrome. J Infect Dev Ctries 5:459–464. https://doi.org/10.3855/jidc.1847.
252. Socan M, Ravnik I, Bencina D, Dovc P, Zakotnik B, Jazbec J. 2001. Neurological symptoms in patients whose cerebrospinal fluid is culture- and/or polymerase chain reaction-positive for *Mycoplasma pneumoniae*. Clin Infect Dis 32:E31–E35. https://doi.org/10.1086/318446.
253. Canavan TN, Mathes EF, Frieden I, Shinkai K. 2015. *Mycoplasma pneumoniae*-induced rash and mucositis as a syndrome distinct from Stevens-Johnson syndrome and erythema multiforme: a systematic review. J Am Acad Dermatol 72:239–245. https://doi.org/10.1016/j.jaad.2014.06.026.

254. Narita M. 2010. Pathogenesis of extrapulmonary manifestations of Mycoplasma pneumoniae infection with special reference to pneumonia. J Infect Chemother 16:162–169. https://doi.org/10.1007/s10156-010-0044-X.
255. Khan FY, Ayassin M. 2009. *Mycoplasma pneumoniae* associated with severe autoimmune hemolytic anemia: case report and literature review. Braz J Infect Dis 13:77–79. https://doi.org/10.1590/S1413-86702009000100018.
256. Zafer MH, Gamel AS, Ansari MA, Hamid ME. 2009. Anemic crisis due to Mycoplasma pneumoniae complication in sickle cell patients. Saudi Med J 30:157–158.
257. Bar Meir E, Amital H, Levy Y, Kneller A, Bar-Dayan Y, Shoenfeld Y. 2000. *Mycoplasma-pneumoniae*-induced thrombotic thrombocytopenic purpura. Acta Haematol 103:112–115. https://doi.org/10.1159/000041030.
258. Aviner S, Miskin H, London D, Horowitz S, Schlesinger M. 2011. *Mycoplasma pneumoniae* infection: a possible trigger for immune thrombocytopenia. Indian J Hematol Blood Transfus 27:46–50. https://doi.org/10.1007/s12288-011-0054-6.
259. Cunha BA, Pherez FM. 2009. *Mycoplasma pneumoniae* community-acquired pneumonia (CAP) in the elderly: diagnostic significance of acute thrombocytosis. Heart Lung 38:444–449. https://doi.org/10.1016/j.hrtlng.2008.10.005.
260. Taylor-Robinson D. 2013. Immunopathological aspects of *Mycoplasma pneumoniae* infection. Curr Pediatr Rev 9:273–278. https://doi.org/10.2174/15733963090413122310411 7.
261. Oishi T, Narita M, Ohya H, Yamanaka T, Aizawa Y, Matsuo M, Matsunaga M, Tsukano S, Taguchi T. 2012. Rhabdomyolysis associated with antimicrobial drug-resistant *Mycoplasma pneumoniae*. Emerg Infect Dis 18:849–851. https://doi.org/10.3201/eid1805.111149.
262. Weng WC, Peng SS, Wang SB, Chou YT, Lee WT. 2009. *Mycoplasma pneumoniae*-associated transverse myelitis and rhabdomyolysis. Pediatr Neurol 40:128–130. https://doi.org/10.1016/j.pediatrneurol.2008.10.009.
263. Laso MDC, Cadario ME, Haymes L, Grimoldi I, Balbarrey Z, Casanueva E. 2006. *Mycoplasma pneumoniae* detection with PCR in renal tissue of a patient with acute glomerulonephritis. Pediatr Nephrol 21:1483–1486. https://doi.org/10.1007/s00467-006-0179-2.
264. Andrews PA, Lloyd CM, Webb MC, Sacks SH. 1994. Acute interstitial nephritis associated with *Mycoplasma pneumoniae* infection. Nephrol Dial Transplant 9:564–566. https://doi.org/10.1093/ndt/9.5.564.
265. Said MH, Layani MP, Colon S, Faraj G, Glastre C, Cochat P. 1999. *Mycoplasma pneumoniae*-associated nephritis in children. Pediatr Nephrol 13:39–44. https://doi.org/10.1007/s004670050559.
266. Adra AL, Vigue MG, Dalla Vale F, Ichay L, Raynaud P, Mariani A, Morin D. 2010. Favorable outcome in a case of *Mycoplasma pneumoniae*-associated crescentic glomerulonephritis. Pediatr Nephrol 25:1765–1769. https://doi.org/10.1007/s00467-010-1491-4.
267. Beersma MF, Dirven K, van Dam AP, Templeton KE, Claas EC, Goossens H. 2005. Evaluation of 12 commercial tests and the complement fixation test for *Mycoplasma pneumoniae*-specific immunoglobulin G (IgG) and IgM antibodies, with PCR used as the "gold standard." J Clin Microbiol 43:2277–2285. https://doi.org/10.1128/JCM.43.5.2277-2285.2005.
268. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, Dowell SF, File TM, Jr, Musher DM, Niederman MS, Torres A, Whitney CG, Infectious Diseases Society of America, American Thoracic Society. 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 44(Suppl 2):S27–S72. https://doi.org/10.1086/511159.
269. Bradley JS, Byington CL, Shah SS, Alverson B, Carter ER, Harrison C, Kaplan SL, Mace SE, McCracken GH, Jr, Moore MR, St Peter SD, Stockwell JA, Swanson JT, Pediatric Infectious Diseases Society, Infectious Diseases Society of America. 2011. The management of community-acquired pneumonia in infants and children older than 3 months of age: clinical practice guidelines by the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America. Clin Infect Dis 53:e25–e76. https://doi.org/10.1093/cid/cir531.
270. Loens K, Van Heirstraeten L, Malhotra-Kumar S, Goossens H, Ieven M. 2009. Optimal sampling sites and methods for detection of pathogens possibly causing community-acquired lower respiratory tract infections. J Clin Microbiol 47:21–31. https://doi.org/10.1128/JCM.02037-08.
271. Raty R, Ronkko E, Kleemola M. 2005. Sample type is crucial to the diagnosis of *Mycoplasma pneumoniae* pneumonia by PCR. J Med Microbiol 54:287–291. https://doi.org/10.1099/jmm.0.45888-0.
272. Cho MC, Kim H, An D, Lee M, Noh SA, Kim MN, Chong YP, Woo JH. 2012. Comparison of sputum and nasopharyngeal swab specimens for molecular diagnosis of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila*. Ann Lab Med 32:133–138. https://doi.org/10.3343/alm.2012.32.2.133.
273. Honda J, Yano T, Kusaba M, Yonemitsu J, Kitajima H, Masuoka M, Hamada K, Oizumi K. 2000. Clinical use of capillary PCR to diagnose Mycoplasma pneumonia. J Clin Microbiol 38:1382–1384.
274. Reznikov M, Blackmore TK, Finlay-Jones JJ, Gordon DL. 1995. Comparison of nasopharyngeal aspirates and throat swab specimens in a polymerase chain reaction-based test for *Mycoplasma pneumoniae*. Eur J Clin Microbiol Infect Dis 14:58–61. https://doi.org/10.1007/BF02112622.
275. Xu D, Li S, Chen Z, Du L. 2011. Detection of *Mycoplasma pneumoniae* in different respiratory specimens. Eur J Pediatr 170:851–858. https://doi.org/10.1007/s00431-010-1360-y.
276. Waites KB, Duffy LB, Schwartz S, Talkington DF. 2010. *Mycoplasma* and *Ureaplasma*, p 3.15.11–13.15.17. *In* Garcia L (ed), Clinical microbiology procedure handbook, 3rd ed. ASM Press, Washington, DC.
277. Waites KB, Taylor-Robinson D. 2015. *Mycoplasma* and *Ureaplasma*, p 1088–1105. *In* Jorgensen J, Pfaller MA, Carroll KC, Funke G, Landry ML, Richter SS, Warnock DW (ed), Manual of clinical microbiology, 11th ed. ASM Press, Washington, DC.
278. Simmons WL, Dybvig K. 2015. Catalase enhances growth and biofilm production of *Mycoplasma pneumoniae*. Curr Microbiol 71:190–194. https://doi.org/10.1007/s00284-015-0822-x.
279. CLSI. 2011. Methods for antimicrobial susceptibility testing of human mycoplasmas. Approved guideline. CLSI document M43-A. Clinical and Laboratory Standards Institute, Wayne, PA.
280. Wreghitt TG, Sillis M. 1985. A micro-capture ELISA for detecting *Mycoplasma pneumoniae* IgM: comparison with indirect immunofluorescence and indirect ELISA. J Hyg 94:217–227. https://doi.org/10.1017/S0022172400061428.
281. Nir-Paz R, Michael-Gayego A, Ron M, Block C. 2006. Evaluation of eight commercial tests for *Mycoplasma pneumoniae* antibodies in the absence of acute infection. Clin Microbiol Infect 12:685–688. https://doi.org/10.1111/j.1469-0691.2006.01469.x.
282. Talkington DF, Shott S, Fallon MT, Schwartz SB, Thacker WL. 2004. Analysis of eight commercial enzyme immunoassay tests for detection of antibodies to *Mycoplasma pneumoniae* in human serum. Clin Diagn Lab Immunol 11:862–867.
283. Ozaki T, Nishimura N, Ahn J, Watanabe N, Muto T, Saito A, Koyama N, Nakane K, Funahashi K. 2007. Utility of a rapid diagnosis kit for *Mycoplasma pneumoniae* pneumonia in children, and the antimicrobial susceptibility of the isolates. J Infect Chemother 13:204–207. https://doi.org/10.1007/s10156-007-0519-6.
284. Liu FC, Chen PY, Huang FL, Tsai CR, Lee CY, Lin CF. 2008. Do serological tests provide adequate rapid diagnosis of *Mycoplasma pneumoniae* infection? Jpn J Infect Dis 61:397–399.
285. Granstrom M, Holme T, Sjogren AM, Ortqvist A, Kalin M. 1994. The role of IgA determination by ELISA in the early serodiagnosis of *Mycoplasma pneumoniae* infection, in relation to IgG and mu-capture IgM methods. J Med Microbiol 40:288–292. https://doi.org/10.1099/00222615-40-4-288.
286. Watkins-Riedel T, Stanek G, Daxboeck F. 2001. Comparison of SeroMP IgA with four other commercial assays for serodiagnosis of *Mycoplasma pneumoniae* pneumonia. Diagn Microbiol Infect Dis 40:21–25. https://doi.org/10.1016/S0732-8893(01)00250-4.
287. Narita M. 2005. Evaluation of ELISA kits for detection of *Mycoplasma pneumoniae*-specific IgG, IgA, IgM antibodies on the diagnosis of Mycoplasma pneumoniae infection in children. Kansenshogaku Zasshi 79:457–463. https://doi.org/10.11150/kansenshogakuzasshi1970.79.457.
288. Lee WJ, Huang EY, Tsai CM, Kuo KC, Huang YC, Hsieh KS, Niu CK, Yu HR. 2017. The role of serum *Mycoplasma pneumoniae* IgA, IgM, and IgG for the diagnosis of *Mycoplasma pneumoniae*-related pneumonia in school-age children and adolescents. Clin Vaccine Immunol 24:e00471-16. https://doi.org/10.1128/CVI.00471-16.
289. Csango PA, Pedersen JE, Hess RD. 2004. Comparison of four *Mycoplasma pneumoniae* IgM-, IgG- and IgA-specific enzyme immunoassays in blood donors and patients. Clin Microbiol Infect 10:1094–1098. https://doi.org/10.1111/j.1469-0691.2004.00993.x.

290. Chang HY, Chang LY, Shao PL, Lee PI, Chen JM, Lee CY, Lu CY, Huang LM. 2014. Comparison of real-time polymerase chain reaction and serological tests for the confirmation of *Mycoplasma pneumoniae* infection in children with clinical diagnosis of atypical pneumonia. J Microbiol Immunol Infect 47:137–144. https://doi.org/10.1016/j.jmii.2013.03.015.
291. Busson L, Van den Wijngaert S, Dahma H, Decolvenaer M, Di Cesare L, Martin A, Vasseur L, Vandenberg O. 2013. Evaluation of 10 serological assays for diagnosing *Mycoplasma pneumoniae* infection. Diagn Microbiol Infect Dis 76:133–137. https://doi.org/10.1016/j.diagmicrobio.2013.02.027.
292. Medjo B, Atanaskovic-Markovic M, Radic S, Nikolic D, Lukac M, Djukic S. 2014. *Mycoplasma pneumoniae* as a causative agent of community-acquired pneumonia in children: clinical features and laboratory diagnosis. Ital J Pediatr 40:104. https://doi.org/10.1186/s13052-014-0104-4.
293. Duffy MF, Whithear KG, Noormohammadi AH, Markham PF, Catton M, Leydon J, Browning GF. 1999. Indirect enzyme-linked immunosorbent assay for detection of immunoglobulin G reactive with a recombinant protein expressed from the gene encoding the 116-kilodalton protein of *Mycoplasma pneumoniae*. J Clin Microbiol 37:1024–1029.
294. Montagnani F, De Paolis F, Beghetto E, Gargano N. 2010. Use of recombinant chimeric antigens for the serodiagnosis of *Mycoplasma pneumoniae* infection. Eur J Clin Microbiol Infect Dis 29:1377–1386. https://doi.org/10.1007/s10096-010-1010-3.
295. Ieven MM, Loens K. 2013. Should serology be abolished in favor of PCR for the diagnosis of *Mycoplasma pneumoniae* infections. Curr Pediatr Rev 9:304–313. https://doi.org/10.2174/15733963090413122311050l.
296. Dumke R, Strubel A, Cyncynatus C, Nuyttens H, Herrmann R, Luck C, Jacobs E. 2012. Optimized serodiagnosis of *Mycoplasma pneumoniae* infections. Diagn Microbiol Infect Dis 73:200–203. https://doi.org/10.1016/j.diagmicrobio.2012.02.014.
297. Miyashita N, Kawai Y, Tanaka T, Akaike H, Teranishi H, Wakabayashi T, Nakano T, Ouchi K, Okimoto N. 2015. Diagnostic sensitivity of a rapid antigen test for the detection of *Mycoplasma pneumoniae*: comparison with real-time PCR. J Infect Chemother 21:473–475. https://doi.org/10.1016/j.jiac.2015.02.007.
298. Miyashita N, Kawai Y, Kato T, Tanaka T, Akaike H, Teranishi H, Nakano T, Ouchi K, Okimoto N. 2016. Rapid diagnostic method for the identification of *Mycoplasma pneumoniae* respiratory tract infection. J Infect Chemother 22:327–330. https://doi.org/10.1016/j.jiac.2016.02.005.
299. Li W, Liu Y, Zhao Y, Tao R, Li Y, Shang S. 2015. Rapid diagnosis of *Mycoplasma pneumoniae* in children with pneumonia by an immunochromatographic antigen assay. Sci Rep 5:15539. https://doi.org/10.1038/srep15539.
300. Waites KB, Xiao L, Paralanov V, Viscardi RM, Glass JI. 2012. Molecular methods for the detection of mycoplasma and ureaplasma infections in humans: a paper from the 2011 William Beaumont Hospital Symposium on molecular pathology. J Mol Diagn 14:437–450. https://doi.org/10.1016/j.jmoldx.2012.06.001.
301. Jensen JS, Sondergard-Andersen J, Uldum SA, Lind K. 1989. Detection of *Mycoplasma pneumoniae* in simulated clinical samples by polymerase chain reaction. Brief report. APMIS 97:1046–1048. https://doi.org/10.1111/j.1699-0463.1989.tb00516.x.
302. Ieven M, Ursi D, Van Bever H, Quint W, Niesters HG, Goossens H. 1996. Detection of Mycoplasma pneumoniae by two polymerase chain reactions and role of *M. pneumoniae* in acute respiratory tract infections in pediatric patients. J Infect Dis 173:1445–1452. https://doi.org/10.1093/infdis/173.6.1445.
303. Zhou Z, Li X, Chen X, Yao L, Pan C, Huang H, Luo F, Zheng X, Sun X, Tan F. 2015. Comparison of P1 and 16S rRNA genes for detection of *Mycoplasma pneumoniae* by nested PCR in adults in Zhejiang, China. J Infect Dev Ctries 9:244–253. https://doi.org/10.3855/jidc.5149.
304. Dumke R, Schurwanz N, Lenz M, Schuppler M, Luck C, Jacobs E. 2007. Sensitive detection of *Mycoplasma pneumoniae* in human respiratory tract samples by optimized real-time PCR approach. J Clin Microbiol 45:2726–2730. https://doi.org/10.1128/JCM.00321-07.
305. Diaz MH, Winchell JM. 2016. The evolution of advanced molecular diagnostics for the detection and characterization of *Mycoplasma pneumoniae*. Front Microbiol 7:232. https://doi.org/10.3389/fmicb.2016.00232.
306. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63. https://doi.org/10.1093/nar/28.12.e63.
307. Ratliff A, Duffy LB, Waites KB. 2014. Comparison of *illumi*gene Mycoplasma DNA amplification and culture for the detection of *Mycoplasma pneumoniae*. J Clin Microbiol 52:1060–1063. https://doi.org/10.1128/JCM.02913-13.
308. Saito R, Misawa Y, Moriya K, Koike K, Ubukata K, Okamura N. 2005. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae*. J Med Microbiol 54:1037–1041. https://doi.org/10.1099/jmm.0.46071-0.
309. Gotoh K, Nishimura N, Ohshima Y, Arakawa Y, Hosono H, Yamamoto Y, Iwata Y, Nakane K, Funahashi K, Ozaki T. 2012. Detection of *Mycoplasma pneumoniae* by loop-mediated isothermal amplification (LAMP) assay and serology in pediatric community-acquired pneumonia. J Infect Chemother 18:662–667. https://doi.org/10.1007/s10156-012-0388-5.
310. Kakuya F, Kinebuchi T, Fujiyasu H, Tanaka R, Kano H. 2014. Genetic point-of-care diagnosis of *Mycoplasma pneumoniae* infection using LAMP assay. Pediatr Int 56:547–552. https://doi.org/10.1111/ped.12327.
311. Petrone BL, Wolff BJ, DeLaney AA, Diaz MH, Winchell JM. 2015. Isothermal detection of *Mycoplasma pneumoniae* directly from respiratory clinical specimens. J Clin Microbiol 53:2970–2976. https://doi.org/10.1128/JCM.01431-15.
312. Ishiguro N, Koseki N, Kaiho M, Kikuta H, Togashi T, Watanabe T, Ariga T, Hokkaido Pediatric Respiratory Infection Study G. 2015. Sensitivity and specificity of a loop-mediated isothermal amplification assay for the detection of Mycoplasma pneumoniae from nasopharyngeal swab samples compared with those of real-time PCR. Clin Lab 61:603–606. https://doi.org/10.7754/Clin.Lab.2014.141016.
313. Matsuda C, Taketani T, Takeuchi S, Taniguchi Y, Nagira M, Moriyama H, Shibata H, Nagai A. 2013. Usefulness of the Loopamp Mycoplasma P detecting reagent kit developed based on the LAMP method. Rinsho Biseibutshu Jinsoku Shindan Kenkyukai Shi 23:53–59.
314. Aizawa Y, Oishi T, Tsukano S, Taguchi T, Saitoh A. 2014. Clinical utility of loop-mediated isothermal amplification for rapid diagnosis of Mycoplasma pneumoniae in children. J Med Microbiol 63:248–251. https://doi.org/10.1099/jmm.0.068288-0.
315. Poritz MA, Blaschke AJ, Byington CL, Meyers L, Nilsson K, Jones DE, Thatcher SA, Robbins T, Lingenfelter B, Amiott E, Herbener A, Daly J, Dobrowolski SF, Teng DH, Ririe KM. 2011. FilmArray, an automated nested multiplex PCR system for multi-pathogen detection: development and application to respiratory tract infection. PLoS One 6:e26047. https://doi.org/10.1371/journal.pone.0026047.
316. Thurman KA, Warner AK, Cowart KC, Benitez AJ, Winchell JM. 2011. Detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. in clinical specimens using a single-tube multiplex real-time PCR assay. Diagn Microbiol Infect Dis 70:1–9. https://doi.org/10.1016/j.diagmicrobio.2010.11.014.
317. Touati A, Benard A, Hassen AB, Bebear CM, Pereyre S. 2009. Evaluation of five commercial real-time PCR assays for detection of *Mycoplasma pneumoniae* in respiratory tract specimens. J Clin Microbiol 47:2269–2271. https://doi.org/10.1128/JCM.00326-09.
318. Dumke R, Jacobs E. 2009. Comparison of commercial and in-house real-time PCR assays used for detection of *Mycoplasma pneumoniae*. J Clin Microbiol 47:441–444. https://doi.org/10.1128/JCM.01989-08.
319. Ginevra C, Barranger C, Ros A, Mory O, Stephan JL, Freymuth F, Joannes M, Pozzetto B, Grattard F. 2005. Development and evaluation of Chlamylege, a new commercial test allowing simultaneous detection and identification of *Legionella*, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae* in clinical respiratory specimens by multiplex PCR. J Clin Microbiol 43:3247–3254. https://doi.org/10.1128/JCM.43.7.3247-3254.2005.
320. Pillet S, Lardeux M, Dina J, Grattard F, Verhoeven P, Le Goff J, Vabret A, Pozzetto B. 2013. Comparative evaluation of six commercialized multiplex PCR kits for the diagnosis of respiratory infections. PLoS One 8:e72174. https://doi.org/10.1371/journal.pone.0072174.
321. Dumke R, Jacobs E. 2014. Evaluation of five real-time PCR assays for detection of *Mycoplasma pneumoniae*. J Clin Microbiol 52:4078–4081. https://doi.org/10.1128/JCM.02048-14.
322. Loens K, Ieven M, Ursi D, Foolen H, Sillekens P, Goossens H. 2003. Application of NucliSens Basic kit for the detection of *Mycoplasma pneumoniae* in respiratory specimens. J Microbiol Methods 54:127–130. https://doi.org/10.1016/S0167-7012(03)00011-3.
323. Loens K, Ieven M, Ursi D, Beck T, Overdijk M, Sillekens P, Goossens H. 2003. Detection of *Mycoplasma pneumoniae* by real-time nucleic acid sequence-based amplification. J Clin Microbiol 41:4448–4450. https://doi.org/10.1128/JCM.41.9.4448-4450.2003.
324. Templeton KE, Scheltinga SA, Graffelman AW, Van Schie JM, Crielaard

JW, Sillekens P, Van Den Broek PJ, Goossens H, Beersma MF, Claas EC. 2003. Comparison and evaluation of real-time PCR, real-time nucleic acid sequence-based amplification, conventional PCR, and serology for diagnosis of *Mycoplasma pneumoniae*. J Clin Microbiol 41:4366–4371. https://doi.org/10.1128/JCM.41.9.4366-4371.2003.

325. Loens K, Beck T, Ursi D, Overdijk M, Sillekens P, Goossens H, Ieven M. 2008. Development of real-time multiplex nucleic acid sequence-based amplification for detection of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella* spp. in respiratory specimens. J Clin Microbiol 46:185–191. https://doi.org/10.1128/JCM.00447-07.
326. Lodes MJ, Suciu D, Wilmoth JL, Ross M, Munro S, Dix K, Bernards K, Stover AG, Quintana M, Iihoshi N, Lyon WJ, Danley DL, McShea A. 2007. Identification of upper respiratory tract pathogens using electrochemical detection on an oligonucleotide microarray. PLoS One 2:e924. https://doi.org/10.1371/journal.pone.0000924.
327. Khanna M, Fan J, Pehler-Harrington K, Waters C, Douglass P, Stallock J, Kehl S, Henrickson KJ. 2005. The pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, Mycoplasma pneumoniae, Chlamydia (Chlamydophila) pneumoniae, Legionella pneumophila, Legionella micdadei, and Bordetella pertussis, and its real-time counterpart. J Clin Microbiol 43:565–571. https://doi.org/10.1128/JCM.43.2.565-571.2005.
328. Wang Y, Kong F, Yang Y, Gilbert GL. 2008. A multiplex PCR-based reverse line blot hybridization (mPCR/RLB) assay for detection of bacterial respiratory pathogens in children with pneumonia. Pediatr Pulmonol 43:150–159. https://doi.org/10.1002/ppul.20749.
329. Roth SB, Jalava J, Ruuskanen O, Ruohola A, Nikkari S. 2004. Use of an oligonucleotide array for laboratory diagnosis of bacteria responsible for acute upper respiratory infections. J Clin Microbiol 42:4268–4274. https://doi.org/10.1128/JCM.42.9.4268-4274.2004.
330. Loens K, Mackay WG, Scott C, Goossens H, Wallace P, Ieven M. 2010. A multicenter pilot external quality assessment programme to assess the quality of molecular detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. J Microbiol Methods 82:131–135. https://doi.org/10.1016/j.mimet.2010.05.006.
331. Loens K, Bergs K, Ursi D, Goossens H, Ieven M. 2007. Evaluation of NucliSens easyMAG for automated nucleic acid extraction from various clinical specimens. J Clin Microbiol 45:421–425. https://doi.org/10.1128/JCM.00894-06.
332. Loens K, Ursi D, Goossens H, Ieven M. 2007. Evaluation of the NucliSens miniMAG RNA extraction and real-time NASBA applications for the detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in throat swabs. J Microbiol Methods 72:217–219. https://doi.org/10.1016/j.mimet.2007.11.008.
333. Bessede E, Renaudin H, Clerc M, de Barbeyrac B, Bebear C, Pereyre S. 2010. Evaluation of the combination of the NucliSENS easyMAG and the EasyQ applications for the detection of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in respiratory tract specimens. Eur J Clin Microbiol Infect Dis 29:187–190. https://doi.org/10.1007/s10096-009-0839-9.
334. Thurman KA, Cowart KC, Winchell JM. 2009. Comparison of nucleic acid extraction methods for the detection of *Mycoplasma pneumoniae*. Diagn Microbiol Infect Dis 65:435–438. https://doi.org/10.1016/j.diagmicrobio.2009.08.001.
335. Ursi D, Ieven M, Noordhoek GT, Ritzler M, Zandleven H, Altwegg M. 2003. An interlaboratory comparison for the detection of *Mycoplasma pneumoniae* in respiratory samples by the polymerase chain reaction. J Microbiol Methods 53:289–294. https://doi.org/10.1016/S0167-7012(02)00230-0.
336. Loens K, Beck T, Ursi D, Pattyn S, Goossens H, Ieven M. 2006. Two quality control exercises involving nucleic acid amplification methods for detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* and carried out 2 years apart (in 2002 and 2004). J Clin Microbiol 44:899–908. https://doi.org/10.1128/JCM.44.3.899-908.2006.
337. Nubling CM, Baylis SA, Hanschmann KM, Montag-Lessing T, Chudy M, Kress J, Ulrych U, Czurda S, Rosengarten R, Mycoplasma Collaborative Study Group. 2015. World Health Organization international standard to harmonize assays for detection of mycoplasma DNA. Appl Environ Microbiol 81:5694–5702. https://doi.org/10.1128/AEM.01150-15.
338. Pereyre S, Tardy F, Renaudin H, Cauvin E, Del Pra Netto Machado L, Tricot A, Benoit F, Treilles M, Bebear C. 2013. Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 51:3314–3323. https://doi.org/10.1128/JCM.01573-13.
339. Xiao D, Zhao F, Zhang H, Meng F, Zhang J. 2014. Novel strategy for typing *Mycoplasma pneumoniae* isolates by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry coupled with ClinProTools. J Clin Microbiol 52:3038–3043. https://doi.org/10.1128/JCM.01265-14.
340. Patel IS, Premasiri WR, Moir DT, Ziegler LD. 2008. Barcoding bacterial cells: a SERS based methodology for pathogen identification. J Raman Spectrosc 39:1660–1672. https://doi.org/10.1002/jrs.2064.
341. Hennigan SL, Driskell JD, Dluhy RA, Zhao Y, Tripp RA, Waites KB, Krause DC. 2010. Detection of *Mycoplasma pneumoniae* in simulated and true clinical throat swab specimens by nanorod array-surface-enhanced Raman spectroscopy. PLoS One 5:e13633. https://doi.org/10.1371/journal.pone.0013633.
342. Henderson KC, Sheppard ES, Rivera-Betancourt OE, Choi JY, Dluhy RA, Thurman KA, Winchell JM, Krause DC. 2014. The multivariate detection limit for *Mycoplasma pneumoniae* as determined by nanorod array-surface enhanced Raman spectroscopy and comparison with limit of detection by qPCR. Analyst 139:6426–6434. https://doi.org/10.1039/C4AN01141D.
343. Henderson KC, Benitez AJ, Ratliff AE, Crabb DM, Sheppard ES, Winchell JM, Dluhy RA, Waites KB, Atkinson TP, Krause DC. 2015. Specificity and strain-typing capabilities of nanorod array-surface enhanced Raman spectroscopy for *Mycoplasma pneumoniae* detection. PLoS One 10:e0131831. https://doi.org/10.1371/journal.pone.0131831.
344. Maquelin K, Hoogenboezem T, Jachtenberg JW, Dumke R, Jacobs E, Puppels GJ, Hartwig NG, Vink C. 2009. Raman spectroscopic typing reveals the presence of carotenoids in *Mycoplasma pneumoniae*. Microbiology 155:2068–2077. https://doi.org/10.1099/mic.0.026724-0.
345. Dumke R, Luck PC, Noppen C, Schaefer C, von Baum H, Marre R, Jacobs E. 2006. Culture-independent molecular subtyping of *Mycoplasma pneumoniae* in clinical samples. J Clin Microbiol 44:2567–2570. https://doi.org/10.1128/JCM.00495-06.
346. Waites KB, Lysynyansky I, Bebear CM. 2014. Emerging antimicrobial resistance in mycoplasmas of humans and animals, p 289–322. Caister Academic Press, Norfolk, UK.
347. Waites KB, Crabb DM, Duffy LB. 2009. Comparative in vitro susceptibilities of human mycoplasmas and ureaplasmas to a new investigational ketolide, CEM-101. Antimicrob Agents Chemother 53:2139–2141. https://doi.org/10.1128/AAC.00090-09.
348. Pereyre S, Guyot C, Renaudin H, Charron A, Bebear C, Bebear CM. 2004. In vitro selection and characterization of resistance to macrolides and related antibiotics in *Mycoplasma pneumoniae*. Antimicrob Agents Chemother 48:460–465. https://doi.org/10.1128/AAC.48.2.460-465.2004.
349. Li BB, Shen JZ, Cao XY, Wang Y, Dai L, Huang SY, Wu CM. 2010. Mutations in 23S rRNA gene associated with decreased susceptibility to tiamulin and valnemulin in *Mycoplasma gallisepticum*. FEMS Microbiol Lett 308:144–149. https://doi.org/10.1111/j.1574-6968.2010.02003.x.
350. Niitu Y, Hasegawa S, Suetake T, Kubota H, Komatsu S, Horikawa M. 1970. Resistance of *Mycoplasma pneumoniae* to erythromycin and other antibiotics. J Pediatr 76:438–443. https://doi.org/10.1016/S0022-3476(70)80485-1.
351. Stopler T, Gerichter CB, Branski D. 1980. Antibiotic-resistant mutants of *Mycoplasma pneumoniae*. Isr J Med Sci 16:169–173.
352. Lucier TS, Heitzman K, Liu SK, Hu PC. 1995. Transition mutations in the 23S rRNA of erythromycin-resistant isolates of *Mycoplasma pneumoniae*. Antimicrob Agents Chemother 39:2770–2773. https://doi.org/10.1128/AAC.39.12.2770.
353. Okazaki N, Narita M, Yamada S, Izumikawa K, Umetsu M, Kenri T, Sasaki Y, Arakawa Y, Sasaki T. 2001. Characteristics of macrolide-resistant *Mycoplasma pneumoniae* strains isolated from patients and induced with erythromycin in vitro. Microbiol Immunol 45:617–620. https://doi.org/10.1111/j.1348-0421.2001.tb01293.x.
354. Matsuoka M, Narita M, Okazaki N, Ohya H, Yamazaki T, Ouchi K, Suzuki I, Andoh T, Kenri T, Sasaki Y, Horino A, Shintani M, Arakawa Y, Sasaki T. 2004. Characterization and molecular analysis of macrolide-resistant *Mycoplasma pneumoniae* clinical isolates obtained in Japan. Antimicrob Agents Chemother 48:4624–4630. https://doi.org/10.1128/AAC.48.12.4624-4630.2004.
355. Okazaki N, Ohya H, Sasaki T. 2007. *Mycoplasma pneumoniae* isolated from patients with respiratory infection in Kanagawa Prefecture in 1976-2006: emergence of macrolide-resistant strains. Jpn J Infect Dis 60:325–326.

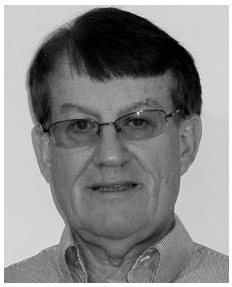
356. Wang Y, Qiu S, Yang G, Song L, Su W, Xu Y, Jia L, Wang L, Hao R, Zhang C, Liu J, Fu X, He J, Zhang J, Li Z, Song H. 2012. An outbreak of *Mycoplasma pneumoniae* caused by a macrolide-resistant isolate in a nursery school in China. Antimicrob Agents Chemother 56:3748–3752. https://doi.org/10.1128/AAC.00142-12.
357. Ho PL, Law PY, Chan BW, Wong CW, To KK, Chiu SS, Cheng VC, Yam WC. 2015. Emergence of macrolide-resistant *Mycoplasma pneumoniae* in Hong Kong is linked to increasing macrolide resistance in multilocus variable-number tandem-repeat analysis type 4-5-7-2. J Clin Microbiol 53:3560–3564. https://doi.org/10.1128/JCM.01983-15.
358. Kogoj R, Mrvic T, Praprotnik M, Kese D. 2015. Prevalence, genotyping and macrolide resistance of *Mycoplasma pneumoniae* among isolates of patients with respiratory tract infections, Central Slovenia, 2006 to 2014. Euro Surveill 20(37):pii=30018. https://doi.org/10.2807/1560-7917.ES.2015.20.37.30018.
359. Dumke R, Luck C, Jacobs E. 2013. Low rate of macrolide resistance in *Mycoplasma pneumoniae* strains in Germany between 2009 and 2012. Antimicrob Agents Chemother 57:3460. https://doi.org/10.1128/AAC.00706-13.
360. Pereyre S, Touati A, Petitjean-Lecherbonnier J, Charron A, Vabret A, Bebear C. 2013. The increased incidence of *Mycoplasma pneumoniae* in France in 2011 was polyclonal, mainly involving *M. pneumoniae* type 1 strains. Clin Microbiol Infect 19:E212–E217. https://doi.org/10.1111/1469-0691.12107.
361. Cardinale F, Chironna M, Dumke R, Binetti A, Daleno C, Sallustio A, Valzano A, Esposito S. 2011. Macrolide-resistant *Mycoplasma pneumoniae* in paediatric pneumonia. Eur Respir J 37:1522–1524. https://doi.org/10.1183/09031936.00172510.
362. Averbuch D, Hidalgo-Grass C, Moses AE, Engelhard D, Nir-Paz R. 2011. Macrolide resistance in *Mycoplasma pneumoniae*, Israel, 2010. Emerg Infect Dis 17:1079–1082. https://doi.org/10.3201/eid/1706.101558.
363. Spuesens EB, Meijer A, Bierschenk D, Hoogenboezem T, Donker GA, Hartwig NG, Koopmans MP, Vink C, van Rossum AM. 2012. Macrolide resistance determination and molecular typing of *Mycoplasma pneumoniae* in respiratory specimens collected between 1997 and 2008 in The Netherlands. J Clin Microbiol 50:1999–2004. https://doi.org/10.1128/JCM.00400-12.
364. Critchley IA, Jones ME, Heinze PD, Hubbard D, Engler HD, Evangelista AT, Thornsberry C, Karlowsky JA, Sahm DF. 2002. In vitro activity of levofloxacin against contemporary clinical isolates of *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* from North America and Europe. Clin Microbiol Infect 8:214–221. https://doi.org/10.1046/j.1469-0691.2002.00392.x.
365. Caballero JDD, del Campo R, Mafe MDC, Galvez M, Rodriguez-Dominguez M, Canton R, Meseguer MA, Hermida JM. 2014. First report of macrolide resistance in a *Mycoplasma pneumoniae* isolate causing community-acquired pneumonia in Spain. Antimicrob Agents Chemother 58:1265–1266. https://doi.org/10.1128/AAC.02325-13.
366. Saegeman V, Proesmans M, Dumke R. 2012. Management of macrolide-resistant *Mycoplasma pneumoniae* infection. Pediatr Infect Dis J 31:1210–1211. https://doi.org/10.1097/INF.0b013e3182611cee.
367. Li X, Atkinson TP, Hagood J, Makris C, Duffy LB, Waites KB. 2009. Emerging macrolide resistance in *Mycoplasma pneumoniae* in children: detection and characterization of resistant isolates. Pediatr Infect Dis J 28:693–696. https://doi.org/10.1097/INF.0b013e31819e3f7a.
368. Wolff BJ, Thacker WL, Schwartz SB, Winchell JM. 2008. Detection of macrolide resistance in *Mycoplasma pneumoniae* by real-time PCR and high-resolution melt analysis. Antimicrob Agents Chemother 52:3542–3549. https://doi.org/10.1128/AAC.00582-08.
369. Yamada M, Buller R, Bledsoe S, Storch GA. 2011. Rising rates of macrolide-resistant *Mycoplasma pneumoniae* in the central United States. Pediatr Infect Dis J https://doi.org/10.1097/INF.0b013e318247f3e0.
370. Eshaghi A, Memari N, Tang P, Olsha R, Farrell DJ, Low DE, Gubbay JB, Patel SN. 2013. Macrolide-resistant *Mycoplasma pneumoniae* in humans, Ontario, Canada, 2010-2011. Emerg Infect Dis https://doi.org/10.3201/eid1909.121466.
371. Chironna M, Sallustio A, Esposito S, Perulli M, Chinellato I, Di Bari C, Quarto M, Cardinale F. 2011. Emergence of macrolide-resistant strains during an outbreak of *Mycoplasma pneumoniae* infections in children. J Antimicrob Chemother 66:734–737. https://doi.org/10.1093/jac/dkr003.
372. Itagaki T, Suzuki Y, Seto J, Abiko C, Mizuta K, Matsuzaki Y. 2013. Two cases of macrolide resistance in *Mycoplasma pneumoniae* acquired during the treatment period. J Antimicrob Chemother 68:724–725. https://doi.org/10.1093/jac/dks454.
373. Dumke R, Stolz S, Jacobs E, Juretzek T. 2014. Molecular characterization of macrolide resistance of a *Mycoplasma pneumoniae* strain that developed during therapy of a patient with pneumonia. Int J Infect Dis 29:197–199. https://doi.org/10.1016/j.ijid.2014.07.014.
374. Hantz S, Garnier F, Peuchant O, Menetrey C, Charron A, Ploy MC, Bebear C, Pereyre S. 2012. Multilocus variable-number tandem-repeat analysis-confirmed emergence of a macrolide resistance-associated mutation in *Mycoplasma pneumoniae* during macrolide therapy for interstitial pneumonia in an immunocompromised child. J Clin Microbiol 50:3402–3405. https://doi.org/10.1128/JCM.01248-12.
375. Nilsson AC, Jensen JS, Bjorkman P, Persson K. 2014. Development of macrolide resistance in *Mycoplasma pneumoniae*-infected Swedish patients treated with macrolides. Scand J Infect Dis 46:315–319. https://doi.org/10.3109/00365548.2013.866268.
376. Degrange S, Cazanave C, Charron A, Renaudin H, Bebear C, Bebear CM. 2009. Development of multiple-locus variable-number tandem-repeat analysis for molecular typing of *Mycoplasma pneumoniae*. J Clin Microbiol 47:914–923. https://doi.org/10.1128/JCM.01935-08.
377. Liu JR, Peng Y, Yang HM, Li HM, Zhao SY, Jiang ZF. 2012. Clinical characteristics and predictive factors of refractory *Mycoplasma pneumoniae* pneumonia. Zhonghua Er Ke Za Zhi 50:915–918.
378. Zhao F, Liu G, Wu J, Cao B, Tao X, He L, Meng F, Zhu L, Lv M, Yin Y, Zhang J. 2013. Surveillance of macrolide-resistant *Mycoplasma pneumoniae* in Beijing, China, from 2008 to 2012. Antimicrob Agents Chemother 57:1521–1523. https://doi.org/10.1128/AAC.02060-12.
379. Zhao F, Liu G, Cao B, Wu J, Gu Y, He L, Meng F, Zhu L, Yin Y, Lv M, Zhang J. 2013. Multiple-locus variable-number tandem-repeat analysis of 201 *Mycoplasma pneumoniae* isolates from Beijing, China, from 2008 to 2011. J Clin Microbiol 51:636–639. https://doi.org/10.1128/JCM.02567-12.
380. Zhao F, Lv M, Tao X, Huang H, Zhang B, Zhang Z, Zhang J. 2012. Antibiotic sensitivity of 40 *Mycoplasma pneumoniae* isolates and molecular analysis of macrolide-resistant isolates from Beijing, China. Antimicrob Agents Chemother 56:1108–1109. https://doi.org/10.1128/AAC.05627-11.
381. Spuesens EB, Meyer Sauteur PM, Vink C, van Rossum AM. 2014. *Mycoplasma pneumoniae* infections—does treatment help? J Infect 69(Suppl 1):S42–S46. https://doi.org/10.1016/j.jinf.2014.07.017.
382. Suzuki S, Yamazaki T, Narita M, Okazaki N, Suzuki I, Andoh T, Matsuoka M, Kenri T, Arakawa Y, Sasaki T. 2006. Clinical evaluation of macrolide-resistant *Mycoplasma pneumoniae*. Antimicrob Agents Chemother 50:709–712. https://doi.org/10.1128/AAC.50.2.709-712.2006.
383. Matsubara K, Morozumi M, Okada T, Matsushima T, Komiyama O, Shoji M, Ebihara T, Ubukata K, Sato Y, Akita H, Sunakawa K, Iwata S. 2009. A comparative clinical study of macrolide-sensitive and macrolide-resistant *Mycoplasma pneumoniae* infections in pediatric patients. J Infect Chemother 15:380–383. https://doi.org/10.1007/s10156-009-0715-7.
384. Kawai Y, Miyashita N, Yamaguchi T, Saitoh A, Kondoh E, Fujimoto H, Teranishi H, Inoue M, Wakabayashi T, Akaike H, Ogita S, Kawasaki K, Terada K, Kishi F, Ouchi K. 2012. Clinical efficacy of macrolide antibiotics against genetically determined macrolide-resistant *Mycoplasma pneumoniae* pneumonia in paediatric patients. Respirology 17:354–362. https://doi.org/10.1111/j.1440-1843.2011.02102.x.
385. Cao B, Zhao CJ, Yin YD, Zhao F, Song SF, Bai L, Zhang JZ, Liu YM, Zhang YY, Wang H, Wang C. 2010. High prevalence of macrolide resistance in *Mycoplasma pneumoniae* isolates from adult and adolescent patients with respiratory tract infection in China. Clin Infect Dis 51:189–194. https://doi.org/10.1086/653535.
386. Wu HM, Wong KS, Huang YC, Lai SH, Tsao KC, Lin YJ, Lin TY. 2013. Macrolide-resistant *Mycoplasma pneumoniae* in children in Taiwan. J Infect Chemother 19:782–786. https://doi.org/10.1007/s10156-012-0523-3.
387. Cardinale F, Chironna M, Chinellato I, Principi N, Esposito S. 2013. Clinical relevance of *Mycoplasma pneumoniae* macrolide resistance in children. J Clin Microbiol 51:723–724. https://doi.org/10.1128/JCM.02840-12.
388. Yoo SJ, Kim HB, Choi SH, Lee SO, Kim SH, Hong SB, Sung H, Kim MN. 2012. Differences in the frequency of 23S rRNA gene mutations in *Mycoplasma pneumoniae* between children and adults with community-acquired pneumonia: clinical impact of mutations confer-

ring macrolide resistance. Antimicrob Agents Chemother 56:6393–6396. https://doi.org/10.1128/AAC.01421-12.
389. Ma Z, Zheng Y, Deng J, Ma X, Liu H. 2014. Characterization of macrolide resistance of *Mycoplasma pneumoniae* in children in Shenzhen, China. Pediatr Pulmonol 49:695–700. https://doi.org/10.1002/ppul.22851.
390. Matsuda K, Narita M, Sera N, Maeda E, Yoshitomi H, Ohya H, Araki Y, Kakuma T, Fukuoh A, Matsumoto K. 2013. Gene and cytokine profile analysis of macrolide-resistant *Mycoplasma pneumoniae* infection in Fukuoka, Japan. BMC Infect Dis 13:591. https://doi.org/10.1186/1471-2334-13-591.
391. Morozumi M, Iwata S, Hasegawa K, Chiba N, Takayanagi R, Matsubara K, Nakayama E, Sunakawa K, Ubukata K, Acute Respiratory Diseases Study Group. 2008. Increased macrolide resistance of Mycoplasma pneumoniae in pediatric patients with community-acquired pneumonia. Antimicrob Agents Chemother 52:348–350. https://doi.org/10.1128/AAC.00779-07.
392. Miyashita N, Oka M, Atypical Pathogen Study G, Kawai Y, Yamaguchi T, Ouchi K. 2010. Macrolide-resistant *Mycoplasma pneumoniae* in adults with community-acquired pneumonia. Int J Antimicrob Agents 36:384–385. https://doi.org/10.1016/j.ijantimicag.2010.06.009.
393. Zhou Z, Li X, Chen X, Luo F, Pan C, Zheng X, Tan F. 2015. Macrolide-resistant *Mycoplasma pneumoniae* in adults in Zhejiang, China. Antimicrob Agents Chemother 59:1048–1051. https://doi.org/10.1128/AAC.04308-14.
394. Miyashita N, Kawai Y, Akaike H, Ouchi K, Hayashi T, Kurihara T, Okimoto N, Atypical Pathogen Study Group. 2012. Macrolide-resistant *Mycoplasma pneumoniae* in adolescents with community-acquired pneumonia. BMC Infect Dis 12:126. https://doi.org/10.1186/1471-2334-12-126.
395. Zhou Y, Zhang Y, Sheng Y, Zhang L, Shen Z, Chen Z. 2014. More complications occur in macrolide-resistant than in macrolide-sensitive *Mycoplasma pneumoniae* pneumonia. Antimicrob Agents Chemother 58:1034–1038. https://doi.org/10.1128/AAC.01806-13.
396. Narita M, Tanaka H. 2007. Cytokines involved in the severe manifestations of pulmonary diseases caused by *Mycoplasma pneumoniae*. Pediatr Pulmonol 42:397. https://doi.org/10.1002/ppul.20445.
397. Hsieh YC, Tsao KC, Huang CG, Tong S, Winchell JM, Huang YC, Shia SH, Lai SH, Lin TY. 2012. Life-threatening pneumonia caused by macrolide-resistant *Mycoplasma pneumoniae*. Pediatr Infect Dis J 31:208–209. https://doi.org/10.1097/INF.0b013e318234597c.
398. Koga S, Ishiwada N, Honda Y, Okunushi T, Hishiki H, Ouchi K, Kohno Y. 2012. A case of meningoencephalitis associated with macrolide-resistant *Mycoplasma pneumoniae* infection. Pediatr Int 54:724–726. https://doi.org/10.1111/j.1442-200X.2012.03588.x.
399. Gardiner SJ, Gavranich JB, Chang AB. 2015. Antibiotics for community-acquired lower respiratory tract infections secondary to Mycoplasma pneumoniae in children. Cochrane Database Syst Rev 1:CD004875. https://doi.org/10.1002/14651858.CD004875.pub5.
400. Biondi E, McCulloh R, Alverson B, Klein A, Dixon A. 2014. Treatment of mycoplasma pneumonia: a systematic review. Pediatrics 133:1081–1090. https://doi.org/10.1542/peds.2013-3729.
401. Morozumi M, Takahashi T, Ubukata K. 2010. Macrolide-resistant *Mycoplasma pneumoniae*: characteristics of isolates and clinical aspects of community-acquired pneumonia. J Infect Chemother 16:78–86. https://doi.org/10.1007/s10156-009-0021-4.
402. To KK, Chan KH, Fung YF, Yuen KY, Ho PL. 2010. Azithromycin treatment failure in macrolide-resistant *Mycoplasma pneumoniae* pneumonia. Eur Respir J 36:969–971. https://doi.org/10.1183/09031936.00041910.
403. Isozumi R, Yoshimine H, Morozumi M, Ubukata K, Ariyoshi K. 2009. Adult community-acquired pneumonia caused by macrolide resistant *Mycoplasma pneumoniae*. Respirology 14:1206–1208. https://doi.org/10.1111/j.1440-1843.2009.01619.x.
404. Atkinson TP, Boppana S, Theos A, Clements LS, Xiao L, Waites K. 2011. Stevens-Johnson syndrome in a boy with macrolide-resistant *Mycoplasma pneumoniae* pneumonia. Pediatrics 127:e1605–e1609. https://doi.org/10.1542/peds.2010-2624.
405. Cheong KN, Chiu SS, Chan BW, To KK, Chan EL, Ho PL. 2016. Severe macrolide-resistant *Mycoplasma pneumoniae* pneumonia associated with macrolide failure. J Microbiol Immunol Infect 49:127–130. https://doi.org/10.1016/j.jmii.2014.11.003.
406. Kawai Y, Miyashita N, Kubo M, Akaike H, Kato A, Nishizawa Y, Saito A, Kondo E, Teranishi H, Ogita S, Tanaka T, Kawasaki K, Nakano T, Terada K, Ouchi K. 2013. Therapeutic efficacy of macrolides, minocycline, and tosufloxacin against macrolide-resistant *Mycoplasma pneumoniae* pneumonia in pediatric patients. Antimicrob Agents Chemother 57:2252–2258. https://doi.org/10.1128/AAC.00048-13.
407. Waites KB, Crabb DM, Bing X, Duffy LB. 2003. In vitro susceptibilities to and bactericidal activities of garenoxacin (BMS-284756) and other antimicrobial agents against human mycoplasmas and ureaplasmas. Antimicrob Agents Chemother 47:161–165. https://doi.org/10.1128/AAC.47.1.161-165.2003.
408. Izumikawa K, Watanabe A, Miyashita N, Ishida T, Hosono H, Kushimoto S, Kohno S. 2014. Efficacy and safety of garenoxacin tablets on clinically diagnosed atypical pneumonia: postmarketing surveillance in Japan. J Infect Chemother 20:541–548. https://doi.org/10.1016/j.jiac.2014.05.002.
409. Bebear C, Pereyre S, Peuchant O. 2011. *Mycoplasma pneumoniae*: susceptibility and resistance to antibiotics. Future Microbiol 6:423–431. https://doi.org/10.2217/fmb.11.18.
410. Harris M, Clark J, Coote N, Fletcher P, Harnden A, McKean M, Thomson A, British Thoracic Society Standards of Care Committee. 2011. British Thoracic Society guidelines for the management of community acquired pneumonia in children: update 2011. Thorax 66(Suppl 2):ii1–ii23. https://doi.org/10.1136/thoraxjnl-2011-200598.
411. Uehara S, Sunakawa K, Eguchi H, Ouchi K, Okada K, Kurosaki T, Suzuki H, Tsutsumi H, Haruta T, Mitsuda T, Yamazaki T. 2011. Japanese guidelines for the management of respiratory infectious diseases in children 2007 with focus on pneumonia. Pediatr Int 53:264–276. https://doi.org/10.1111/j.1442-200X.2010.03316.x.
412. Principi N, Esposito S. 2013. Macrolide-resistant *Mycoplasma pneumoniae*: its role in respiratory infection. J Antimicrob Chemother 68:506–511. https://doi.org/10.1093/jac/dks457.
413. Bebear C. 2012. Editorial commentary: infections due to macrolide-resistant *Mycoplasma pneumoniae*: now what? Clin Infect Dis 55:1650–1651. https://doi.org/10.1093/cid/cis791.
414. Waites KB. 2013. Antimicrobial susceptibilities and treatment options for *Mycoplasma pneumoniae* infections in children—is macrolide resistance clinically important? Curr Pediatr Rev 9:279–288. https://doi.org/10.2174/15733963090413122310431 2.
415. Sendi P, Graber P, Lepere F, Schiller P, Zimmerli W. 2008. *Mycoplasma pneumoniae* infection complicated by severe mucocutaneous lesions. Lancet Infect Dis 8:268. https://doi.org/10.1016/S1473-3099(08)70068-9.
416. You SY, Jwa HJ, Yang EA, Kil HR, Lee JH. 2014. Effects of methylprednisolone pulse therapy on refractory *Mycoplasma pneumoniae* pneumonia in children. Allergy Asthma Immunol Res 6:22–26. https://doi.org/10.4168/aair.2014.6.1.22.
417. Luo Z, Luo J, Liu E, Xu X, Liu Y, Zeng F, Li S, Fu Z. 2014. Effects of prednisolone on refractory *Mycoplasma pneumoniae* pneumonia in children. Pediatr Pulmonol 49:377–380. https://doi.org/10.1002/ppul.22752.
418. Lu A, Wang L, Zhang X, Zhang M. 2011. Combined treatment for child refractory *Mycoplasma pneumoniae* pneumonia with ciprofloxacin and glucocorticoid. Pediatr Pulmonol 46:1093–1097. https://doi.org/10.1002/ppul.21481.
419. Oh JW. 2014. The efficacy of glucocorticoid on macrolide resistant *Mycoplasma pneumoniae* in children. Allergy Asthma Immunol Res 6:3–5. https://doi.org/10.4168/aair.2014.6.1.3.
420. Waites KB, Crabb DM, Duffy LB. 2016. In vitro activities of investigational ketiolide WCK 4873 (nafithromycin) and other antimicrobial agents against human mycoplasmas and ureaplasmas, abstr 4912. ASM Microbe, Boston, MA.
421. Pandya M, Chakrabarti A, Rathy S, Katoch R, Venkataraman R, Bhateja P, Mathur T, Kumar GR, Malhotra S, Rao M, Bhadauria T, Barman TK, Das B, Upadhyay D, Bhatnagar PK. 2010. Activity of a novel series of acylides active against community-acquired respiratory pathogens. Int J Antimicrob Agents 36:169–174. https://doi.org/10.1016/j.ijantimicag.2010.03.026.
422. Homma T, Fujimura T, Maki H, Yamano Y, Shimada J, Kuwahara S. 2010. In vitro antibacterial activities of S-013420, a novel bicyclolide, against respiratory tract pathogens. J Antimicrob Chemother 65:1433–1440. https://doi.org/10.1093/jac/dkq147.
423. Sader HS, Paukner S, Ivezic-Schoenfeld Z, Biedenbach DJ, Schmitz FJ, Jones RN. 2012. Antimicrobial activity of the novel pleuromutilin antibiotic BC-3781 against organisms responsible for community-acquired respiratory tract infections (CARTIs). J Antimicrob Chemother 67:1170–1175. https://doi.org/10.1093/jac/dks001.
424. Waites KB, Crabb DM, Duffy LB, Jensen JS, Liu Y, Paukner S. 2017. *In*

vitro activities of lefamulin and other antimicrobial agents against macrolide-susceptible and macrolide-resistant *Mycoplasma pneumoniae* from the United States, Europe, and China. Antimicrob Agents Chemother, 61:e02008-16. https://doi.org/10.1128/AAC.02008-16.
425. Waites KB, Crabb DM, Duffy LB, Huband MD. 2015. In vitro antibacterial activity of AZD0914 against human mycoplasmas and ureaplasmas. Antimicrob Agents Chemother 59:3627–3629. https://doi.org/10.1128/AAC.04945-14.
426. Pucci MJ, Podos SD, Thanassi JA, Leggio MJ, Bradbury BJ, Deshpande M. 2011. In vitro and in vivo profiles of ACH-702, an isothiazoloquinolone, against bacterial pathogens. Antimicrob Agents Chemother 55:2860–2871. https://doi.org/10.1128/AAC.01666-10.
427. Waites KB, Crabb DM, Liu Y, Duffy LB. 2016. In vitro activities of omadacycline (PTK 0796) and other antimicrobial agents against human mycoplasmas and ureaplasmas. Antimicrob Agents Chemother https://doi.org/10.1128/AAC.01734-16.
428. Sun R, Wang L. 2013. Inhibition of *Mycoplasma pneumoniae* growth by FDA-approved anticancer and antiviral nucleoside and nucleobase analogs. BMC Microbiol 13:184. https://doi.org/10.1186/1471-2180-13-184.
429. Balish MF, Distelhorst SL. 2016. Potential molecular targets for narrow-spectrum agents to combat *Mycoplasma pneumoniae* infection and disease. Front Microbiol 7:205. https://doi.org/10.3389/fmicb.2016.00205.
430. Peuchant O, Menard A, Renaudin H, Morozumi M, Ubukata K, Bebear CM, Pereyre S. 2009. Increased macrolide resistance of *Mycoplasma pneumoniae* in France directly detected in clinical specimens by real-time PCR and melting curve analysis. J Antimicrob Chemother 64:52–58. https://doi.org/10.1093/jac/dkp160.
431. Spuesens EB, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C. 2010. Macrolide resistance determination and molecular typing of *Mycoplasma pneumoniae* by pyrosequencing. J Microbiol Methods 82:214–222. https://doi.org/10.1016/j.mimet.2010.06.004.
432. Nummi M, Mannonen L, Puolakkainen M. 2015. Development of a multiplex real-time PCR assay for detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and mutations associated with macrolide resistance in *Mycoplasma pneumoniae* from respiratory clinical specimens. Springerplus 4:684. https://doi.org/10.1186/s40064-015-1457-x.
433. Liu Y, Ye X, Zhang H, Wu Z, Xu X. 2014. Rapid detection of Mycoplasma pneumoniae and its macrolide-resistance mutation by Cycleave PCR. Diagn Microbiol Infect Dis 78:333–337. https://doi.org/10.1016/j.diagmicrobio.2013.12.002.
434. Lin C, Li S, Sun H, Zhao H, Feng Y, Cao L, Yuan Y, Zhang T. 2010. Nested PCR-linked capillary electrophoresis and single-strand conformation polymorphisms for detection of macrolide-resistant *Mycoplasma pneumoniae* in Beijing, China. J Clin Microbiol 48:4567–4572. https://doi.org/10.1128/JCM.00400-10.
435. Ji M, Lee NS, Oh JM, Jo JY, Choi EH, Yoo SJ, Kim HB, Hwang SH, Choi SH, Lee SO, Kim MN, Sung H. 2014. Single-nucleotide polymorphism PCR for the detection of *Mycoplasma pneumoniae* and determination of macrolide resistance in respiratory samples. J Microbiol Methods 102:32–36. https://doi.org/10.1016/j.mimet.2014.04.009.
436. Chan KH, To KK, Chan BW, Li CP, Chiu SS, Yuen KY, Ho PL. 2013. Comparison of pyrosequencing, Sanger sequencing, and melting curve analysis for detection of low-frequency macrolide-resistant Mycoplasma pneumoniae quasispecies in respiratory specimens. J Clin Microbiol 51:2592–2598. https://doi.org/10.1128/JCM.00785-13.
437. Li SL, Sun HM, Zhao HQ, Cao L, Yuan Y, Feng YL, Xue GH. 2012. A single tube modified allele-specific-PCR for rapid detection of erythromycin-resistant *Mycoplasma pneumoniae* in Beijing. Chin Med J (Engl) 125:2671–2676.
438. Touati A, Blouin Y, Sirand-Pugnet P, Renaudin H, Oishi T, Vergnaud G, Bebear C, Pereyre S. 2015. Molecular epidemiology of *Mycoplasma pneumoniae:* genotyping using single nucleotide polymorphisms and SNaPshot technology. J Clin Microbiol 53:3182–3194. https://doi.org/10.1128/JCM.01156-15.
439. Zhao F, Cao B, Li J, Song S, Tao X, Yin Y, He L, Zhang J. 2011. Sequence analysis of the p1 adhesin gene of *Mycoplasma pneumoniae* in clinical isolates collected in Beijing in 2008 to 2009. J Clin Microbiol 49:3000–3003. https://doi.org/10.1128/JCM.00105-11.
440. Dorigo-Zetsma JW, Dankert J, Zaat SA. 2000. Genotyping of *Mycoplasma pneumoniae* clinical isolates reveals eight P1 subtypes within two genomic groups. J Clin Microbiol 38:965–970.
441. Cousin-Allery A, Charron A, de Barbeyrac B, Fremy G, Skov Jensen J, Renaudin H, Bebear C. 2000. Molecular typing of *Mycoplasma pneumoniae* strains by PCR-based methods and pulsed-field gel electrophoresis. Application to French and Danish isolates. Epidemiol Infect 124:103–111. https://doi.org/10.1017/S0950268899003313.
442. Touati A, Cazanave C, Bebear CM. 2013. Strain typing of *Mycoplasma pneumoniae* and its value in epidemiology. Curr Pediatr Rev 9:334–342. https://doi.org/10.2174/15733963090413122311163 2.
443. Sasaki T, Kenri T, Okazaki N, Iseki M, Yamashita R, Shintani M, Sasaki Y, Yayoshi M. 1996. Epidemiological study of *Mycoplasma pneumoniae* infections in Japan based on PCR-restriction fragment length polymorphism of the P1 cytadhesin gene. J Clin Microbiol 34:447–449.
444. Ursi D, Ieven M, van Bever H, Quint W, Niesters HG, Goossens H. 1994. Typing of *Mycoplasma pneumoniae* by PCR-mediated DNA fingerprinting. J Clin Microbiol 32:2873–2875.
445. Ovyn C, van Strijp D, Ieven M, Ursi D, van Gemen B, Goossens H. 1996. Typing of *Mycoplasma pneumoniae* by nucleic acid sequence-based amplification, NASBA. Mol Cell Probes 10:319–324. https://doi.org/10.1006/mcpr.1996.0043.
446. Lluch-Senar M, Cozzuto L, Cano J, Delgado J, Llorens-Rico V, Pereyre S, Bebear C, Serrano L. 2015. Comparative "-omics" in *Mycoplasma pneumoniae* clinical Isolates reveals key virulence factors. PLoS One 10:e0137354. https://doi.org/10.1371/journal.pone.0137354.
447. Dumke R, Catrein I, Pirkil E, Herrmann R, Jacobs E. 2003. Subtyping of *Mycoplasma pneumoniae* isolates based on extended genome sequencing and on expression profiles. Int J Med Microbiol 292:513–525. https://doi.org/10.1078/1438-4221-00231.
448. Catrein I, Dumke R, Weiner J, III, Jacobs E, Herrmann R. 2004. Cross-complementation between the products of the genes P1 and ORF6 of *Mycoplasma pneumoniae* subtypes 1 and 2. Microbiology 150:3989–4000. https://doi.org/10.1099/mic.0.27506-0.
449. Dumke R, Von Baum H, Luck PC, Jacobs E. 2010. Subtypes and variants of Mycoplasma pneumoniae: local and temporal changes in Germany 2003-2006 and absence of a correlation between the genotype in the respiratory tract and the occurrence of genotype-specific antibodies in the sera of infected patients. Epidemiol Infect 138:1829–1837. https://doi.org/10.1017/S0950268810000622.
450. Dumke R, Jacobs E. 2011. Culture-independent multi-locus variable-number tandem-repeat analysis (MLVA) of *Mycoplasma pneumoniae*. J Microbiol Methods 86:393–396. https://doi.org/10.1016/j.mimet.2011.06.008.
451. Xue G, Wang Q, Yan C, Jeoffreys N, Wang L, Li S, Gilbert GL, Sun H. 2014. Molecular characterizations of PCR-positive Mycoplasma pneumoniae specimens collected from Australia and China. J Clin Microbiol 52:1478–1482. https://doi.org/10.1128/JCM.03366-13.
452. Benitez AJ, Diaz MH, Wolff BJ, Pimentel G, Njenga MK, Estevez A, Winchell JM. 2012. Multilocus variable-number tandem-repeat analysis of *Mycoplasma pneumoniae* clinical isolates from 1962 to the present: a retrospective study. J Clin Microbiol 50:3620–3626. https://doi.org/10.1128/JCM.01755-12.
453. Sun H, Xue G, Yan C, Li S, Cao L, Yuan Y, Zhao H, Feng Y, Wang L, Fan Z. 2013. Multiple-locus variable-number tandem-repeat analysis of *Mycoplasma pneumoniae* clinical specimens and proposal for amendment of MLVA nomenclature. PLoS One 8:e64607. https://doi.org/10.1371/journal.pone.0064607.
454. Chalker VJ, Pereyre S, Dumke R, Winchell J, Khosla P, Sun H, Yan C, Vink C, Bebear C. 2015. International Mycoplasma pneumoniae typing study: interpretation of M. pneumoniae multilocus variable-number tandem-repeat analysis. New Microbes New Infect 7:37–40. https://doi.org/10.1016/j.nmni.2015.05.005.
455. Yan C, Sun H, Lee S, Selvarangan R, Qin X, Tang YW, Waites KB, Zheng X. 2015. Comparison of molecular characteristics of *Mycoplasma pneumoniae* collected from U.S. and China. J Clin Microbiol https://doi.org/10.1128/JCM.02468-15.
456. Yan C, Sun H, Xue G, Zhao H, Wang L, Feng Y, Li S. 2014. A single-tube multiple-locus variable-number tandem-repeat analysis of *Mycoplasma pneumoniae* clinical specimens by use of multiplex PCR-capillary electrophoresis. J Clin Microbiol 52:4168–4171. https://doi.org/10.1128/JCM.02178-14.
457. Unemo M, Dillon JA. 2011. Review and international recommendation of methods for typing *Neisseria gonorrhoeae* isolates and their implications for improved knowledge of gonococcal epidemiology, treatment, and biology. Clin Microbiol Rev 24:447–458. https://doi.org/10.1128/CMR.00040-10.

458. Brown RJ, Holden MT, Spiller OB, Chalker VJ. 2015. Development of a multilocus sequence typing scheme for molecular typing of *Mycoplasma pneumoniae*. J Clin Microbiol 53:3195–3203. https://doi.org/10.1128/JCM.01301-15.
459. Wodke JA, Alibes A, Cozzuto L, Hermoso A, Yus E, Lluch-Senar M, Serrano L, Roma G. 2015. MyMpn: a database for the systems biology model organism Mycoplasma pneumoniae. Nucleic Acids Res 43:D618–623. https://doi.org/10.1093/nar/gku1105.
460. Dandekar T, Huynen M, Regula JT, Ueberle B, Zimmermann CU, Andrade MA, Doerks T, Sanchez-Pulido L, Snel B, Suyama M, Yuan YP, Herrmann R, Bork P. 2000. Re-annotating the Mycoplasma pneumoniae genome sequence: adding value, function and reading frames. Nucleic Acids Res 28:3278–3288. https://doi.org/10.1093/nar/28.17.3278.
461. Rechnitzer H, Rottem S, Herrmann R. 2013. Reconstitution of an active arginine deiminase pathway in *Mycoplasma pneumoniae* M129. Infect Immun 81:3742–3749. https://doi.org/10.1128/IAI.00441-13.
462. Li S, Sun H, Liu F, Feng Y, Zhao H, Xue G, Yan C. 2016. Two case reports: whole genome sequencing of two clinical macrolide-resistant *Mycoplasma pneumoniae* isolates with different responses to azithromycin. Medicine (Baltimore, MD) 95:e4963. https://doi.org/10.1097/MD.0000000000004963.
463. Shahbaaz M, Bisetty K, Ahmad F, Hassan MI. 2015. In silico approaches for the identification of virulence candidates amongst hypothetical proteins of *Mycoplasma pneumoniae* 309. Comput Biol Chem 59A:67–80. https://doi.org/10.1016/j.compbiolchem.2015.09.007.
464. Zhang J, Song X, Ma MJ, Xiao L, Kenri T, Sun H, Ptacek T, Li S, Waites KB, Atkinson TP, Shibayama K, Dybvig K, Feng Y. 2016. Inter- and intra-strain variability of tandem repeats in *Mycoplasma pneumoniae* based on next-generation sequencing data. Future Microbiol https://doi.org/10.2217/fmb-2016-0111.
465. Lluch-Senar M, Luong K, Llorens-Rico V, Delgado J, Fang G, Spittle K, Clark TA, Schadt E, Turner SW, Korlach J, Serrano L. 2013. Comprehensive methylome characterization of *Mycoplasma genitalium* and *Mycoplasma pneumoniae* at single-base resolution. PLoS Genet 9:e1003191. https://doi.org/10.1371/journal.pgen.1003191.
466. Weiner J, III, Herrmann R, Browning GF. 2000. Transcription in Mycoplasma pneumoniae. Nucleic Acids Res 28:4488–4496. https://doi.org/10.1093/nar/28.22.4488.
467. Guell M, van Noort V, Yus E, Chen WH, Leigh-Bell J, Michalodimitrakis K, Yamada T, Arumugam M, Doerks T, Kuhner S, Rode M, Suyama M, Schmidt S, Gavin AC, Bork P, Serrano L. 2009. Transcriptome complexity in a genome-reduced bacterium. Science 326:1268–1271. https://doi.org/10.1126/science.1176951.
468. Kuhner S, van Noort V, Betts MJ, Leo-Macias A, Batisse C, Rode M, Yamada T, Maier T, Bader S, Beltran-Alvarez P, Castano-Diez D, Chen WH, Devos D, Guell M, Norambuena T, Racke I, Rybin V, Schmidt A, Yus E, Aebersold R, Herrmann R, Bottcher B, Frangakis AS, Russell RB, Serrano L, Bork P, Gavin AC. 2009. Proteome organization in a genome-reduced bacterium. Science 326:1235–1240. https://doi.org/10.1126/science.1176343.
469. Llorens-Rico V, Cano J, Kamminga T, Gil R, Latorre A, Chen WH, Bork P, Glass JI, Serrano L, Lluch-Senar M. 2016. Bacterial antisense RNAs are mainly the product of transcriptional noise. Sci Adv 2:e1501363. https://doi.org/10.1126/sciadv.1501363.
470. Junier I, Unal EB, Yus E, Llorens-Rico V, Serrano L. 2016. Insights into the mechanisms of basal coordination of transcription using a genome-reduced bacterium. Cell Syst 2:391–401. https://doi.org/10.1016/j.cels.2016.04.015.
471. van Noort V, Seebacher J, Bader S, Mohammed S, Vonkova I, Betts MJ, Kuhner S, Kumar R, Maier T, O'Flaherty M, Rybin V, Schmeisky A, Yus E, Stulke J, Serrano L, Russell RB, Heck AJ, Bork P, Gavin AC. 2012. Cross-talk between phosphorylation and lysine acetylation in a genome-reduced bacterium. Mol Syst Biol 8:571. https://doi.org/10.1038/msb.2012.4.
472. Linchevski I, Klmenet E, Nir-Paz R. 2009. Mycoplasma pneumoniae vaccine protective efficacy and adverse reactions—systematic review and meta-analysis. Vaccine 27:2437–2446. https://doi.org/10.1016/j.vaccine.2009.01.135.
473. Bose SR, Balish MF, Krause DC. 2009. *Mycoplasma pneumoniae* cytoskeletal protein HMW2 and the architecture of the terminal organelle. J Bacteriol 191:6741–6748. https://doi.org/10.1128/JB.01486-08.
474. Boonmee A, Ruppert T, Herrmann R. 2009. The gene mpn310 (hmw2) from *Mycoplasma pneumoniae* encodes two proteins, HMW2 and HMW2-s, which differ in size but use the same reading frame. FEMS Microbiol Lett 290:174–181. https://doi.org/10.1111/j.1574-6968.2008.01422.x.
475. Cimolai N, Cheong AC. 1996. An assessment of a new diagnostic indirect enzyme immunoassay for the detection of anti-*Mycoplasma pneumoniae* IgM. Am J Clin Pathol 105:205–209. https://doi.org/10.1093/ajcp/105.2.205.
476. Alexander TS, Gray LD, Kraft JA, Leland DS, Nikaido MT, Willis DH. 1996. Performance of Meridian ImmunoCard Mycoplasma test in a multicenter clinical trial. J Clin Microbiol 34:1180–1183.
477. Matas L, Dominguez J, De Ory F, Garcia N, Gali N, Cardona PJ, Hernandez A, Rodrigo C, Ausina V. 1998. Evaluation of Meridian ImmunoCard Mycoplasma test for the detection of *Mycoplasma pneumoniae*-specific IgM in paediatric patients. Scand J Infect Dis 30:289–293. https://doi.org/10.1080/00365549850160954.
478. Thacker WL, Talkington DF. 2000. Analysis of complement fixation and commercial enzyme immunoassays for detection of antibodies to *Mycoplasma pneumoniae* in human serum. Clin Diagn Lab Immunol 7:778–780.
479. Thurman KA, Walter ND, Schwartz SB, Mitchell SL, Dillon MT, Baughman AL, Deutscher M, Fulton JP, Tongren JE, Hicks LA, Winchell JM. 2009. Comparison of laboratory diagnostic procedures for detection of *Mycoplasma pneumoniae* in community outbreaks. Clin Infect Dis 48:1244–1249. https://doi.org/10.1086/597775.
480. Thacker WL, Talkington DF. 1995. Comparison of two rapid commercial tests with complement fixation for serologic diagnosis of *Mycoplasma pneumoniae* infections. J Clin Microbiol 33:1212–1214.
481. Fedorko DP, Emery DD, Franklin SM, Congdon DD. 1995. Evaluation of a rapid enzyme immunoassay system for serologic diagnosis of *Mycoplasma pneumoniae* infection. Diagn Microbiol Infect Dis 23:85–88. https://doi.org/10.1016/0732-8893(95)00175-1.
482. Sobieszczanska BM, Kasprzykowska U, Duda-Madej A, Secewicz A, Marciniak J, Gosciniak G. 2014. Relevance of serology for *Mycoplasma pneumoniae* infection among children with persistent cough. Adv Clin Exp Med 23:185–190. https://doi.org/10.17219/acem/37046.
483. Rastawicki W, Kaluzewski S, Jagielski M, Gierczynski R. 2002. Evaluation of commercial usefulness for microparticle agglutination Serodia-Myco II test for serodiagnosis of *Mycoplasma pneumoniae* infections. Med Dosw Mikrobiol 54:67–73.
484. Lieberman D, Horowitz S, Horovitz O, Schlaeffer F, Porath A. 1995. Microparticle agglutination versus antibody-capture enzyme immunoassay for diagnosis of community-acquired *Mycoplasma pneumoniae* pneumonia. Eur J Clin Microbiol Infect Dis 14:577–584. https://doi.org/10.1007/BF01690728.
485. Barker CE, Sillis M, Wreghitt TG. 1990. Evaluation of Serodia Myco II particle agglutination test for detecting *Mycoplasma pneumoniae* antibody: comparison with mu-capture ELISA and indirect immunofluorescence. J Clin Pathol 43:163–165. https://doi.org/10.1136/jcp.43.2.163.
486. Pierce VM, Elkan M, Leet M, McGowan KL, Hodinka RL. 2012. Comparison of the Idaho Technology FilmArray system to real-time PCR for detection of respiratory pathogens in children. J Clin Microbiol 50:364–371. https://doi.org/10.1128/JCM.05996-11.
487. Morozumi M, Hasegawa K, Kobayashi R, Inoue N, Iwata S, Kuroki H, Kawamura N, Nakayama E, Tajima T, Shimizu K, Ubukata K. 2005. Emergence of macrolide-resistant *Mycoplasma pneumoniae* with a 23S rRNA gene mutation. Antimicrob Agents Chemother 49:2302–2306. https://doi.org/10.1128/AAC.49.6.2302-2306.2005.
488. Kawai Y, Miyashita N, Kubo M, Akaike H, Kato A, Nishizawa Y, Saito A, Kondo E, Teranishi H, Wakabayashi T, Ogita S, Tanaka T, Kawasaki K, Nakano T, Terada K, Ouchi K. 2013. Nationwide surveillance of macrolide-resistant *Mycoplasma pneumoniae* infection in pediatric patients. Antimicrob Agents Chemother 57:4046–4049. https://doi.org/10.1128/AAC.00663-13.
489. Suzuki Y, Itagaki T, Seto J, Kaneko A, Abiko C, Mizuta K, Matsuzaki Y. 2013. Community outbreak of macrolide-resistant *Mycoplasma pneumoniae* in Yamagata, Japan in 2009. Pediatr Infect Dis J 32:237–240. https://doi.org/10.1097/INF.0b013e31827aa7bd.
490. Komatsu H, Tsunoda T, Inui A, Sogo T, Fujisawa T. 2014. Characteristics of hospitalized children infected with macrolide-resistant *Mycoplasma pneumoniae*. Braz J Infect Dis 18:294–299. https://doi.org/10.1016/j.bjid.2013.09.004.
491. Akaike H, Miyashita N, Kubo M, Kawai Y, Tanaka T, Ogita S, Kawasaki K, Nakano T, Terada K, Ouchi K, Atypical Pathogen Study Group. 2012. In vitro activities of 11 antimicrobial agents against macrolide-resistant Mycoplasma pneumoniae isolates from pediatric patients: results from

a multicenter surveillance study. Jpn J Infect Dis 65:535–538. https://doi.org/10.7883/yoken.65.535.
492. Katsushima Y, Katsushima F, Suzuki Y, Seto J, Mizuta K, Nishimura H, Matsuzaki Y. 2015. Characteristics of *Mycoplasma pneumoniae* infection identified on culture in a pediatric clinic. Pediatr Int 57:247–252. https://doi.org/10.1111/ped.12513.
493. Liu Y, Ye X, Zhang H, Xu X, Li W, Zhu D, Wang M. 2009. Antimicrobial susceptibility of *Mycoplasma pneumoniae* isolates and molecular analysis of macrolide-resistant strains from Shanghai, China. Antimicrob Agents Chemother 53:2160–2162. https://doi.org/10.1128/AAC.01684-08.
494. Xin D, Mi Z, Han X, Qin L, Li J, Wei T, Chen X, Ma S, Hou A, Li G, Shi D. 2009. Molecular mechanisms of macrolide resistance in clinical isolates of *Mycoplasma pneumoniae* from China. Antimicrob Agents Chemother 53:2158–2159. https://doi.org/10.1128/AAC.01563-08.
495. Liu Y, Ye X, Zhang H, Xu X, Wang M. 2012. Multiclonal origin of macrolide-resistant *Mycoplasma pneumoniae* isolates as determined by multilocus variable-number tandem-repeat analysis. J Clin Microbiol 50:2793–2795. https://doi.org/10.1128/JCM.00678-12.
496. Liu Y, Ye X, Zhang H, Xu X, Li W, Zhu D, Wang M. 2010. Characterization of macrolide resistance in *Mycoplasma pneumoniae* isolated from children in Shanghai, China. Diagn Microbiol Infect Dis 67:355–358. https://doi.org/10.1016/j.diagmicrobio.2010.03.004.
497. Bao F, Qu JX, Liu ZJ, Qin XG, Cao B. 2013. The clinical characteristics, treatment and outcome of macrolide-resistant *Mycoplasma pneumoniae* pneumonia in children. Zhonghua Jie He He Hu Xi Za Zhi 36:756–761.
498. Lung DC, Yip EK, Lam DS, Que TL. 2013. Rapid defervescence after doxycycline treatment of macrolide-resistant *Mycoplasma pneumoniae*-associated community-acquired pneumonia in children. Pediatr Infect Dis J 32:1396–1399. https://doi.org/10.1097/INF.0b013e3182a25c71.
499. Hong KB, Choi EH, Lee HJ, Lee SY, Cho EY, Choi JH, Kang HM, Lee J, Ahn YM, Kang YH, Lee JH. 2013. Macrolide resistance of *Mycoplasma pneumoniae*, South Korea, 2000-2011. Emerg Infect Dis 19:1281–1284. https://doi.org/10.3201/eid1908.121455.
500. Uh Y, Hong JH, Oh KJ, Cho HM, Park SD, Kim J, Yoon KJ. 2013. Macrolide resistance of *Mycoplasma pneumoniae* and its detection rate by real-time PCR in primary and tertiary care hospitals. Ann Lab Med 33:410–414. https://doi.org/10.3343/alm.2013.33.6.410.
501. Seo YH, Kim JS, Seo SC, Seo WH, Yoo Y, Song DJ, Choung JT. 2014. Predictive value of C-reactive protein in response to macrolides in children with macrolide-resistant *Mycoplasma pneumoniae* pneumonia. Korean J Pediatr 57:186–192. https://doi.org/10.3345/kjp.2014.57.4.186.
502. Dumke R, von Baum H, Luck PC, Jacobs E. 2010. Occurrence of macrolide-resistant *Mycoplasma pneumoniae* strains in Germany. Clin Microbiol Infect 16:613–616. https://doi.org/10.1111/j.1469-0691.2009.02968.x.



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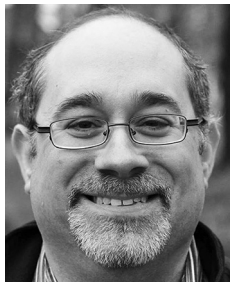

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