MYCOPLASMA PNEUMONIAE CLINICAL MANIFESTATIONS, MICROBIOLOGY, AND IMMUNOLOGY

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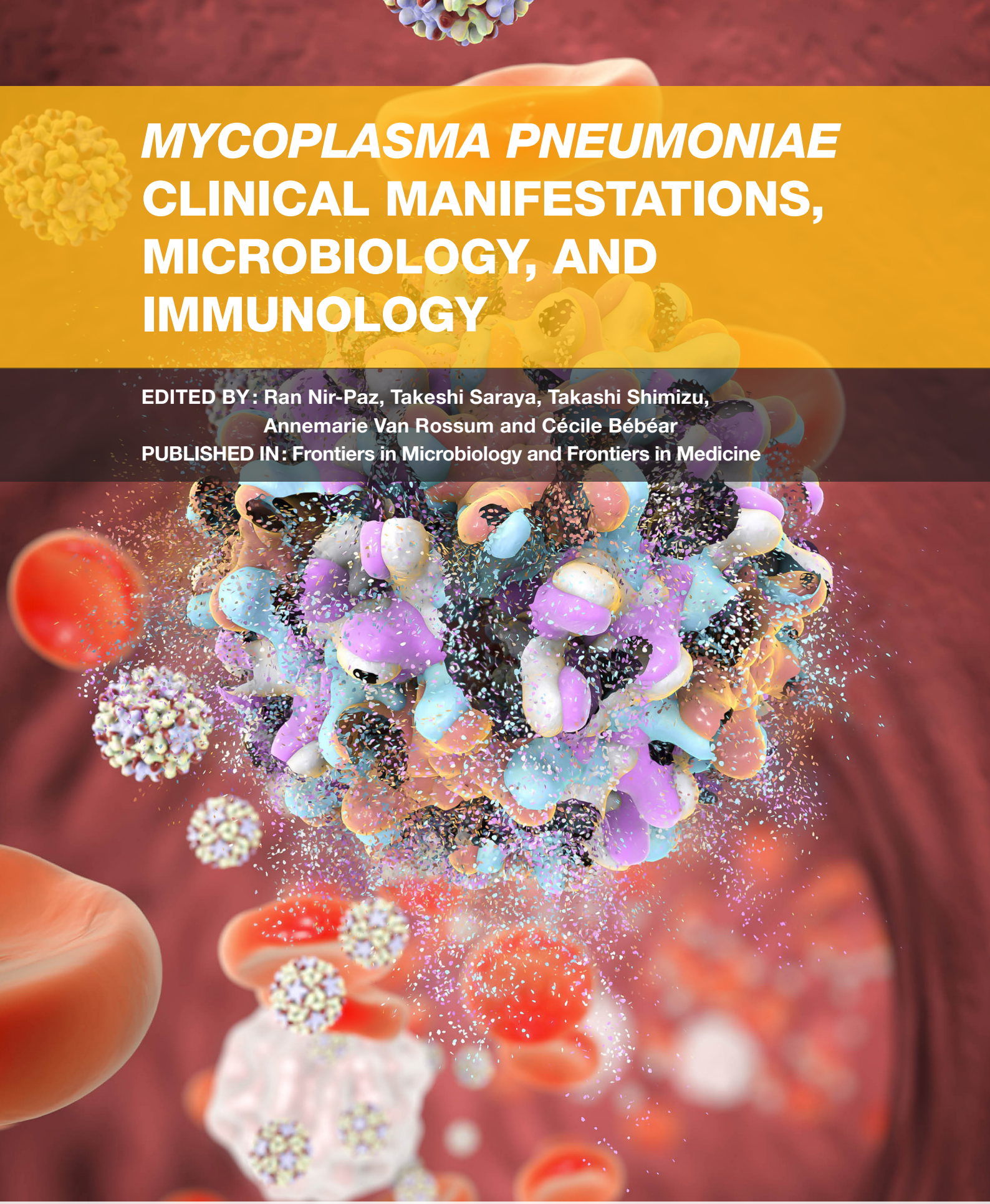
PUBLISHED IN: Frontiers in Microbiology and Frontiers in Medicine



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ISSN 1664-8714
ISBN 978-2-88945-362-7
DOI 10.3389/978-2-88945-362-7

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MYCOPLASMA PNEUMONIAE CLINICAL MANIFESTATIONS, MICROBIOLOGY, AND IMMUNOLOGY

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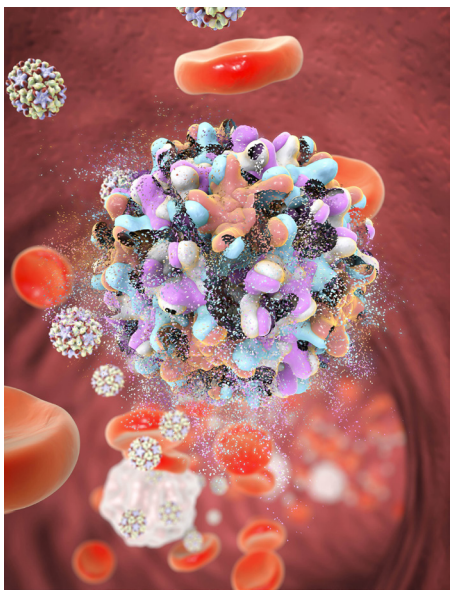

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Mycoplasma pneumoniae (Mp) is a major human pathogen that causes both upper and lower respiratory infections, and is one of the leading causes of community acquired pneumonia (CAP), accounting for 11–15% of CAP throughout the world. Additionally it is known to induce an inflammatory process which depends on several mechanisms such as virulence of Mp (lipoproteins, community acquired respiratory distress syndrome (CARDS) toxin, oxidative products) and host defenses (cellular immunity and humoral immunity). Although it is a common pathogen, the pathogenesis for Mp infections is not yet fully understood. From the clinical point of view, since the pioneer studies in the 1960s and 1970s on the clinical presentation of Mp associated

disease, the diagnostics approaches have changed dramatically leading to a better understanding of the clinical presentation and new issues have emerged - such as antibiotics resistance.

The purpose of this Frontiers ebook is to thoroughly review and discuss the clinical presentation in view of the improved diagnostics, microbiological and immunological analysis of Mp infections, with focus on the history of Mp, clinical features of disease, bacterial structure of Mp and mechanism of gliding, clinical and laboratory diagnostics, the role of lipoproteins and Toll-like receptor, CARDS toxin, subtyping of Mp isolates and genome analysis, macrolide resistance and treatment.

Citation: Nir-Paz, R., Saraya, T., Shimizu, T., Van Rossum, A., Bébéar, C., eds. (2017). *Mycoplasma pneumoniae* Clinical Manifestations, Microbiology, and Immunology. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-362-7

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frontiers in Microbiology

EDITORIAL
published: 29 September 2017
doi: 10.3389/fmicb.2017.01916

Editorial: *Mycoplasma pneumoniae* Clinical Manifestations, Microbiology, and Immunology

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Keywords: ***Mycoplasma pneumoniae*, mycoplasma infections, epidemiology, pneumonia, respiratory tract infections**

Editorial on the Research Topic

Mycoplasma pneumoniae Clinical Manifestations, Microbiology, and Immunology

OPEN ACCESS

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *01 September 2017*
Accepted: *20 September 2017*
Published: *29 September 2017*

Citation:
Nir-Paz R, Saraya T, Shimizu T, Van Rossum A and Bébéar C (2017) Editorial: Mycoplasma pneumoniae Clinical Manifestations, Microbiology, and Immunology. Front. Microbiol. 8:1916. doi: 10.3389/fmicb.2017.01916

Mycoplasma pneumoniae is a major human pathogen that causes both upper and lower respiratory infections, and is one of the leading causes of community-acquired pneumonia (CAP), accounting for 11–15% of CAP throughout the world (Brown et al.; Kishaba; Meyer Sauteur et al.; Saraya). *M. pneumoniae* is also one of the smallest free-living human pathogens, with a genome size of less than 700 genes (Spuesens et al.). In this volume of Frontiers we have explored few of many aspects related to this major human pathogen. The purpose of this topic was to thoroughly review and to create a body of knowledge encompassing both medical and biological information gathered in both the western world and Asia.

Saraya contributed a nice historical perspective on the discovery of *M. pneumoniae* and a few authors contributed an updated and fresh look on the clinical manifestations of *M. pneumoniae* (Kishaba; Meyer Sauteur et al.; Izumikawa; Narita; Tanaka; Parrott et al.). The unique epidemiological challenges presented by this pathogen include both outbreaks and the "Olympic" (3–5 years) intervals between epidemics, which reported here in both Japan and the UK (Brown et al.; Parrott et al.; Yamazaki and Kenri). Since its discovery, the main challenge for diagnostic labs and clinicians was its fastidious growth that led to a reliance on serology, and later on, amplification techniques for diagnosis. Two reviews cover diagnostics here (Diaz and Winchell, Loens and Ieven). An additional review combines the antibody response to epidemiological aspects as a means to explain the epidemiology (Dumke and Jacobs). Pathogenesis is covered by 2 topics: One on the bacterial aspect and the other on the host inflammatory response to *M. pneumoniae* infection (Miyata and Hamaguchi; Shimizu). Additionally, Spuesens et al. explored the possible role of genomics in elucidating the differentiation between sick and carrier patients. Obviously, for each infectious agent, treatment and cure is the goal, if prevention is not possible (yet). However, since *M. pneumoniae* is lacking a cell wall, antibiotic choices are limited to the use of macrolides and related antibiotics, tetracyclines and quinolones, and some of those classes have limited use in the pediatric populations (Meyer Sauteur et al.; Pereyre et al.). Pereyre et al. summarized the emerging macrolide resistance and Balish and Distelhorst explored the possible use of new agents in the future.

Obviously, not everything can be covered in depth and many of the topics explored here raise many new questions that remains to be answered, by the scientific community.

AUTHOR CONTRIBUTIONS

RN wrote the first draft; TSa, TSh, AV, and CB reviewed commented and edited the manuscript. All authors have approved the manuscript.

ACKNOWLEDGMENTS

This work was part of the ESGMI—The ESCMID study group on mycoplasma infections initiative.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 22 March 2016
doi: 10.3389/fmicb.2016.00364

The History of *Mycoplasma pneumoniae* Pneumonia

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In the United States in the 1930s, although the pathogen was not known, atypical pneumonia was clinically distinguished from pneumococcal pneumonia by its resistance to sulfonamides. Reimann (1938) reported seven patients with an unusual form of tracheo bronchopneumonia and severe constitutional symptoms. He believed the clinical picture of this disease differed from that of the disease caused by influenza viruses or known bacteria and instead suspected "primary atypical pneumonia." For many years, the responsible infectious agent was tentatively classified as a filterable virus that could pass through a Seitz filter to remove bacteria and was reported to be a psittacosis-like or new virus. After that, Eaton et al. (1942, 1944, 1945) identified an agent that was the principal cause of primary atypical pneumonia using cotton rats, hamsters, and chick embryos. Eaton et al. (1942, 1944, 1945) did not perform an inoculation study in human volunteers. During the 1940s, there were three groups engaged in discovering the etiology of the primary atypical pneumonia. (1) Commission on Acute Respiratory Diseases Diseases directed by John Dingle, (2) Dr. Monroe Eaton's group, the Virus Research Laboratory of the California State Public Health Department, (3) The Hospital of the Rockefeller Institute for Medical Research directed by Horsfall. During 1940s, the members of the Commission on Acute Respiratory Diseases concluded that the bacteria-free filtrates obtained from the patients, presumably containing a virus, could induce primary atypical pneumonia in human volunteers via Pinehurst trials. During 1950s, serological approaches for identification of the Eaton agent developed such as Fluorescent-Stainable Antibody, and at the beginning of the1960s, the Eaton agent successfully grew in media, and finally accepted as a cause of primary atypical pneumonia. Thus, technical difficulties with visualizing the agent and failure to recognize the full significance of the Pinehurst transmission experiments resulted in a lapse of 20 years before acceptance of the Eaton agent as *Mycoplasma pneumoniae*. This review describes the history of *M. pneumoniae* pneumonia with a special focus on the recognition between the 1930 and 1960s of the Eaton agent as the infectious cause.

Keywords: *Mycoplasma pneumoniae* pneumonia, Eaton agent, Pinehurst trials, primary atypical pneumonia, history

OPEN ACCESS

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *21 November 2015*
Accepted: *07 March 2016*
Published: *22 March 2016*

Citation:
Saraya T (2016) The History of Mycoplasma pneumoniae Pneumonia. Front. Microbiol. 7:364. doi: 10.3389/fmicb.2016.00364

INTRODUCTION

Atypical bacterial pneumonia is caused by atypical organisms that are not detectable on Gram stain and cannot be cultured using standard methods, and characterized by a symptom includes headache, low-grade fever, cough, and malaise. The most common organisms are *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila.* The history

of *C. pneumoniae* began in Taiwan in 1965, which was first isolated from the eye of a child in a trachoma vaccine study and first isolated from the respiratory tract in 1983 from a University of Washington student (Grayston et al., 1986; Grayston, 2000). Among them, *M. pneumoniae* is one of the leading causes of community acquired pneumonia. The term mycoplasma emerged in the 1950s and means "mykes" (fungus) and "plasma" (formed) in Greek. Isolation of the first mycoplasma was the bovine pleuropneumonia agent, now known as *M. mycoides* subsp. *mycoides*, which was reported initially in Nocard and Roux (1898). This bacterium became to know over the next 50 years as pleuropneumonia-like organisms (PPLO) in various animals. Dienes and Edsall (1937) detected first *Mycoplasma* isolated from humans in a Bartholin's gland abscess, known as *M. hominis*. Regarding with *M. pneumoniae*, it was first isolated in tissue culture from the sputum of a patient with primary atypical pneumonia by Eaton et al. (1944) as Eaton agent. However, its taxonomy remained obscure until the early 1960s when it was clearly identified as a bacterium. The cell volume of *M. pneumoniae*, is less than 5% of that of a typical bacillus and rarely exceed 100 μm in diameter. *M. pneumoniae*, lacks a cell wall, which makes it intrinsically resistant to antimicrobials, such as β-lactams. In this regards, identification of the *M. pneumoniae* was a challenging issue for pioneers. This review focus on the history of discovering and acceptance the Eaton agent as the cause of primary atypical pneumonia.

FIGURE 1 | Photograph of Dr. Eaton Eaton, Monroe D., U.S. microbiologist, 1904–1958. The photograph of Dr. Eaton in the manuscript (*Rev Infect Dis*, 12, 338–353), which taken in the early 1960s and reprinted permission was obtained.

ATYPICAL PNEUMONIA-DISCOVERY OF A NEW CLINICAL SYNDROME (1940s)

Reimann (1938, 1984) reported several patients with similar clinical features such as mild symptoms of hoarseness, sore throat, pyrexia with relative bradycardia, and persistent dry cough. The fever lasted from 10 to 43 days in the cases of severe involvement but most typically only lasted about 3 weeks. He believed that those symptoms were strikingly similar to those of patients in a report by Scadding (1937) from London, characterized as gradual onset, malaise, shivering, dyspnea, dry cough, marked sweating, slight leukocytosis, and roentgenographic shadows of diffuse pneumonia. Reimann also indicated that colleagues in other East Coast cities had recognized this syndrome, but it was usually diagnosed as influenza.

Indeed, Meiklejohn et al. described primary atypical pneumonia as being caused by psittacosis-like viruses (Meiklejohn et al., 1944) and/or a new atypical pneumonia virus (Meiklejohn et al., 1945). Around the same time, Dingle described that primary atypical pneumonia of unknown etiology was a more common disease than previously thought (Finland and Dingle, 1942).

DISCOVERY OF THE EATON AGENT AND ASSOCIATED ANIMAL MODELS

Eaton et al. (1942) (**Figure 1**) also reported that an infectious agent obtained from a total of 78 patients with atypical pneumonia was apparently transmissible to cotton rats. Most of the inoculation materials were retrieved from sputum or lung samples from patients with atypical pneumonia and were intranasally inoculated to the cotton rats. Among the total of 131 cotton rats receiving material, 35 developed pneumonia and lung lesions described as patchy and reddish-gray with maximum intensity of illness at 6–8 days after inoculation. The etiological agent was presumably a filterable virus as large as 180–250 μm (infectivity was retained by a membrane of an average pore diameter of 300 μm) that differed from the psittacosis-like virus or other known viruses that were known to infect cotton rats by the intranasal route.

Eaton et al. (1944) demonstrated that both infected chick embryo tissues and instillation of infective human material (sputum from 128 persons having atypical pneumonia and lung tissue from 15 patients who had died of the disease) generated similar pulmonary lesions in the animal models of cotton rats and hamsters. In addition, the agent propagated in chick embryos was specifically neutralized by serum from patients who had recovered from primary atypical pneumonia but was not neutralized by acute phase specimens (Eaton et al., 1944).

Eaton (1950) studied antibiotic therapy in his virus-infected cotton rats and described that the agents causing primary atypical pneumonia were sensitive to aureomycin but were somewhat smaller than viruses of the psittacosis-lymphogranuloma group, which were also inhibited by this drug. Unfortunately, however, the virus inoculated into human volunteers was not studied for its

ability to grow in chicken embryos, and no inoculations of human volunteers were performed with either the virus propagated in chick embryos or chick embryo lung suspensions infected with the Eaton agent. Thus, the organism was identified in Eaton et al. (1944) but its taxonomy remained obscure until the early 1960s when it was clearly identified as a bacterium.

PINEHURST TRIALS

During World War II, management of atypical pneumonia was a serious problem for the United States Army, and the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board performed a series of experiments to investigate this problem (Commission on Respiratory Diseases, 1946d). In October 1943, the Commission on Acute Respiratory Diseases group performed a first transmission study of primary atypical pneumonia to human volunteers (Commission on Acute Respiratory Diseases, 1945) at Fort Bragg, North Carolina, so-called Pinehurst area and demonstrated that unfiltered throat washings and sputa obtained from patients early in the course of the disease caused a respiratory illness in 10 of 12 volunteers.

Next, second and third transmission experiments were conducted during the summer of 1944 (Commission on Respiratory Diseases, 1946a,b,c). The inoculum consisted of throat washings and sputum from patients admitted to Fort Bragg Regional Hospital with atypical pneumonia. Inoculation material was arranged into three patterns (untreated, filtered through Corning sintered glass or Seitz filters, or autoclaved at 15 pounds pressure for 10 min), which was introduced into the nose and throat of each volunteer in synchronization with deep inspiration by means of an atomizer and nebulized three times in a single day.

In the second experiment, each group comprised 12 men, and primary atypical pneumonia occurred equally in the filtered ($n = 4$, 33.3%), untreated ($n = 3$, 25%), and autoclaved ($n = 3$, 25%) groups. The latter group was considered to be due to either contamination of the inner surface of the air pump or cross infection after inoculation.

The third experiment consisted of an autoclaved group ($n = 18$), filtered group ($n = 12$), and untreated group ($n = 12$). The resulting incidence of primary atypical pneumonia in each group was 0%, 25% ($n = 3$), and 25% ($n = 3$), respectively. No cases of pneumonia developed in healthy volunteers who received autoclaved inoculum using rigid precautions during inoculation. The members of the Commission on Acute Respiratory Diseases concluded that the bacteria-free filtrates, presumably containing a virus, could induce primary atypical pneumonia in human volunteers.

Of note, they did not perform the following experiments: (1) direct inoculation of Eaton agent to volunteers, (2) analysis of inoculation materials obtained from patients with primary atypical pneumonia, (3) preinoculation and postinoculation sera from the Pinehurst volunteers in the chick embryo lung/hamster neutralizing antibody assay, (4) determination of cross immunity to Eaton agent in patients with pneumonia in the Pinehurst trial, or (5) neutralization by pretreatment with rabbit antisera to the Eaton agent in further volunteer experiments.

Thus, the failure of collaboration in 1944 between the Commission on Acute Respiratory Diseases members (Dingle, et al.) and the Eaton group left the full significance of the Pinehurst transmission experiments unrealized for 20 years (**Table 1**).

SEROLOGICAL APPROACHES FOR IDENTIFICATION OF THE EATON AGENT

Cold Hemagglutinins

Peterson et al. (1943) reported that the development of cold agglutinins could serve as a criterion for segregating some of

TABLE 1 | History of acceptance of the Eaton agent as a cause of primary atypical pneumonia.

Author	Summary
Dienes and Edsall (1937)	First isolation of *Mycoplasma (Mycoplasma hominis)* from humans
Reimann (1938)	Recognition of symptoms of "atypical pneumonia"
Eaton et al. (1944)	Discovery of Eaton agent
Commission on Acute Respiratory Diseases (1945) directed by Dingle et al.	Pinehurst trials: first trial
Commission on Respiratory Diseases (1946b,c) directed by Dingle et al.	Pinehurst trials: second trial
Commission on Respiratory Diseases (1946b,c) directed by Dingle et al.	Pinehurst trials: third trial
Liu et al. (1959)	Establishment of IF technique
Liu et al. (1959)	
Chanock et al. (1960a)	Eaton agent causes lower respiratory tract infection
Chanock et al. (1960b)	Eaton agent grow in cell culture, monkey kidney tissue culture
Chanock et al. (1961b)	Eaton agent causes lower respiratory tract infection
Clyde et al. (1961)	Fluorescent-stainable antibody to the Eaton agent positive for primary atypical pneumonia
Marmion and Goodburn (1961)	Eaton agent is not a virus
NIH conference (1961)	Acceptance of Eaton agent as a cause of primary atypical pneumonia
Rifkind et al. (1962)	Inoculation of volunteers with Eaton agent
Chanock et al. (1962a)	Successful culture of the Eaton agent on cell-free medium
Chanock (1963)	Taxonomic designation of *M. pneumoniae*

the prevalent cases of primary atypical pneumonia until definite etiological agents could be established. The maximum titer of cold agglutinins (in most cases 1:160 or 1:320 at 0°C) was usually obtained at or near the end of the febrile period, and a rapid decline in titer occurred during convalescence. Dingle and Jordan demonstrated a rise in the titer of cold hemagglutinins in over 80% of inoculated healthy volunteers who had atypical pneumonia or a minor respiratory illness, but the titer was elevated in only one of the patients who did not develop an illness (Commission on Respiratory Diseases, 1946c).

Moreover, correlation of maximum cold hemagglutinin titers with (1) extent of pulmonary involvement, (2) height and duration of fever (Meiklejohn, 1943), and (3) other indices of severity of illness showed no constant trends. Furthermore, Cook et al. (1960) reported that both the hemagglutinin test and streptococcus MG agglutinins frequently failed to develop in patients with atypical pneumonia if the fluorescent antibody test for the Eaton agent was positive.

Streptococcus MG Agglutinins

Serum streptococcus MG agglutinins will rise in some cases of primary atypical pneumonia. However, the Pinehurst trial (Commission on Respiratory Diseases, 1946c) showed that a rise in the titer of agglutinins for streptococcus MG was not associated with primary atypical pneumonia.

Neutralizing Antibody for Eaton's Pleuropneumonia-Like Organisms

Convalescent-phase sera from patients with infections caused by Eaton's pleuropneumonia-like organism (PPLO) had the ability to inhibit growth of the organism (Eaton et al., 1945; Clyde, 1963). However, this test has little diagnostic role in most instances (Horsfall et al., 1943).

Fluorescent-Stainable Antibody for Eaton's PPLO

Liu (1957) described a technique which provided greater facility in making a serologic diagnosis of Eaton agent-related infections. Unlike cold hemagglutinins, fluorescent-stainable antibody elevations develop in the 3rd–4th week of illness, persist for 12–18 months, and appear to be quite sensitive and specific (Liu et al., 1959). In 1960, among patients with primary atypical pneumonia, Cook et al. (1960) established a rise in Eaton fluorescent antibody (FA) titer in 85% of 26 patients with cold and/or streptococcus MG agglutinins and in 26% of 69 patients without cold agglutinins.

Complement Fixation with Eaton's PPLO

Lind et al. (1997) studied the diagnostic yield of the Complement Fixation (CF) test using serum samples from an over 50-year period from 1946 to 1995 in Denmark and reported that the sensitivity and specificity of the CF test were 78 and 92%, respectively, if the patient was considered to have a current or recent *M. pneumoniae* infection when the *M. pneumoniae* CF test demonstrated a ≥fourfold rise in titer to ≥64 in at least two consecutive sera (Lind et al., 1997).

VISUALIZATION OF THE EATON AGENT (1960s)

For many years, the agent was considered to be a virus. However, Marmion and Goodburn (1961) successfully visualized the small coccobacillary bodies on the mucous layer covering the bronchial epithelium of the Eaton agent-infected chick embryo, which suggested that the Eaton agent was not a virus.

Chanock et al. (1960b) demonstrated that propagation in a tissue culture system was possible, but they were unable to visualize the agent directly. In this regard, Clyde et al. (1961) was able to subculture the tissue culture materials obtained from infected chick embryos into monkey kidney cells and finally visualized the brightly stained, rounded, granular structures using Liu's indirect fluorescent antibody procedure (Liu, 1957). They appeared quite similar to those of the PPLO family.

Next, Chanock et al. (1962a) described the successful growth of the Eaton agent in cell-free media, incorporating 2.5% yeast extract and 20% horse serum. The colonies that formed on agar were granular, with the center embedded, which occasionally presented as a "fried egg" appearance (**Figure 2**) with a dense center and a less dense periphery.

Taken together, the properties previously defined for the Eaton agent included the following points: (1) size between 180 and 250 μm, (2) sensitivity to tetracyclines and organic gold salts, and (3) occurrence of coccobacillary bodies on infected chick embryo bronchial epithelium. These characteristics were consistent with the contention that the organism was of the PPLO genus. This accumulated evidence strongly suggested that the Eaton agent was a member of, or shared many properties with, the genus *Mycoplasma*.

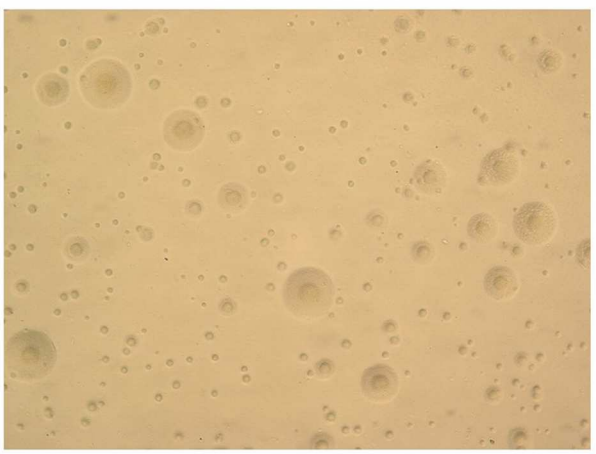

FIGURE 2 | Appearance of colonies of *Mycoplasma pneumoniae*. Colonies of *M. pneumoniae* on an agar plate typically have a unique "fried egg" appearance.

RE-EVALUATION OF THE EATON AGENT AS A POSSIBLE CAUSE OF PRIMARY ATYPICAL PNEUMONIA VIA TRANSMISSION EXPERIMENTS TO HUMAN VOLUNTEERS

After the Pinehurst trials, the stalemate over the acceptance of the evidence was eventually overcome by observations of the nature of the Eaton agent or virus and its recognition as a mycoplasma (Marmion, 1990). However, Chanock et al. (1961b) recovered the Eaton agent with monkey and human kidney tissue and the transmission study was performed by coarse spray and instillation into the noses and mouths of 52 healthy adults (21–36 years of age) from the federal prison system. Among 52 volunteers, the Eaton agent infected all 27 seronegative volunteers (fluorescent antibody titer prior to challenge was less than 1:10), and 17 of 25 individuals who possessed antibody (1:10 or greater) prior to the challenge. This suggested that in the second tissue culture passage, the Eaton agent itself was responsible for initiating the sequence of events which led to pneumonia, otitis, or febrile respiratory disease, irrespective of the presence of a positive fluorescent antibody titer for the Eaton agent (Chanock et al., 1961b; Rifkind et al., 1962).

Clyde et al. (1961) examined the preserved sera from 70 volunteers participating in two primary atypical pneumonia transmission experiments (Pinehurst trials) with regard to fluorescent-stainable antibodies to the Eaton agent. He found that fluorescent-stainable antibody responses were associated with cases of primary atypical pneumonia (Clyde et al., 1961).

In other studies, Chanock et al. (1960a) also revealed evidence that the Eaton agent had developed in 16% of patients with etiologically undiagnosed lower respiratory tract illness using fluorescent-stainable antibody (Eaton antibody). Similarly, other reports of Eaton agent–pneumonia in the 1960s showed that the Eaton agent was certainly considered to be a cause of primary atypical pneumonia (Chanock et al., 1961a; Evans and Brobst, 1961; Kingston et al., 1961; Goodburn et al., 1963).

TAXONOMIC DESIGNATION *M. pneumoniae*

An accumulation of studies have demonstrated evidence that the organism previously known as "primary atypical pneumonia virus" or "Eaton agent" is not a virus (Clyde and Denny, 1963), but rather, a member of the genus *Mycoplasma* (PPLO; Marmion and Goodburn, 1961; Chanock et al., 1962a,b).

ACCEPTANCE OF THE EATON AGENT AS A CAUSE OF ATYPICAL PNEUMONIA

In 1926, a Conference on Newer Respiratory Disease Viruses, mycoplasmas, and PPLOs was held at the National Institutes of Health (NIH), and Dr. Dingle finally accepted the Eaton agent as the cause of primary atypical pneumonia (USPHS, 1962).

After being convinced of the data as described in the paragraph of "Re-evaluation of the Eaton agent as a possible cause of primary atypical pneumonia via transmission experiments to human volunteers," Chanock (1963) finally proposed the nomenclature for the atypical pneumonia organism (Eaton agent) as *M. pneumoniae*.

In the history of *M. pneumoniae* pneumonia, acceptance of the Eaton agent as a cause of the disease required nearly 20 years (Clyde, 1993). Most of the pioneers were lonely and belonged to the small scale laboratories, except for the Pinehurst trials which were supported by numerous workers already well known. Therefore, the delay of acceptance of the Eaton agent possibly due to institutional or group competitiveness (Marmion, 1990), and turned out to be a long journey for Dr. Eaton.

AUTHOR CONTRIBUTIONS

TS generated the manuscript and figures.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 27 October 2016
doi: 10.3389/fmicb.2016.01701

Comparison of *Mycoplasma pneumoniae* Genome Sequences from Strains Isolated from Symptomatic and Asymptomatic Patients

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OPEN ACCESS

Edited by:
Miguel Cacho Teixeira, University of Lisbon, Portugal

Reviewed by:
Claire Bertelli, Simon Fraser University, Canada
Andre Ducati Luchessi, Federal University of Rio Grande do Norte, Brazil

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *14 May 2016*
Accepted: *12 October 2016*
Published: *27 October 2016*

Citation:
Spuesens EBM, Brouwer RWW, Mol KHJM, Hoogenboezem T, Kockx CEM, Jansen R, Van IJcken WFJ, Van Rossum AMC and Vink C (2016) Comparison of Mycoplasma pneumoniae Genome Sequences from Strains Isolated from Symptomatic and Asymptomatic Patients. Front. Microbiol. 7:1701. doi: 10.3389/fmicb.2016.01701

Mycoplasma pneumoniae is a common cause of respiratory tract infections (RTIs) in children. We recently demonstrated that this bacterium can be carried asymptomatically in the respiratory tract of children. To identify potential genetic differences between *M. pneumoniae* strains that are carried asymptomatically and those that cause symptomatic infections, we performed whole-genome sequence analysis of 20 *M. pneumoniae* strains. The analyzed strains included 3 reference strains, 3 strains isolated from asymptomatic children, 13 strains isolated from clinically well-defined patients suffering from an upper ($n = 4$) or lower ($n = 9$) RTI, and one strain isolated from a follow-up patient who recently recovered from an RTI. The obtained sequences were each compared to the sequences of the reference strains. To find differences between strains isolated from asymptomatic and symptomatic individuals, a variant comparison was performed between the different groups of strains. Irrespective of the group (asymptomatic vs. symptomatic) from which the strains originated, subtype 1 and subtype 2 strains formed separate clusters. We could not identify a specific genotype associated with *M. pneumoniae* virulence. However, we found marked genetic differences between clinical isolates and the reference strains, which indicated that the latter strains may not be regarded as appropriate representatives of circulating *M. pneumoniae* strains.

Keywords: *Mycoplasma pneumoniae*, whole genome sequencing, infection, colonization, respiratory tract infections

INTRODUCTION

Mycoplasma pneumoniae is a human bacterial pathogen that has been estimated to cause pneumonia in up to 40% of children hospitalized because of community-acquired pneumonia (Waites and Talkington, 2004). In addition, *M. pneumoniae* is associated with extra-pulmonary manifestations of which the most common are central nervous system complications

(e.g., Guillain-Barré Syndrome and encephalitis), haemolytic anemia, non-specific myalgias or arthralgias, and renal complications, such as glomerulonephritis. *M. pneumoniae* infections are commonly treated with antibiotics that target the bacterial DNA metabolism or protein synthesis. However, evidence for effectiveness of these antibiotics against *M. pneumoniae* infections is scarce and based on comparative studies (Mulholland et al., 2012).

A recent study has shown that *M. pneumoniae* can also be carried asymptomatically: the bacterium was detected in the upper respiratory tract of 21% of children that did not show any signs of a respiratory tract infection (RTI) (Spuesens et al., 2013). Asymptomatic carriage of potential pathogens is a well-known, common phenomenon in the general population. For instance, potential pathogens like *Streptococcus pneumoniae, Staphylococcus aureu*s and rhinovirus can cause RTIs, but can also be carried asymptomatically. Clearly, it is important to identify the determining factors, both from pathogen and host, involved either in control of an asymptomatic carriage status or in triggering symptomatic infection. In the case of *S. pneumoniae*, specific bacterial gene clusters were found to be associated with invasive disease (Wulff-Burchfield et al., 2010). Several *M. pneumoniae* virulence factors have been identified in previous studies (Waites and Talkington, 2004). The first step to invasive disease is the attachment of *M. pneumoniae* to its human host using an elaborate attachment organelle, which consists of several attachment and accessory proteins. Without an intact attachment organelle, *M. pneumoniae* is unable to cause disease. Several other virulence mechanisms include direct cytotoxicity (e.g., by production of H_2O_2) and activation of the inflammatory cascade leading to cytokine-mediated tissue injury (Waites and Talkington, 2004). In 2005, the Community-Acquired Respiratory Distress Syndrome (CARDS) toxin was discovered as another potential virulence factor (Kannan et al., 2011). It is unknown whether all *M. pneumoniae* strains express these factors or whether there are genes in *M. pneumoniae* associated with carriage and/or pathogenicity.

Until a few years ago, only a single genome sequence was available for *M. pneumonia* (Himmelreich et al., 1996). This sequence was derived from laboratory strain M129. Since 2013, several other genomic sequences have been published (Lluch-Senar et al., 2015; Simmons et al., 2013; Xiao et al., 2015). Most of these sequences were obtained in two independent studies. One of these studies reported the comparative genome analysis of 15 *M. pneumoniae* strains isolated form respiratory tract samples and cerebrospinal fluid samples collected from patients between 1940 and 2009 in the USA, China, and England (Xiao et al., 2015). The other study reported the sequences of 23 clinical *M. pneumoniae* strains isolated between 1964 and 2011 in six different countries (Lluch-Senar et al., 2015). In both studies, the genomes of *M. pneumoniae* strains seem very stable over time and at different locations in the world. The sequences of these studies show a low number of non-synonymous single-nucleotide polymorphisms, but a high rate of variation among repetitive elements (Lluch-Senar et al., 2015; Xiao et al., 2015).

Despite the availability of a significant set of *M. pneumoniae* genome sequences, it is currently difficult to determine the association between *M. pneumoniae* genotype and virulence. This is mainly due to the fact that all known *M. pneumoniae* sequences were exclusively obtained from strains isolated from patients with RTI symptoms. Clearly, this precludes determination of the putative genetic differences between strains causing symptomatic infections and strains carried by asymptomatic individuals. The recent isolation of a set of *M. pneumoniae* strains from both asymptomatic children and children suffering from RTI (Spuesens et al., 2013), however, allows a direct comparison of the genetic make-up of strains associated with bacterial carriage and those involved in symptomatic infection. We therefore set out to determine the genome sequence of 3 reference strains (M129, FH, and R003), 3 strains from asymptomatic individuals, and 13 strains from clinically well-defined patients suffering from either an upper or lower RTI. The analysis and comparison of the obtained sequences did not reveal a specific genotype associated with *M. pneumoniae* virulence. However, we did find striking differences between the genomes of clinical isolates and those of the *M. pneumoniae* reference strains.

METHODS AND MATERIALS

Patient Samples

Most of the patient samples were collected as part of a clinical study designed to investigate the existence of *M. pneumoniae* asymptomatic carriage in children (Spuesens et al., 2013). This study was carried out in Rotterdam, the Netherlands between 2008 and 2012. Patient information was collected and documented prospectively as part of the study. All *M. pneumoniae* culture-positive samples from the different groups (asymptomatic, symptomatic, and follow-up) were selected for analysis as part of this study. The other samples from symptomatic patients were collected by the Regional Laboratory of Public Health Kennemerland, Haarlem, The Netherlands. These samples were taken from patients with suspected *M. pneumoniae* infection as part of the medical work-up ordered by the treating physician. All samples with a positive culture for *M. pneumoniae* were selected and used in this study. The Medical Ethics Review Board of the Erasmus MC approved the study on asymptomatic carriage (NL20418.078.08) and written informed consent was obtained in this study from all parents and children above the age of 12 years. The Medical Ethics Review Board of the Erasmus MC approved the use of the samples collected during routine medical work-up in the Regional Laboratory of Public Health Kennemerland (MEC 2013-344).

Culture and DNA Isolation

The culturing of *M. pneumoniae* was performed in the laboratory of pediatrics of the Erasmus MC, as previously described (Sluijter et al., 2008). In short, 100 µl original sample and 10-fold dilutions were used for culturing. Culturing was performed in *Mycoplasma* medium containing 1.4% Difco™ PPLO broth (Becton Dickinson), 0.15% Difco™ TC Yeastolate, UF (Becton Dickinson), 1.4% glucose, 20% horse serum, 1,000 U/ml Penicillin G, 500 U/ml Polymyxine B, and 0.02 mg/ml phenol red. The pH of the medium was adjusted to 7.8–8.0 using a solution of 2 N NaOH, followed by filter-sterilization. Cells were harvested upon color change of the medium (from red/orange to yellow). The cells were added to Mycoplasma medium agar

plates and single colonies were harvested. These were grown in 3 ml of medium at 37°C/5% CO_2 in 25 cm^2 tissue culture flasks (Greiner). Cells were harvested upon color change of the medium, and DNA was isolated from the cells as described previously (Spuesens et al., 2009).

Grouping of the Strains

The strains were divided into 4 groups as indicated in **Table 1**. Group 1 includes reference strains *M. pneumoniae* M129 (subtype 1, ATCC 29342), *M. pneumoniae* FH (subtype 2, ATCC 15531) and *M. pneumoniae* R003 (subtype 2a). The other groups consisted of strains isolated from asymptomatic children (Group 2), patients with an upper RTI (Group 3) and patients with a lower RTI (Group 4).

Sequencing

The sequencing of all strains including re-sequencing of the reference strains was performed at the Center for Biomics of the Erasmus MC using an Illumina HiSeq2000 sequencer. DNA libraries were prepared according to the Illumina TruSeq DNA protocol. The libraries were sequenced using the TruSeq V2 protocol with paired-end 100-bp reads. Between 0.9 and 2.5 gigabases of DNA sequence was generated for each of the isolates and a 1224- to 3300-fold genome coverage was obtained. The generated short-read datasets were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP081446.

De novo Assembly

To check the quality of the raw sequence data we used FastQC. This analysis showed that the majority of the sequenced data had a PHRED quality score exceeding Q30, which is considered as good quality (**Supplementary File 1**). Reads were purged from the Illumina sequence adapter and renamed according to the standards expected by Abyss (Software Abyss 1.3.6). Bases with a PHRED quality score below 30 (base call accuracy $\geq$ 99.9%) were not used in the assembly. After the initial processing, Abyss was run for each of the samples individually. For each sample, the optimal k-mer was determined by varying k between 25 and 100 in steps of 5 (**Supplementary Figure 1**). The assembly with the largest N50 contig was considered optimal.

The assembled sequences were separately reported by Abyss as unitigs, contigs, and scaffolds. The unitig files hold the assemblies that were generated without taking the pair information into account. The contigs are the assemblies generated with the pair information taken into account. Scaffolds consist of merged contigs based on read pairs, and differ from the contigs in that they may contain unresolved repeats and spacers. For the

TABLE 1 | Sample ID, patient information, strain information and *de novo* assembly information.

Group	Strain/sample ID	Diagnose group	Sequence ID	Size (bp)[b]	Subtype[c]	Largest contig (bp)	N50 contig (bp)	M129 genome covered (%)[d]
1	M129	Reference	01	802,479	1	135,172	59,602	99.89
	FH	Reference	02	803,911	2	98,770	50,959	99.29
	R003	Reference	03	800,612	2	123,538	54,253	99.21
2	B174	Asymptomatic	11	805,944	1	135,177	53,512	99.83
	B247	Asymptomatic	12	801,799	1	96,961	66,934	99.63
	B406	Asymptomatic	14	804,626	2	98,962	51,557	99.06
3	A016	Upper RTI	04	800,489	2	123,550	54,265	99.18
	A058	Upper RTI	06	800,399	1	135,171	54,703	99.73
	C024	Upper RTI	15	809,160	1	135,170	59,355	99.85
	H030	Upper RTI	19	806,820	1	135,258	59,621	99.81
4	A035	Lower RTI	05	805,945	2	123,540	54,255	99.04
	A103	Lower RTI	07	821,841	1	92,532	45,886	99.73
	HAP111	Lower RTI	10	808,319	2	135,217	54,427	99.23
	HAP157	Lower RTI	09	800,436	1	92,731	51,728	99.73
	H010	Lower RTI	18	805,135	1	191,508	66,444	99.67
	H016	Lower RTI	20	805,824	1	135,161	59,300	99.77
	H026	Lower RTI	13	790,390	2	98,587	48,754	98.80
	C036-1[a]	Lower RTI	16	807,258	1	135,171	59,440	99.77
	C036-2[a]	Lower RTI	17	804,586	1	191,498	66,641	99.73
	HAP157FUP	Follow-up	08	802,814	1	191,496	66,615	99.75

[a] *Morphologically different M. pneumoniae colonies originating from the same patient sample. Both a large (C036-1) and a small colony (C036-2) were sequenced.*
[b] *Total assembly (bp). Contigs over 500 bp in length are considered in the assembly size.*
[c] *Subtype 1 and 2 as described previously by Spuesens et al. (2013).*
[d] *All contigs, including contigs below 500 bp in length, were aligned to the M129 reference genome with MUMmer.*

downstream analysis, the contigs from the assemblies with the largest N50 contigs were used. These contigs are presented in **Supplementary File 3**.

Larger rearrangements, insertions and deletions were determined by comparing the assembled contigs with the *M. pneumoniae* M129 reference genome (NCBI accession number NC_000912.1). This alignment was performed with MUMmer (version 3.23) (Kurtz et al., 2004). These alignments are presented in **Supplementary File 2**. Analysis of the alignments was performed in R (version 3.2.2).

SNV and InDel Comparisons

In addition to the *de novo* assembly, the reads were aligned to the *M. pneumoniae* M129 reference genome (NCBI accession number NC_000912.1) using BWA (Li and Durbin, 2009) (version 0.5.9) to detect smaller variants. Single-nucleotide variants (SNVs) and short insertions and deletions (InDels) were determined relative to the reference strain with SAMtools mpileup (Li et al., 2009) (version 0.1.16) (**Supplementary Table 1** and **Supplementary File 4**). For each sample, the frequencies of the SNVs and InDels relative to the total number of reads at that position were determined as well.

The strains were clustered based on their SNV/InDel profiles. SNV/InDels that were not present in at least 20% of the reads at a position in a single strain were removed from the analysis. These filtered SNV/InDels were considered low-abundant and may be caused by intrastrain variations or technical errors. The strains were then clustered based on their SNV/InDels profiles using hierarchical clustering. The distances between the SNV/InDels profiles were calculated with the Euclidean distance measure. These analyses were performed with standard facilities present in R (version 3.2.2) (Kurtz et al., 2004; Wickham, 2009; R Development Core Team, 2011; de Vries and Ripley, 2015).

RESULTS

Selection of Strains and Isolates of *M. pneumoniae*

We have determined the complete genome sequences of a total of 20 strains or isolates of *M. pneumoniae*. The isolates were obtained either from asymptomatic children ($n = 3$), from patients with an upper RTI (URTI; $n = 4$), or from patients with a lower RTI (LRTI; $n = 9$). The names (ID) and origins of the isolates are listed in **Table 1**. Two of the isolates from the LRTI group were obtained at the same time from a single patient (C036-1 and C036-2). These two isolates were selected because they differed in colony morphology on agar plates. Two other samples from the LRTI group were also collected from a single patient, one at the moment of RTI (HAP157) and one collected 4 weeks later, after recovery of the clinical symptoms (HAP157FUP). In addition to the clinical isolates, three reference laboratory strains were included in this study, i.e., subtype 1 strain M129, and subtype 2 strains FH and R003.

DNA Sequencing and *de novo* Genome Sequence Assembly

The genomic sequences of the 20 *M. pneumoniae* strains were determined using a paired-end 100-bp sequencing protocol on the HiSeq2000 platform (Illumina). For each of the strains, genome assemblies were generated that ranged from 790 kb (H026) to 821 kb (A103) in length (**Table 1**). These cumulative contig lengths are similar to the published genome length of reference strain M129 (816 kb) (Himmelreich et al., 1996). For strain M129, which was also included in our study, the total amount of sequence contained in contigs over 500 bp was found to be ∼802 kb, which is 14 kb shorter than the previously published genome size of this strain (**Table 1**). Thus, ∼2% of the M129 genome could not be reliably retrieved with the procedures used in this study. When contigs under 500 bp were considered as well, 99.9% of the M129 genome was covered (**Table 1**; **Supplementary Table 1**). The gaps are likely caused by repetitive sequences (RepMP sequences) (Spuesens et al., 2009) in the genome of *M. pneumoniae,* which are known to pose problems in short read sequencing. Gaps similar to those in the M129 genome were also found in the other strains (**Table 1**). Strikingly, the type 1 strains covered ∼0.5% of the M129 genome more than the type 2 strains did. This difference is centered around a single locus at position 558,624–561,515, which is present in type 1 strains but is absent from type 2 strains (**Supplementary File 2**).

We identified one sequence stretch within the assembled contigs that was exclusively present in type 2 strains. The length of this stretch is 5.3 kb (in strains R003, A016, and A035) or 5.7 kb (in strains FH, B406, HAP111, and H026). The 5.3-kb fragment completely overlaps with the 5.7-kb fragment and corresponds to the sequence at position 704,213–709,505 of the FH genome. The 5.7-kb fragment corresponds to position 703,813–709,505 of this genome.

Sequence Differences between Strains Isolated from Asymptomatic and Symptomatic Individuals

To compare the genomic sequences of the different *M. pneumoniae* strains, their SNV/InDels profiles were subjected to hierarchical clustering analysis (**Figure 1**). This analysis clearly distinguished two major families of strains, which correspond to the known major subtypes of *M. pneumoniae*, i.e., subtypes 1 and 2. However, genetically similar strains did not cluster together with regard to the clinical groups of patients they were isolated from (Groups 1–4 in **Table 1**). For example, strain B406, which was isolated form an asymptomatic child (Group 2), shows a high degree of similarity with strain H026, which was isolated from an adult with an LRTI (Group 4). The hierarchical clustering based on SNV/InDels therefore does not indicate that the overall genome similarity is greater among the strains within each group than between strains from different groups.

To investigate whether the strains within each of the four groups share specific single nucleotide polymorphisms, small insertions or deletions in their genomes, a subtractive comparative approach was performed in which the following criteria were applied: (1) all variants with a low coverage (less than 21 times covered sequence) are filtered in each sample, (2) SNV/InDels present in the asymptomatic group are filtered from those in the other groups except the reference group (since SNV/InDels in the asymptomatic group are unlikely to cause a "phenotype"), (3) all SNV/InDels present in the

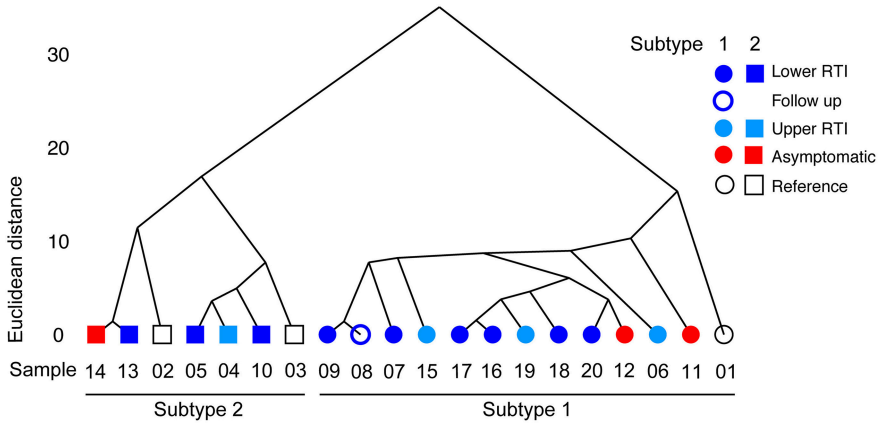

FIGURE 1 | Hierarchical clustering based on Euclidean distance. The Euclidean distance between variant frequencies (on the y-axis) is plotted against the different strains sequenced in this study. The strains are indicated by their sequence ID listed in **Table 1**. Only the variants with a frequency above 0.2 were used in this analysis (see the text for details). Hierarchical clustering was applied based on Euclidean distance and the average linkage method (hclust).

reference group are filtered from those in the other groups except the asymptomatic group, and (4) only SNV/InDels are analyzed that are present in all samples in one group. Using this procedure, we were unable to identify variants that were shared between all samples from the same group. Although a number of variants were shared between some samples from the same group (**Table 2**), we did not find specific sequence differences between the strains from the group of symptomatic patients (Groups 3 and 4) and those from the other groups (Groups 1 and 2). Variant frequencies for the different comparisons are shown in **Tables 2–4**.

Variation in Follow-Up Samples of a Single Patient

Two of the isolates that were sequenced were acquired at two different time-points from the same patient. The first isolate, HAP157, was obtained when this patient suffered from an LRTI. The other isolate, HAP157FUP, was taken 4 weeks later, after resolution of the infection following a course of antibiotics (azithromycin). Eight relevant differences were found between strains HAP157 and HAP157FUP (**Table 3**). One of these differences was localized to the P1 gene (MPN141), which encodes the major attachment protein of *M. pneumoniae*; in contrast to strain HAP157, HAP157FUP was found to have a deletion of an AGT triplet at position 182,792–182,794 within the P1 gene. This triplet is part of a previously described tandem repeat of which the biological relevance is yet unknown (Dorigo-Zetsma et al., 2001).

The Sequences of Isolates Displaying Differences in Colony Morphology

Two of the strains (C036-1 and C036-2) were isolated from the same patient at a single time point. These strains were analyzed separately because they displayed different colony morphologies on agar plates. The sequences of these strains were found to be highly similar (**Figure 1**): only 5 SNPs were identified between these strains (**Table 4**).

DISCUSSION

We determined the complete genome sequences of *M. pneumoniae* isolates obtained from asymptomatic *M. pneumoniae* carriers, and from patients suffering from an upper or lower RTI caused by *M. pneumoniae*. In addition, we analyzed the genomes of 3 *M. pneumoniae* reference strains. In a comparison of these sequences, we could not identify a specific genotype that is associated with *M. pneumoniae* virulence or asymptomatic bacterial carriage.

Our previous findings on sequence variation among *M. pneumoniae* strains, which mainly focussed on the P1 gene and repetitive elements (RepMP sequences), showed that variation among repetitive sequences is very common in this bacterium (Spuesens et al., 2009, 2010). In the current analysis of whole-genome sequences of 20 different *M. pneumoniae* isolates, however, we did not identify large genomic rearrangements. These findings are concordant with those of Xiao et al. In their analysis, the sequences of 15 *M. pneumoniae* strains seem very stable over time and at different locations in the world. Similarly, Lluch-Senar et al. (2015) found a relatively low number of non-synonymous SNPs among the genome sequences of 23 strains from 6 different countries. However, they do report a high rate of variation among repetitive elements in the *M. pneumoniae* genomes (Lluch-Senar et al., 2015).

Although we were able to reliably determine the genomic sequences of *M. pneumoniae* isolates from different well-defined groups of patients, the most important drawback of our study is the limited availability of *M. pneumoniae* isolates from asymptomatic patients. In routine microbiological diagnostics, asymptomatic patients are usually not tested for the presence of *M. pneumoniae*. In addition, in our previous study on asymptomatic carriage of *M. pneumoniae*, only a limited number of *M. pneumoniae* PCR-positive samples also turned out to be culture-positive (Spuesens et al., 2013). Clearly, for genomic sequence analysis, culturing is a crucial step in obtaining pure, clonal bacterial isolates. Nevertheless, in future studies, we aim to obtain higher number of *M. pneumoniae* isolates from different

TABLE 2 | SNV/InDels frequencies of the isolates from symptomatic groups.

Upper RTI group

SNV/InDels	Sequence ID				Locus
(NC_000912.1)	05	07	09	10	
111954-111954:C-A	0.28	0.00	0.24	0.20	MPN089
528806-528806:G-GT	0.38	0.42	0.33	0.05	NA
536132-536132:A-ACC	0.67	0.00	0.00	0.71	MPN442
622601-622601:G-GTT	0.38	0.40	0.10	0.33	NA
706408-706408:A-ACC	0.21	0.01	0.00	0.22	NA

Lower RTI group

SNV/InDels	Sequence ID									Locus
(NC_000912.1)	05	07	09	10	13	16	17	18	20	
41006-41006:G-A	0.00	0.00	0.00	0.00	0.00	0.99	1.00	1.00	0.00	MPN034
140960-140960:C-A	0.00	0.00	0.00	0.00	0.00	1.00	0.99	1.00	0.00	MPN108
171528-171528:A-AG	0.00	0.00	0.00	0.00	0.00	0.67	0.68	0.66	0.00	MPN132
171529-171529:C-CCCAAG	0.00	0.00	0.00	0.00	0.00	0.66	0.67	0.65	0.00	MPN132
405692-405692:A-T	0.00	0.00	0.00	0.00	0.00	0.99	0.99	1.00	0.00	MPN341
428005-428005:A-G	0.00	0.00	0.00	0.00	0.00	1.00	0.99	1.00	0.00	MPN358
536132-536132:A-ACC	0.68	0.00	0.00	0.01	0.00	0.73	0.70	0.71	0.67	MPN442
622601-622601:G-GTT	0.18	0.06	0.40	0.39	0.01	0.04	0.06	0.30	0.33	NA
733651-733651:G-A	0.00	0.00	0.00	0.00	0.00	0.99	0.99	1.00	0.00	MPN612

NA, Not applicable.

TABLE 3 | Variant frequencies of strains HAP157 and HAP157FUP.

SNV/InDels	Sequence ID		Locus
(NC_000912.1)	08	09	
182792-182794:AGT-	0.66	0.01	MPN141
195459-195459:C-CA	0.25	0.04	NA
570767-570767:G-T	0.89	0.62	NA
570769-570769:A-G	0.88	0.61	NA
570770-570770:C-G	0.85	0.61	NA
622601-622601:G-GTT	0.04	0.40	NA
622601-622601:G-GT	0.41	0.14	NA
649041-649041:G-T	0.91	0.00	NA

NA, Not applicable.

TABLE 4 | Variant frequencies of the isolates C036-1 and C036-2 with different colony morphologies.

SNV/InDels	Sequence ID		Locus
(NC_000912.1)	16	17	
195459-195459:C-CA	0.03	0.25	NA
626378-626378:G-T	0.00	0.35	NA
649044-649044:G-C	0.99	0.00	NA
649047-649047:G-T	0.00	0.99	NA
716850-716850:C-A	0.00	0.38	MPN594

NA, Not applicable.

groups of asymptomatic individuals. This should provide more insight in the physiology of asymptomatic colonization of the human respiratory tract by *M. pneumoniae*.

Another important issue to consider is whether or not *M. pneumoniae* was the actual causative agent of the RTI in the different groups of symptomatic patients that were included in this study. Recent studies have indicated that multiple pathogens can be present in the respiratory tract of children and adults with RTI (Spuesens et al., 2013; Biesbroek et al., 2014a,b; Dickson et al., 2014). As a consequence, we cannot rule out that pathogens other than *M. pneumoniae* might have caused the symptomatic "phenotype" in at least some of the included symptomatic patients. Clearly, this may have obstructed the identification of potential genomic features that allow discrimination between pathogenic *M. pneumoniae* strains and strains that can be carried asymptomatically by the human host.

In conclusion, in this study we have shown that there is no specific genotype that can be associated with *M. pneumoniae* virulence or asymptomatic carriage. In addition, we found marked genetic differences between clinical isolates and the reference strains, which indicated that the latter strains may not be regarded as appropriate representatives of circulating *M. pneumoniae* strains.

AUTHOR CONTRIBUTIONS

ES, AV, and CV initiated the study and were responsible for the original design. ES, RB, CK, KM, and TH all contributed to the execution of the study (specifically by retrieving patient clinical information, performance of the culture of the *M. pneumoniae* strains, DNA isolation and sequencing). RJ provided the clinical

information and some of the *M. pneumoniae* strains. ES, RB, WV, AV, and CV analyzed and interpreted the data. ES wrote the first draft of the manuscript and was responsible for the subsequent modifications. All authors were involved in the final modifications of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01701/full#supplementary-material

Supplementary File 1 | Fast QC files. HTML files per strain. Each FastQC report includes: Basic Statistics, Per base sequence, quality, Per sequence quality scores, Per base sequence content, Per sequence GC content, Per base N content, Sequence Length Distribution, Sequence Duplication Levels, Overrepresented sequences, Adapter Content, and Kmer Content.

Supplementary File 2 | MUMmer alignments. The alignments of the generated genome assemblies to the M129 reference genome.

Supplementary File 3 | Generated contigs. Generated genome assemblies per strain.

Supplementary File 4 | Variants.

Supplementary Figure 1 | The results of the optimization on the assembly statistics.

Supplementary Table 1 | Differences between the reference sequence and the reads as determined by interpreting the output of samtools mpileup.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

HYPOTHESIS AND THEORY
published: 28 June 2016
doi: 10.3389/fmicb.2016.00960

Integrated Information and Prospects for Gliding Mechanism of the Pathogenic Bacterium *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae forms a membrane protrusion at a cell pole and is known to adhere to solid surfaces, including animal cells, and can glide on these surfaces with a speed up to 1 μm per second. Notably, gliding appears to be involved in the infectious process in addition to providing the bacteria with a means of escaping the host's immune systems. However, the genome of *M. pneumoniae* does not encode any of the known genes found in other bacterial motility systems or any conventional motor proteins that are responsible for eukaryotic motility. Thus, further analysis of the mechanism underlying *M. pneumoniae* gliding is warranted. The gliding machinery formed as the membrane protrusion can be divided into the surface and internal structures. On the surface, P1 adhesin, a 170 kDa transmembrane protein forms an adhesin complex with other two proteins. The internal structure features a terminal button, paired plates, and a bowl (wheel) complex. In total, the organelle is composed of more than 15 proteins. By integrating the currently available information by genetics, microscopy, and structural analyses, we have suggested a working model for the architecture of the organelle. Furthermore, in this article, we suggest and discuss a possible mechanism of gliding based on the structural model, in which the force generated around the bowl complex transmits through the paired plates, reaching the adhesin complex, resulting in the repeated catch of sialylated oligosaccharides on the host surface by the adhesin complex.

Keywords: cell architecture, evolution, electron microscopy, *Mollicute*, motility, sialylated oligosaccharide

OPEN ACCESS

Edited by:
Takeshi Saraya,
Kyorin University School of Medicine, Japan

Reviewed by:
Yunlong Li,
Wadsworth Center, USA
John I. Glass,
J. Craig Venter Institute, USA

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *13 February 2016*
Accepted: *02 June 2016*
Published: *28 June 2016*

Citation:
Miyata M and Hamaguchi T (2016) Integrated Information and Prospects for Gliding Mechanism of the Pathogenic Bacterium Mycoplasma pneumoniae. Front. Microbiol. 7:960. doi: 10.3389/fmicb.2016.00960

INTRODUCTION

Mycoplasmas are parasitic and occasionally commensal bacteria that lack a peptidoglycan layer and have small genomes (Razin et al., 1998; Razin and Hayflick, 2010). *Mycoplasma pneumoniae* is a causative pathogen of human bronchitis and walking pneumonia as discussed in other reviews in this issue. This particular mycoplasma forms a membrane protrusion, called the "attachment organelle" or the "tip structure," at a cell pole, binds to solid surfaces including those on the host, and glides in the direction of the protrusion (**Figure 1**; Video 1; Miyata, 2008; Miyata and Nakane, 2013; Balish, 2014). The gliding speed can reach up to 1 μm, one-half its cell length, per second (Radestock and Bredt, 1977; Kenri et al., 2004; Nakane and Miyata, 2009). This motility, combined

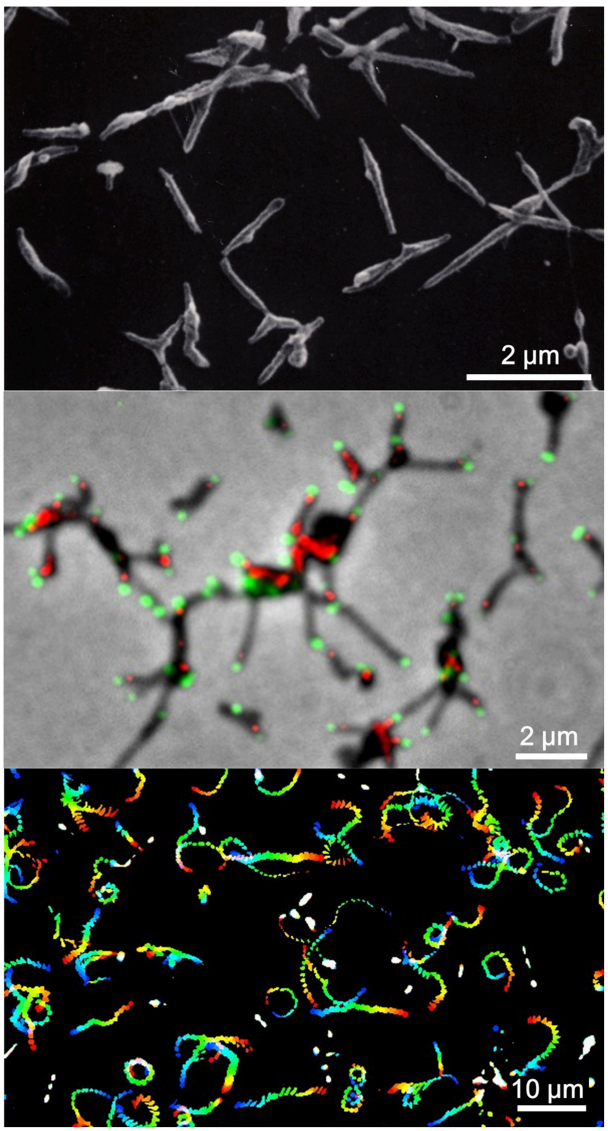

FIGURE 1 | *Mycoplasma pneumoniae* cell structure and gliding. **(Upper)** Scanning electron microscopy (SEM) of *M. pneumoniae*. **(Middle)** Merged image of phase contrast and fluorescence microscopy. Component proteins of the attachment organelle are marked in green and red. **(Lower)** Gliding track. The video frames are colored from red to blue with time and integrated for 20 s. The source video (Video 1) is available.

with its cytadherence capacities, is involved in the *M. pneumoniae* infection process, enabling the cells to translocate from the tips of bronchial cilia to the host cell surface (Krunkosky et al., 2007; Prince et al., 2014). Interestingly, the genome sequence shows that this motility is not related to other known mechanisms of bacterial motility, nor does it involve motor proteins known to be involved in eukaryotic cell motility (Fraser et al., 1995; Dandekar et al., 2000; Jaffe et al., 2004b). In this article, we highlight what is currently understood concerning *M. pneumoniae* gliding and motility, providing insight into the function and mechanism of this process.

MOTILITY IN *MOLLICUTES*

The species in class *Mollicutes* represented by *Mycoplasmas* are classified into four subgroups (**Figure 2**; Grosjean et al., 2014). *Mycoplasma mobile* and *Mycoplasma pulmonis* in the *Hominis* subgroup and most species in the *Pneumoniae* subgroup have gliding capability. They share similar features in terms of the gliding process (i.e., they form a protrusion at a pole, bind to sialylated oligosaccharides fixed on solid surfaces through this protrusion, and glide in the direction of protrusion, at a speed ranging 0.1–4.5 μm per second). However, the proteins involved in the actual mechanisms underlying this motility in the two subgroups do not show any similarities in amino acid sequence or gene arrangement. These observations suggest that these two systems developed independently, achieving similar results through convergent evolution.

The *Spiroplasma* subgroup contains the *Spiroplasma* and other species (**Figure 2**; Grosjean et al., 2014), all of *Spiroplasma* species swim in liquids of high viscosity (Shaevitz et al., 2005; Wada and Netz, 2009). Spiroplasmas propel viscous medium by propagating paired kinks formed at the front end to the back end along the helix axis (**Figure 3**), which is not related to spirochete swimming in which they rotate their flagella around the helix axis in the periplasmic space via the flagellar motors localized at both poles of the cell (Zhao et al., 2014). Notably, the spiroplasma genomes do not contain known genes related to other motility systems, with the exception of five homologs of MreB, known as "bacterial actin," which functions as a component of the cytoskeleton in walled bacteria (Ku et al., 2014). Many species are known to have some type of motility, and all species in the *Pneumoniae*, *Hominis*, and *Spiroplasma* subgroups appear to utilize a common mechanism, although they may have developed autonomously.

DEVELOPMENT OF THREE INDEPENDENT MOTILITY SYSTEMS IN *MOLLICUTES*

When studying the *Mollicute* species, one question that invariably arises is why and how did they develop these motility systems three separate times? Class *Mollicutes* developed from a low GC branch of Gram-positive bacteria i.e., phylum, *Firmicutes* including *Bacillus* and *Clostridium*, through their parasitic lifecycles (Weisburg et al., 1989). It also appears that the *Mollicutes* stopped synthesizing a peptidoglycan layer during their evolution, possibly as a way to evade the natural immune system of host animals, which often use Toll-like receptors to recognize peptidoglycans (Akira and Takeda, 2004). Changing the surface composition in this manner resulted in a subsequent change in cell shape, which became more flexible, as well as a reduction in genome size. Although bacterial flagella are well conserved in many bacterial species, and are effective for translocation through a wide variety of environments, including low and high viscosity liquids, and even liquid-solid interfaces (Kearns, 2010), these systems also require the mechanical support of a peptidoglycan layer. In fact, peptidoglycan binding sites on the flagella machinery are essential for rotation (Blair et al., 1991;

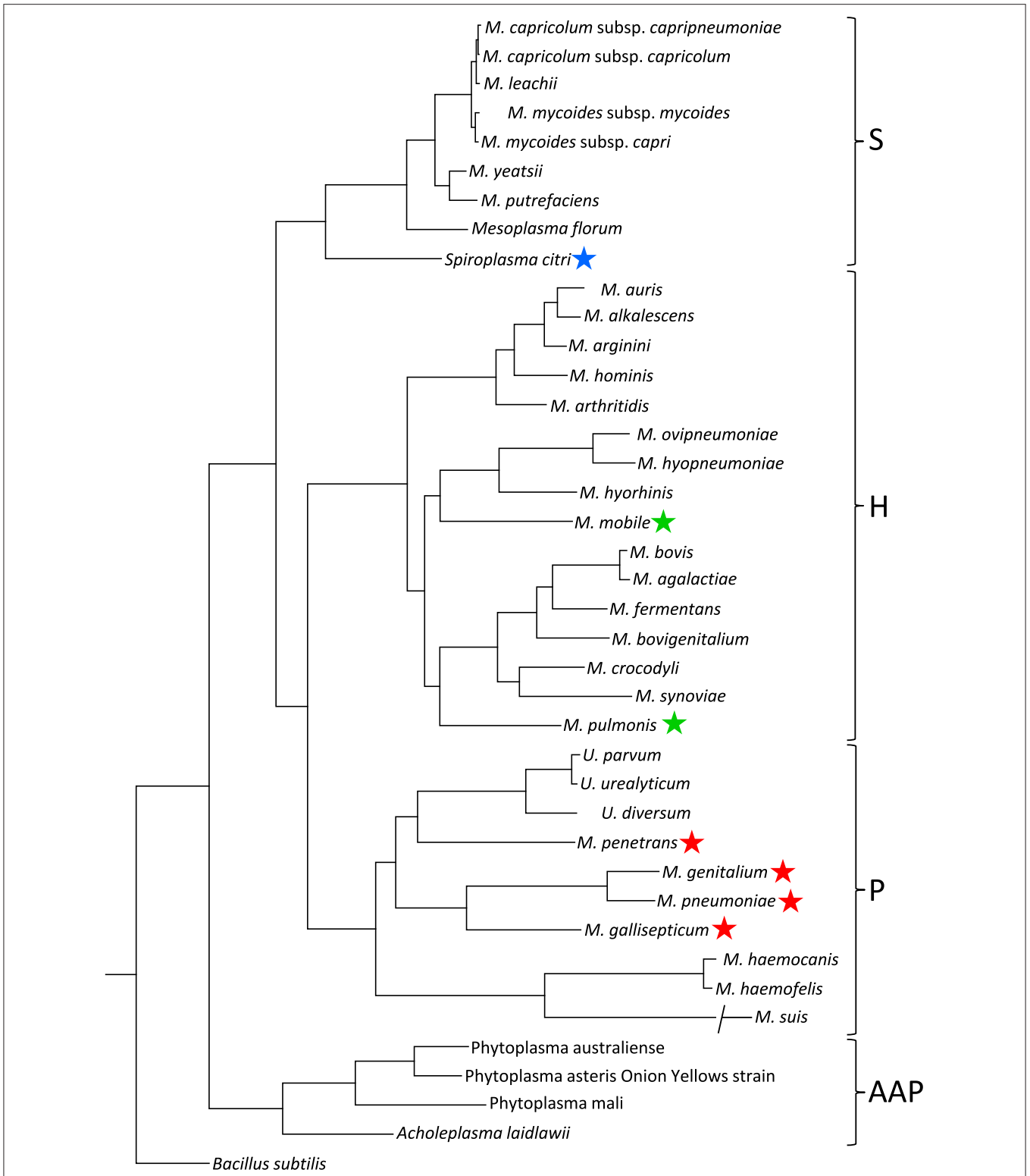

FIGURE 2 | Phylogenetic tree inferred using the multiple alignments of 79 proteins in conjunction with the maximum likelihood method. Main phylogenetic subgroup are indicated: S, *Spiroplasma*; H, *Hominis*; P, *Pneumoniae*; AAP, *Acholeplasma/Anaeroplasma/Phytoplasma*. Motile species are marked by blue, green, and red stars for the motility types represented by *S. citri*, *M. mobile*, and *M. pneumoniae*, respectively. This figure was modified from study (Grosjean et al., 2014) through the courtesy of Dr Pascal Sirand-Pugnet.

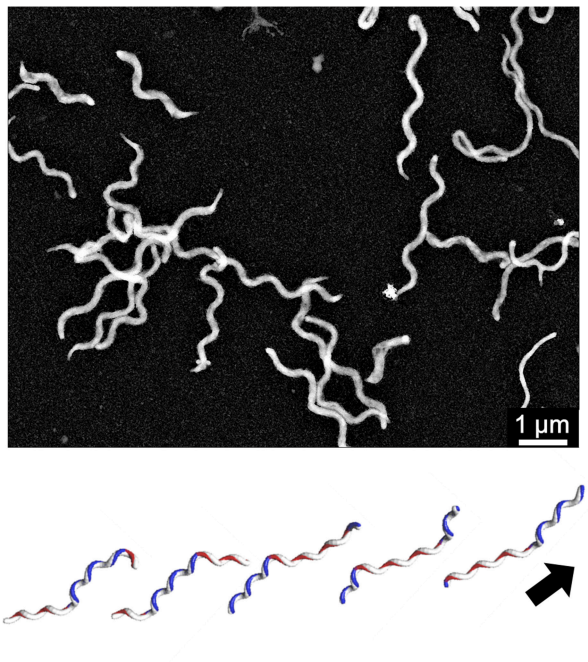

FIGURE 3 | *Spiroplasma* swimming. (Upper) Negative staining electron microscopy of *S. eriocheiris* cells. **(Lower)** Swimming scheme. A pair of kinks, or inversion points of helicity that travel from the front to the back, propel the cell in the direction of the arrow. This image is courtesy of Dr Hirofumi Wada.

De Mot and Vanderleyden, 1994; Koebnik, 1995). In *Mollicutes*, the evolutionary loss of bacterial flagella was again likely done in order to evade the host immune system, because flagellin is also a ligand of Toll-like receptors. However, motility is sometimes critical for parasitic bacteria to survive in host tissues, and in order to compensate for the lack of flagella, *Mollicutes* somehow developed new motility systems (Miyata, 2010; Miyata and Nakane, 2013; Balish, 2014; Miyata and Hamaguchi, 2016). The loss of the peptidoglycan layer may have actually help to mediate the development of this novel type of motility. In a cell, many housekeeping proteins are physically moving around the cell to perform various functions, including DNA replication, DNA repair, RNA synthesis, protein synthesis, protein degradation, ATP synthesis, transport, cell shape maintenance, cytokinesis, peptidoglycan synthesis, and so on. The development of a new motility system may have been achieved by transmitting these movements across the cell membrane to the scaffold outside. In walled bacteria, however, the movements inside cell cannot be transmitted outside, because the rigid peptidoglycan layer blocks the transmission of these movements. Perhaps, *Mollicutes* were capable of independently developing motility as many as three times, based on this advantage. For example, we recently suggested the possibility that *M. mobile* gliding may have originated from an accidental combination of F-type ATPase and adhesin movements (Miyata and Hamaguchi, 2016). Moreover, spiroplasma swimming may have originated from the cell shape maintenance system using MreB, a bacterial ortholog of eukaryotic actin (Kurner et al., 2005; Trachtenberg et al., 2008; Ozyamak et al., 2013). This kind of survival strategy adopted by *Mollicutes* may to some extent mimic that of the *Cephalopoda* species (Albertin et al., 2015), which over the course of their evolution quit making hard shells and instead developed flexible bodies with camouflaging abilities and different types of traveling systems (see other reviews in this issue).

PURPOSE OF MOTILITY

Although motile bacteria generally move to access nutrients and escape from waste and predators using "two-component systems" (Typas and Sourjik, 2015), neither type of mycoplasma gliding exhibits any obvious chemotaxis and no homologs of any two-component system genes have been identified in the mycoplasma genomes (Fraser et al., 1995; Dandekar et al., 2000; Jaffe et al., 2004b). However, we cannot rule out the possibility of chemotaxis in mycoplasmas (Kirchhoff et al., 1987), because the failure to detect chemotaxis may be a result of the experimental conditions used in these studies. Interestingly, the *Spiroplasma* species do show chemotaxis to amino acids, although they do not have any two-component system genes coded by their genomes (Daniels and Longland, 1984; Lo et al., 2013).

Even if the gliding mycoplasmas move randomly, such movements will enable them to reach conditions that are better for propagation, for example other spaces in the same and different animals. Gliding mycoplasmas repeatedly bind sialylated oligosaccharides on host cell surfaces to enable adhesion and gliding, as discussed below (Nagai and Miyata, 2006; Kasai et al., 2013, 2015). The binding affinity and gliding properties depend on structures that vary significantly in different tissues and in different animals. Mycoplasmas may detect the differences in the structure and reach the tissue covered with the sialylated oligosaccharide for which they have the highest affinity. Indeed, *M. mobile* stays longer on sialylated oligosaccharides with higher affinities than ones with lower affinities (Kasai et al., 2015). Prince et al. examined the infection efficiency of *M. pneumoniae* for a non-gliding mutant as well as other reduced gliding mutants with retained binding activity using a cell culture model of the human trachea (Prince et al., 2014). They found that the mutant cells could not establish robust infection like the unaltered *M. pneumoniae,* which efficiently moved from the tip of cilia to the host cell surface. Cytadherence of mycoplasmas, which is linked to gliding motility, has a well-established role in parasitism and pathogenicity (Razin and Jacobs, 1992; Razin et al., 1998). When a mycoplasma strain loses its capacity for cytadherence, it is easily removed by the host.

THE ATTACHMENT ORGANELLE

M. pneumoniae basically has one attachment organelle per cell (**Figures 1**, **4**; Bredt, 1968; Seto et al., 2001; Hasselbring et al., 2006; Nakane et al., 2015), suggesting that the formation of the nascent organelle occurs precisely coupling with cellular growth and division. This connection between attachment organelle formation and cell division has even been traced by fluorescence microscopy in both fixed and live cells (Seto et al., 2001;

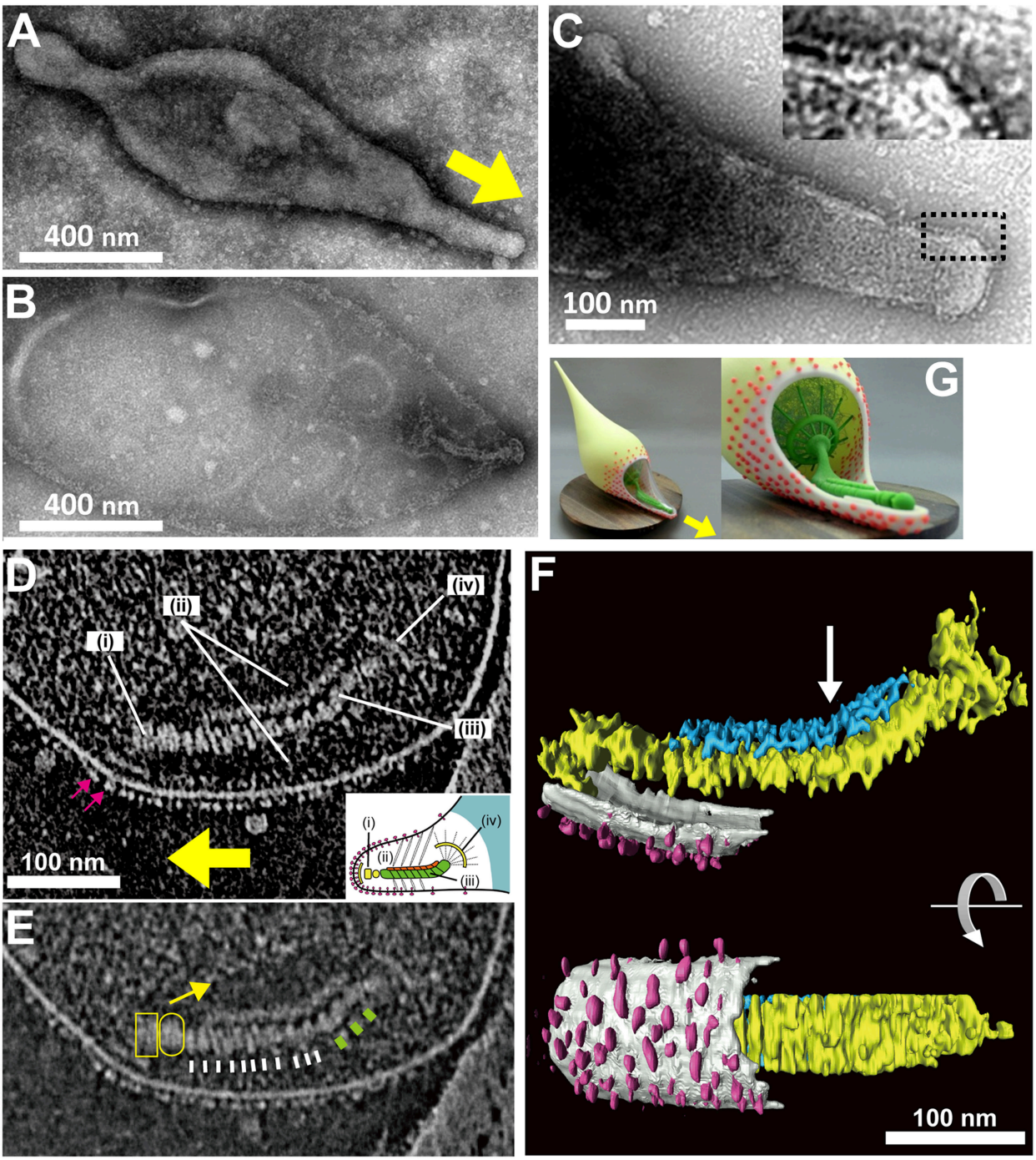

FIGURE 4 | Attachment organelle of *Mycoplasma pneumoniae* visualized by EM. (A) Untreated cell bound to a carbon-coated grid. The membrane protrusion at the right pole is the attachment organelle. The gliding direction is shown by a yellow arrow. **(B)** Image of a cell treated with 1% Tween 20. The cell membrane was partially damaged, and the internal core remained at the right pole of the cell. **(A,B)** were modified from study (Nakane et al., 2015). **(C)** Nap structure. The area outlined by the dashed box is magnified in the inset. This panel was modified from study (Nakane et al., 2011). **(D)** ECT Image with a dense array of the knoblike particles on the membrane surface marked by pink arrows, and the terminal button (i), translucent area (ii), paired plates (iii), and bowl (wheel) complex (iv). The gliding direction is shown by a yellow arrow. Inset: schematic diagram of the attachment organelle. **(E)** Identical image to **(D)** but at lower contrast. The outline of the translucent area is marked with a yellow arrow. The thin and thick striations of the thick plate are marked by white and green bars, respectively. The terminal button is marked by a box and an oval corresponding to those in the inset schematic of **(D)**. **(F)** Rendered three-dimensional image of the tomogram shown in **(D)** depicting the knoblike particles (pink), membranes (light gray), and thin (blue) and thick (yellow) plates of the internal core. The bend is marked with an arrow. **(D–F)** were modified from study (Kawamoto et al., 2016). **(G)** Three dimensionally printed model of cell schematic. The surface and internal structures of the attachment organelle are colored red and green, respectively. The upper side of the cell membrane on the attachment organelle is omitted to show the internal structures. The *stl* format file to print out via 3D printer is available as a supplement. The gliding direction is shown by a yellow arrow.

Hasselbring et al., 2006). These studies indicate that a nascent organelle is formed adjacent to the original one and moves laterally to the other pole. However, the cell images highlighting organelle formation vary in terms of the number of attachment organelles, with the next nascent organelle sometimes emerging before the end of cytokinesis.

It has been shown that the mycoplasma will bind to solid surfaces through the organelle and glide always in the direction of the organelle. In some genetic backgrounds the attachment organelle detaches from the cell body and glides away, suggesting that this organelle performs all of the activities essential for movement (Hasselbring and Krause, 2007a; García-Morales et al., 2016). The attachment organelle is approximately 300–350 nm long and the structures involved can be divided into those on the surface and those that are internal. The surface structures are composed of at least a few proteins that are directly involved in sialylated oligosaccharide binding on the host surfaces. The internal structures have several roles, including organelle formation and maintenance and force generation and transmission. The internal structures of the mycoplasma can be further divided into the translucent area and the core, which is composed of a terminal button, paired plates, and bowl complex (Miyata, 2008; Miyata and Nakane, 2013; Balish, 2014).

BINDING TARGETS FOR GLIDING

To identify the binding targets used during *M. pneumoniae* gliding, we focused on sialylated oligosaccharides (**Figure 5**) for two reasons: (i) these compounds have already been shown to be the binding targets of *M. mobile* gliding (Nagai and Miyata, 2006), and (ii) these compounds have been reported to be the binding target for static adherence of *M. pneumoniae* (Sobeslavsky et al., 1968; Manchee and Taylor-Robinson, 1969; Baseman et al., 1982a; Roberts et al., 1989). Our recent study showed that free molecules of sialylated oligosaccharides blocked the binding of *M. pneumoniae* to glass surface and also removed the gliding mycoplasma cells from the glass surfaces (Kasai et al., 2013). It is therefore probable that the gliding legs bind to the free 3′-*N*-acetylneuraminyl-*N*-acetyllactosamine when they are displaced from the binding target on the glass. We also analyzed the inhibitory effects of 16 chemically synthesized sialylated compounds on the gliding and binding of *M. pneumoniae* and concluded that the recognition of sialylated oligosaccharide by *M. pneumoniae* legs proceeds in a "lock-and-key" fashion, with the binding affinity being dependent on structural differences among the sialylated compounds examined. Further, this study also demonstrated that the binding of the leg and the sialylated oligosaccharide is cooperative, with Hill constants ranging from 1.5 to 2.3.

P1 ADHESIN COMPLEX

The P1 adhesin (MPN141) is a 1627 amino acid protein that localizes over the whole surface of the attachment organelle (Seto et al., 2001; Seto and Miyata, 2003; Nakane et al., 2015) and is responsible for binding to solid surfaces (Baseman et al., 1982b; Feldner et al., 1982; Hu et al., 1982; Krause et al., 1982; Razin and Jacobs, 1992). Although P1 adhesin was identified as the adhesin involved in static binding, this protein also appears to function in the legs of gliding *M. pneumoniae* as monoclonal antibodies against P1 adhesin decrease gliding speed over time and eventually remove gliding cells from glass surfaces (Seto et al., 2005). Isolated P1 adhesin is cleaved at the C-terminal side of the 59th amino acid and forms a complex with a molecular mass of about 480 kDa that contains two molecules of P1 adhesin and two molecules of P90 (protein B, MPN142) (**Figure 6**; Hansen et al., 1979; Layh-Schmitt et al., 2000; Nakane et al., 2011). The complex forms a sphere approximately 20 nm in diameter when analyzed with rotary-shadowing electron microscopy (EM). P90 is encoded in tandem with P1 adhesin and is cleaved from another protein, P40 (protein C), after translation

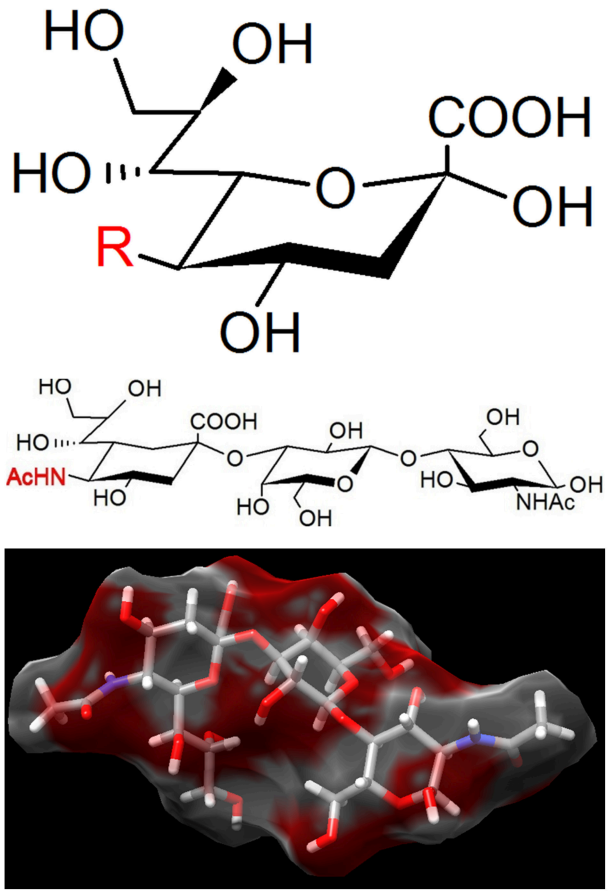

FIGURE 5 | The binding target of *Mycoplasma pneumoniae*. (Upper) Sialic acid is a generic form of the neuraminic acid derivatives, a monosaccharide with a nine-carbon backbone. Three representative structures are *N*-acetylneuraminic acid (R = AcNH), *N*-glycolylneuraminic acid (R = GcNH), and 2-keto-3-deoxynonic acid (KDN; R = OH). *N*-acetylneuraminic acid, the most common member, is occasionally called sialic acid. Sialic acids are found widely as the tip structure of oligosaccharide linked to glycoproteins and glycolipids on animal cell surfaces. **(Middle)** 3′-*N*-acetylneuraminyl-*N*-acetyllactosamine, recognized by *M. pneumoniae*. **(Lower)** The 3D structure of the same compound with **(Middle)** adapted from PubChem (CID: 14367963).

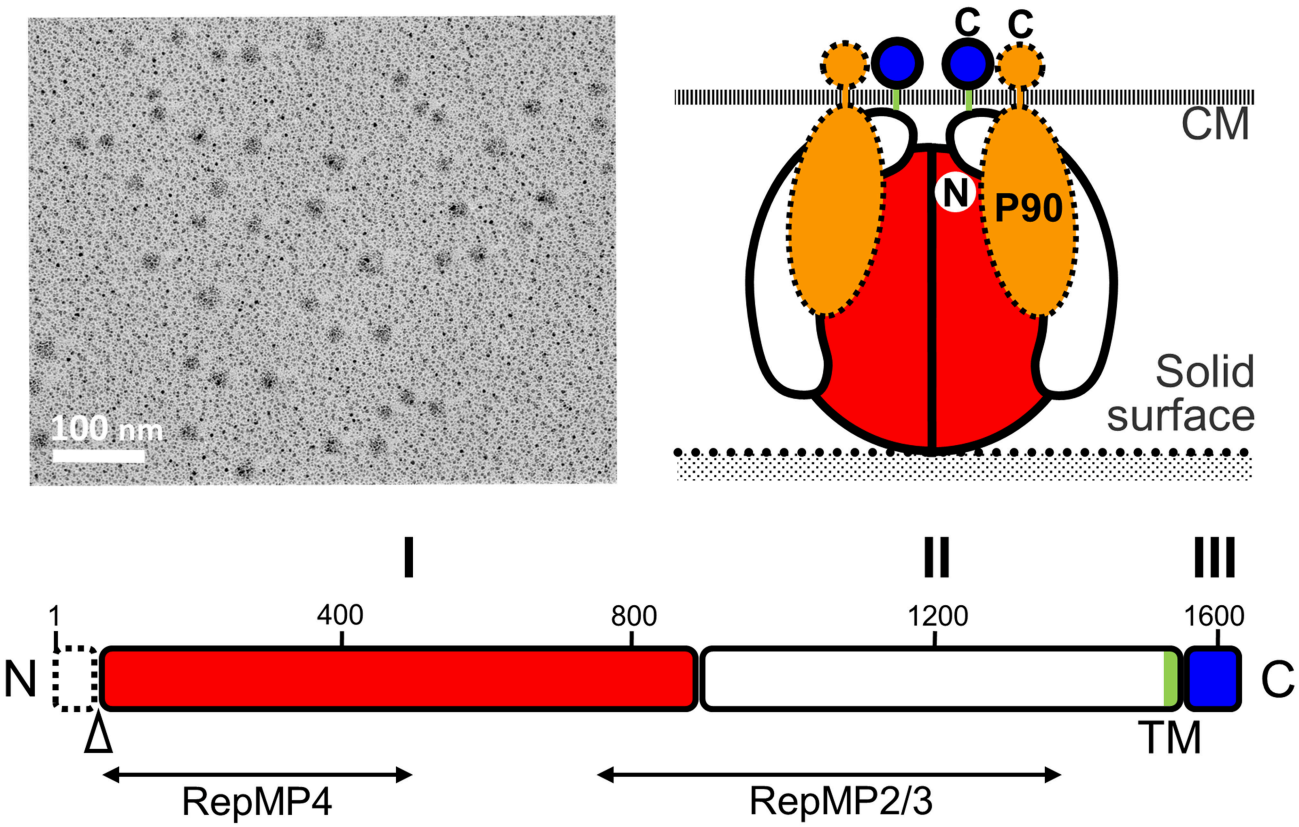

FIGURE 6 | Structure of the P1 adhesin complex. (Upper left) Rotary-shadowing EM of the isolated P1 adhesin complex. **(Lower)** Schematic of the P1 adhesin amino acid sequence. The molecule is divided into domains I, II, and III, which are linked by predicted flexible hinges. Domain I is highly conserved. The transmembrane segment is colored green. The N-terminal 59 residues are removed during the maturation process at the position marked by an open triangle. The regions homologous to repetitive elements coded in different loci are indicated by lines marked RepMP4 and RepMP2/3. Domain III is inside the cell and probably interacts with other proteins in the cell. **(Upper right)** Schematic diagram of the P1 adhesin complex. Two molecules of P1 adhesin are predicted to fold into a globular complex with two molecules of P90. This figure was modified from study (Nakane et al., 2011) on permission.

(Layh-Schmitt and Herrmann, 1992; Catrein et al., 2005). This cleavage is not seen in the ortholog of this protein in *Mycoplasma genitalium*, suggesting that P40 is also involved in the P1 adhesin complex on the organelle surface (Layh-Schmitt and Herrmann, 1994; Layh-Schmitt et al., 2000; Seto et al., 2001).

The P1 adhesin molecule can be divided into three domains (Nakane et al., 2011). Amino acid analysis indicates that domains I and III are well conserved between different species, and that there is a transmembrane region within domain II. It is thought that the binding site for sialylated oligosaccharides may be present in domain I because the amino acid sequences of the binding site would not be expected to evolve rapidly and domain III is predicted to be the internal part. However, the amino acid sequence does not show any clear similarities with any of the known sialylated oligosaccharide receptors, suggesting that P1 adhesin has a novel receptor structure.

The reduction of gliding speed by antibody binding to domain II may suggest that the actual movement of P1 adhesin is involved in the gliding mechanism (Seto et al., 2005). It is possible that the movements generated elsewhere may be transmitted to the P1 adhesin complex (Nakane et al., 2015), with the internal part, domain III, being responsible for force transmission from another internal part of the organelle. Alternatively, the distortion of the whole organelle shape or lateral array of the P1 adhesin complex may play this role (see below).

The sequence of P1 adhesin varies between clinical strains, resulting in structural changes in the immunodominant epitopes of the adhesin, which likely enable host immune system evasion (Nakane et al., 2011). The variation in the P1 adhesin sequence is thought to be generated by intragenomic recombination (Kenri et al., 1999; Spuesens et al., 2009).

SURFACE STRUCTURE

As *M. pneumoniae* binds to solid surfaces at the attachment organelle, surface structures responsible for binding would be expected to be located on this organelle. Nap structures, reminiscent of the raised surface of a cloth, can be observed at the surface of the membrane protrusion/attachment organelle using negatively stained EM (**Figure 4**; Baseman et al., 1982b; Hu et al., 1982). The nap structure on the surface likely corresponds to the

P1 adhesin complex, because they localize on the organelle in a similar pattern, and the dimension and size of the P1 adhesin complex are comparable to those of the nap structures (Hu et al., 1982, 1987; Baseman et al., 1982b; Nakane et al., 2011). Notably, the nap structures cannot be clearly seen, because of the presence of the multiple layers. However, the surface structure of the attachment organelle could be reconstructed using electron cryotomography (ECT), in which the images of frozen specimens are captured at a series of angles relative to the electron beam, and the three dimensional structure is calculated. These structures appear to be "knob" shaped, with a length of 4–8 nm and a diameter of 8 nm (Henderson and Jensen, 2006; Seybert et al., 2006). Recent analysis by ECT suggested that the knob is identical to the nap in negative-staining EM, and showed that it forms a two-dimensional array of limited regularity on the surface (Kawamoto et al., 2016).

INTERNAL STRUCTURE

The internal structure of the attachment organelle can be divided into two parts: the core structure and the translucent area. The core of *M. pneumoniae* was identified in the 1980s (Meng and Pfister, 1980; Gobel et al., 1981; **Figure 4**), and has also been referred to as the "electron dense core" in sectioning images (Wilson and Collier, 1976; Seto and Miyata, 2003), because it has a high electron density compared with other parts of the cell. The core can also be readily observed by EM in the center of the attachment organelle of the species in *Pneumoniae* subgroup after treating the cells with Triton X-100 (Meng and Pfister, 1980; Gobel et al., 1981; Hatchel and Balish, 2008; Nakane and Miyata, 2009; Relich et al., 2009). The core structure can be further divided into three parts: the "terminal button," the "paired plates," and the "bowl (wheel) complex" (**Figure 7**). These features are more evident in the cytoskeletal structure of *Mycoplasma gallisepticum*, which was previously described as an "asymmetrical dumbbell" (Nakane and Miyata, 2009). The details of this structure were further examined by ECT of whole cells (Hasselbring et al., 2006; Seybert et al., 2006; Kawamoto et al., 2016) and by negative staining EM of the core following isolation using centrifugation (**Figure 7**; Nakane et al., 2015).

COMPONENT PROTEINS OF THE ATTACHMENT ORGANELLE

To date, 15 proteins coded on 9 loci on the genome have been identified as components of the attachment organelle as shown in **Figure 8** (Krause and Balish, 2004; Miyata and Nakane, 2013; Balish, 2014; Nakane et al., 2015), and each has recently been mapped on the organelle image systematically in nanometer order by fluorescence microscopy and immuno EM (**Figure 9**; Nakane et al., 2015). The features of this organization are summarized in **Figure 8**, including a new concept, intrinsically disordered region (IDR), which cannot form a stable three dimensional structure but can achieve it when it binds to other structures (Dyson and Wright, 2005). Interestingly, the sequences of P65 (MPN309), HMW1 (MPN447), HMW3 (MPN452), P30 (MPN453), and P200 (MPN567) are mostly predicted as IDR. We tried to assign all of 15 proteins onto the schematic of attachment organelle, based on the information that is currently available (**Figure 9**).

P30, ANOTHER SURFACE PROTEIN

P30 (MPN453), which is comprised of 274 amino acid residues, has a transmembrane segment beginning from amino acid residue 72, with the carboxyl terminus oriented toward the outside of the cell (**Figure 8**; Chang et al., 2011). The subcellular localization of this protein is limited to the end of the attachment organelle, distinct from that of P1 adhesin (**Figure 9**; Seto et al., 2001; Seto and Miyata, 2003; Nakane et al., 2015). Notably, even in-frame deletions of 11 amino acid residues of this protein have been shown to mostly disrupt binding and gliding activities, although the stability of the protein and its localization are not significantly affected, showing that this protein functions as an important player in the gliding mechanism (Hasselbring et al., 2005; Chang et al., 2011). Moreover, chemical cross-linking studies suggest close proximity between P30 and P1 adhesin (Layh-Schmitt et al., 2000). The overexpression of the ortholog in *M. genitalium*, P32 (MG_318), rescues the instability of the P1 adhesin complex in mutant strains (García-Morales et al., 2016). The role of P32 is suspected to be similar to that of P30 in *M. pneumoniae* because P30 can be replaced by P32 in *M. pneumoniae* without significantly disrupting function (Relich and Balish, 2011). P65 (see below), a component of the internal structure likely interacts with P30, because depletion of either of them affects the other one's stability (Jordan et al., 2001; Chang et al., 2011; Hasselbring et al., 2012). These observations may suggest that P30 is linked to the front end of the internal structure, P65 and has a role in the proper alignment and movements of P1 adhesin complexes.

TERMINAL BUTTON

A small piece of the membrane of Triton X-100 treated cells sometimes appears to be attached to the terminal button, suggesting a complex structure that includes the polar cell membrane. The terminal button can be divided into three major parts, the most distal of which is attached to the inner layer of the peripheral membrane proteins (**Figure 7**; Nakane et al., 2015). P65 (MPN309) and HMW3 (MPN452) have been mapped onto this structure (Stevens and Krause, 1992; Jordan et al., 2001; Seto et al., 2001; Seto and Miyata, 2003; Nakane et al., 2015). Considering the close spatial and functional relations between P30 and P65, as well as the positioning of the component proteins, P65 likely interacts with the internal domain of P30 (**Figure 9**; Jordan et al., 2001; Chang et al., 2011; Hasselbring et al., 2012). Further, the tight binding of the terminal button to the front side of the membrane may be achieved through this interaction. P30 may fix dozens of P1 adhesin complexes at the front end to form the initial core of the P1 adhesin complex array covering the organelle through the lateral interactions among the complexes. P65, on the other hand, may determine the gliding direction by modifying the angle of the organelle

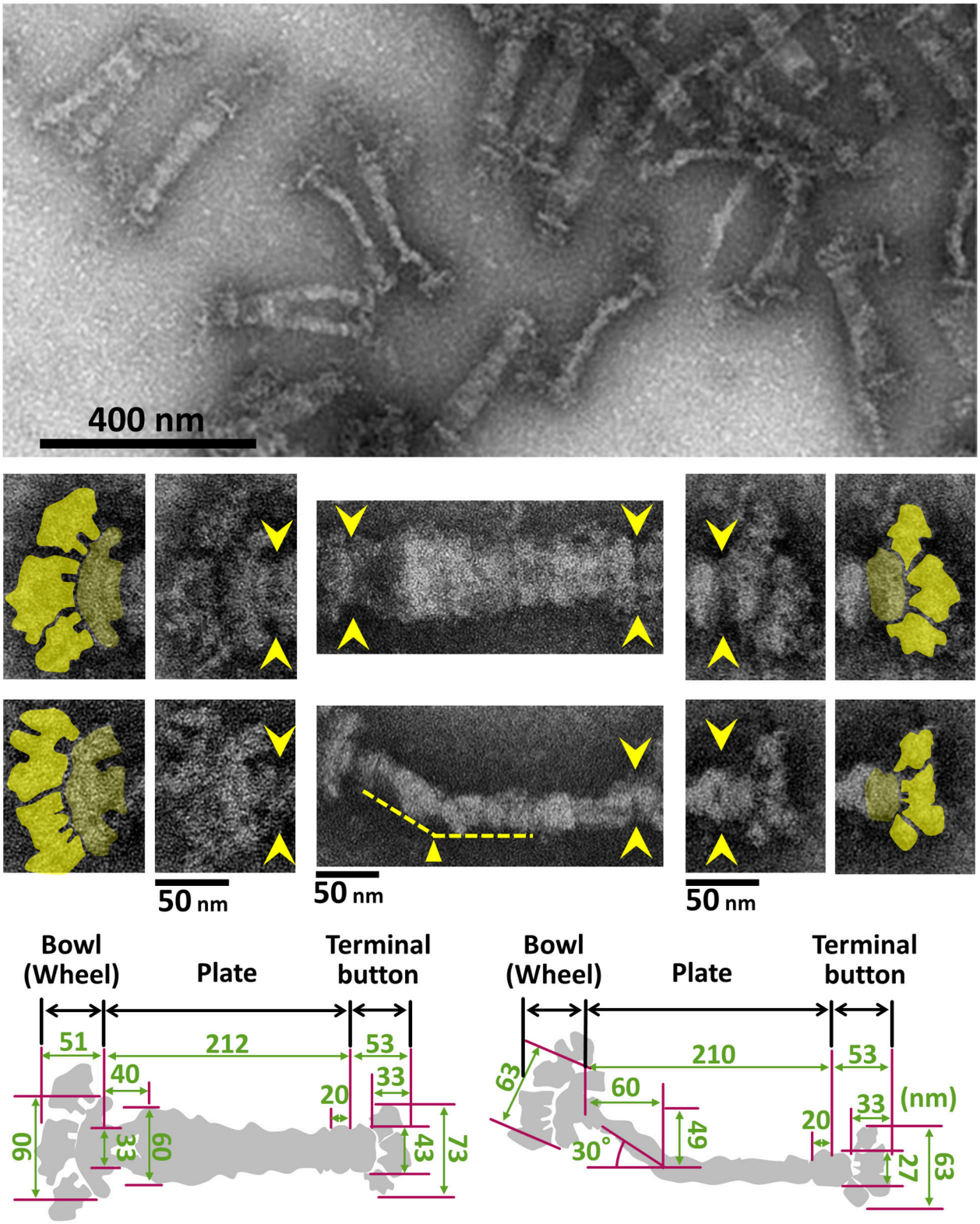

FIGURE 7 | Negative staining EM images of the isolated internal core of the attachment organelle. (Upper) Core fraction. **(Middle)** Structural features of the internal core. Top and side views of the structures are shown in the upper and the lower panels, respectively. Yellow arrowheads indicate the boundaries between the core components. The original and colored images are shown in the adjacent panels for the bowl complex and terminal buttons in the left and the right, respectively. The thin plate is not visible in this preparation. A bend is observed in the side view around 60 nm from the back end, as marked by the yellow triangle. **(Lower)** Schematics and dimensions averaged for 40 structures. This figure was modified from study (Nakane et al., 2015).

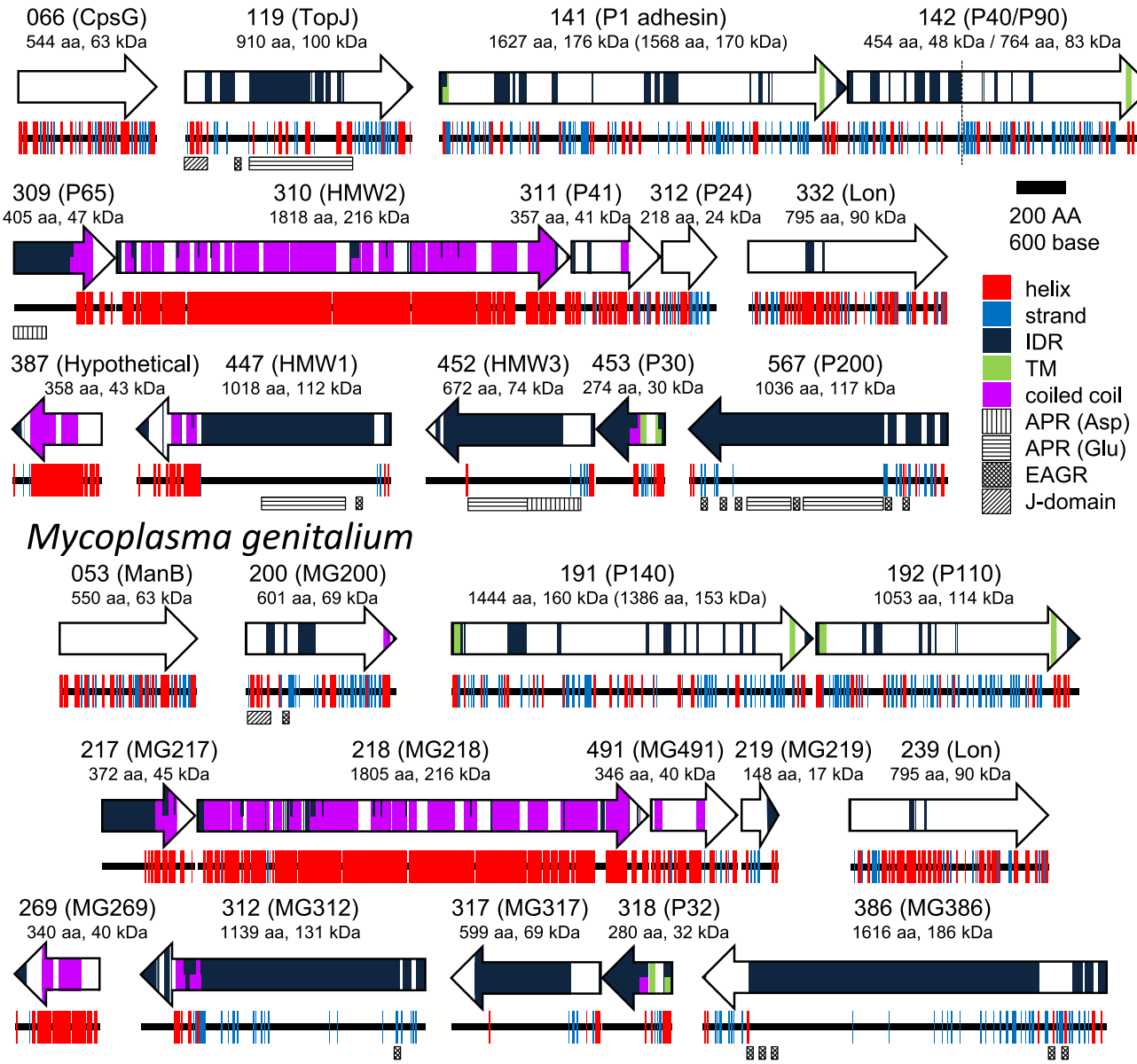

FIGURE 8 | Protein components of the attachment organelle. Fifteen and fourteen proteins coded in nine loci on the genome are presented with their MPN and MG codes , respectively and the protein name. α-helix (helix), β-strand (strand), intrinsically disordered region (IDR), transmembrane segment (TM), coiled coil, Aspartic acid, and Proline rich region (APR Asp), Glutamic acid and Proline rich region (APR Glu), Enriched in Aromatic and Glycine Residues (EAGR), and the J-domain are predicted and marked. The amino acid number and predicted mass are shown beneath the protein name. The size after processing is shown in parentheses for MPN141 (P1 adhesin) and MG191 (P140). The cleavage site between P40 and P90 is shown by a broken line. *M. pneumoniae* and *M. genitalium* are presented as upper and lower panels, respectively.

relative to the cell axis as deletion of MG217 in *M. genitalium*, the ortholog of P65, changes the gliding direction, consistent with this function (Burgos et al., 2008). The N- and C-terminus of HMW2 (MPN310), a coiled-coil protein were also mapped to the terminal button and bowl complex, respectively (Nakane et al., 2015). Probably, HMW3 bundles the array of HMW2 at the back side of terminal button.

PAIRED PLATES

The paired plates are composed of paired striated plates separated by a gap of about 7 nm (Hasselbring et al., 2006; Henderson and Jensen, 2006; Seybert et al., 2006; Kawamoto et al., 2016). The paired plates appear flexible and bend approximately 30 degrees just proximal to their middle (**Figure 7**). This bend suggests that

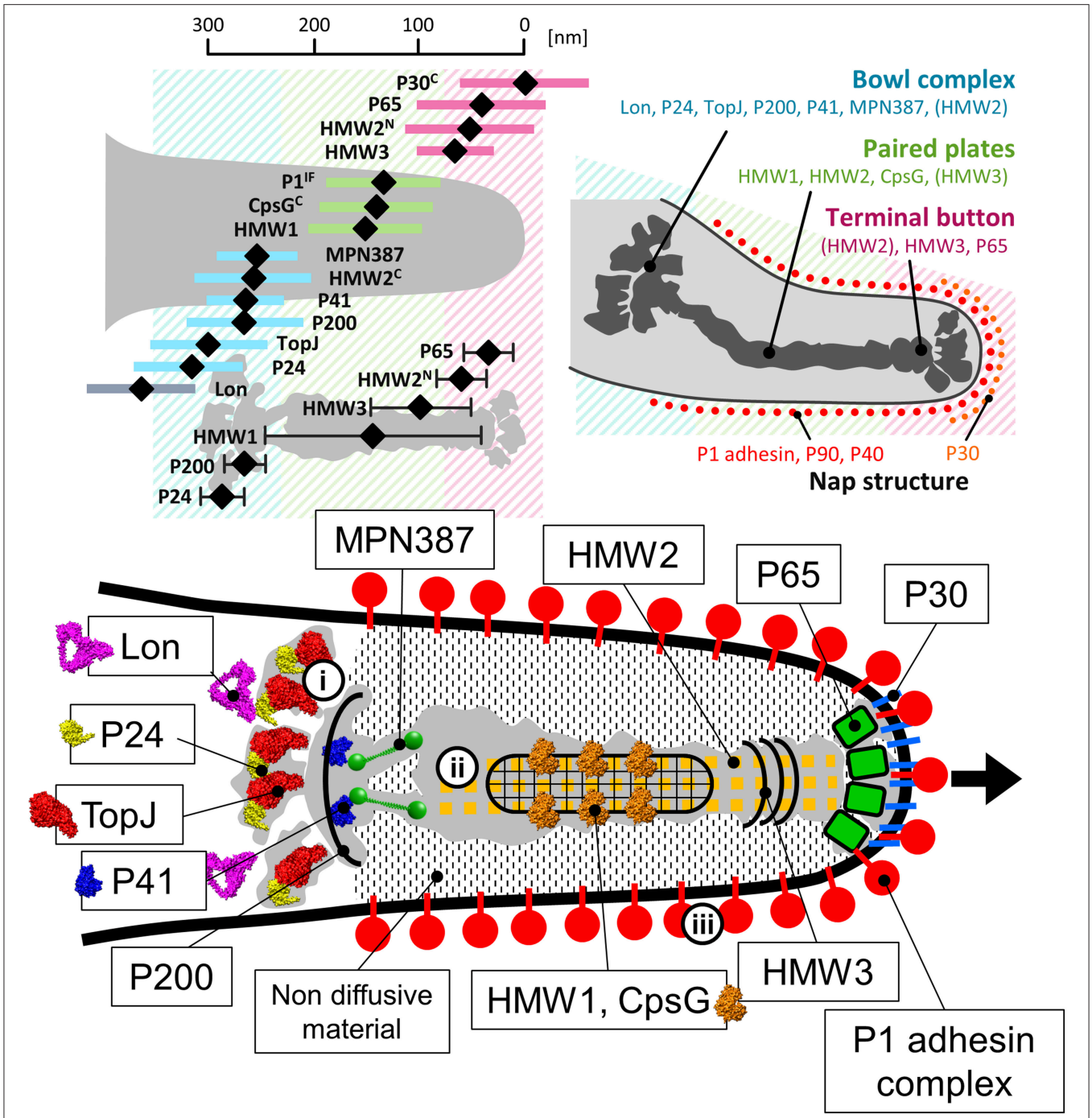

FIGURE 9 | Mapping and assignment of component proteins on the attachment organelle. (Upper left) Protein positions based on fluorescence microscopy. The proteins were labeled by a monoclonal antibody for P1 adhesin, EYFP (Enhanced Yellow Fluorescent Protein) tagging at the C terminus if the protein name is marked "C," or EYFP tagging at the N terminus for others. HMW2 was mapped at both the N- and C-terminus. The average position in individual cells and standard deviation relative to the cell edge are plotted with a black diamond and a colored bar, respectively. The mapping data based on immuno EM are plotted as a black diamond with a black bar in the lower. The proteins are mapped onto three localization groups, the terminal button (pink), the paired plates (green), and the bowl complex (blue). **(Upper right)** The attachment organelle including component proteins. Upper panels were modified from study (Nakane et al., 2015). **(Lower)** Assignment of the component proteins onto the schematic of the attachment organelle based on EM. The protein structures were predicted by Phyre2 (Kelley and Sternberg, 2009) whenever possible. In the gliding mechanism, the force may be generated at the bowl complex (i), transmitted through the paired plates (ii), and modulated by the P1 adhesin complex (iii). The gliding direction is shown by a black arrow.

M. pneumoniae cells have three axes, front-back, upper-lower and left-right. The actual alignment of these axes relative to the solid surface during gliding is unknown. The localization of the N- and C-terminus at the terminal button and bowl complex, respectively, shows that HMW2 protein forms a dimer and a parallel bundle (**Figures 8**, **9**; Krause et al., 1997; Nakane et al., 2015). In the thick plate, striations may correspond to eleven coiled-coil regions intermittently appearing in the sequence of the 1818 amino acids present in HMW2 (Dandekar et al., 2000; Letunic et al., 2015; Kawamoto et al., 2016). The thin plate featured with a stable hexagonal lattice (Kawamoto et al., 2016) is likely composed of HMW1 (MPN447) and CpsG (MPN066).

The paired plates likely function as the scaffold for formation of the attachment organelle, as both of HMW1 (MPN447) and HMW2 (MPN310) are essential for the early stage of organelle formation (Popham et al., 1997; Hahn et al., 1998; Seto and Miyata, 2003; Kenri et al., 2004; Burgos et al., 2007, 2008; Bose et al., 2009). The paired plates are also expected to have a critical role in gliding. Indeed, an *M. genitalium* mutant can glide without MG218, the ortholog of HMW2, if P32 (MG318) the ortholog of P30 (MPN453) was overexpressed, however gliding speed was 100-fold decreased (García-Morales et al., 2016).

BOWL COMPLEX

Based on their examination of EM cryosections, Hegermann et al. have suggested that the striated paired plate is attached at the proximal end to a "wheel (bowl) complex" with fibrils, which connects the complex to the cell body (Hegermann et al., 2002; Mayer, 2006). A similar structure, called the "bowl," has been found at the position of the wheel complex in *M. pneumoniae* by ECT, although fibrils were not observed (Henderson and Jensen, 2006; Seybert et al., 2006; Kawamoto et al., 2016). In contrast, fibrils were observed in negatively stained EM images of the "asymmetrical dumbbell" isolated from *M. gallisepticum* cells, suggesting that fibrils are probably present in the core in *M. pneumoniae* (Nakane and Miyata, 2009). In the reduced contrast ECT images, the identification of thin fibers is sometimes more difficult than in other methods. MPN387, the C terminus of HMW2 (MPN310), P41 (MPN311), P200 (MPN567), TopJ (MPN119), P24 (MPN312), and Lon (MPN332) appear to be localized on the bowl complex (Kenri et al., 2004; Jordan et al., 2007; Cloward and Krause, 2009; Nakane et al., 2015; **Figure 9**). The bowl complex likely has a role in connecting the attachment organelle to other parts of the cell, because in mutant strains of *M. pneumoniae* and *M. genitalium* that lack P41 or MG491, respectively, the attachment organelle occasionally detaches from the cell body and glides independently (Hasselbring and Krause, 2007a,b; García-Morales et al., 2016). The bowl complex may also be responsible for the generation or transmission of force, because mutation of the P200 or TopJ orthologs in *M. genitalium* (MG386 and MG200, respectively) results in mutants that can adhere, but have less gliding capacity, common with the character of P200 mutant of *M. pneumoniae* (Pich et al., 2006; Jordan et al., 2007). Moreover, the mutants depleted for MPN387 cannot glide, although they also retain their cytadherence properties (Hasselbring et al., 2005). The mapped position (Nakane et al., 2015) and the predicted molecular shape of the MPN387 protein suggested that it fits onto the front side of bowl complex. Thus, MPN387 may bridge the bowl complex to the back side of paired plates to transmit force. We have also predicted the molecular shapes of P41, TopJ, P24, and Lon, based on their amino acid sequences (Kelley and Sternberg, 2009), and have assign those images onto the core image based on recent mapping results (**Figure 9**).

TRANSLUCENT AREA

The core is surrounded by an electron lucent area from which the dense complexes are excluded (Wilson and Collier, 1976; Shimizu and Miyata, 2002; Seto and Miyata, 2003). This area is unlikely an artifact of chemical fixation and dehydration because it is also observed following rapid freezing (Henderson and Jensen, 2006; Seybert et al., 2006; Kawamoto et al., 2016). Hegermann et al. examined the structure of this area by treating fixed cells with Triton X-100 and suggest that thin filamentous structures connect the electron-dense core and the periphery of the cell (Hegermann et al., 2002). Henderson and Jensen also proposed that the translucent area was created by the exclusion of macromolecules via the repeated movement of the electron-dense core (Henderson and Jensen, 2006). However, this hypothesis is unlikely because the translucent area can be observed in all cells even if they are not in conditions optimized for gliding (Shimizu and Miyata, 2002; Seto and Miyata, 2003). This area may be occupied by stiff, less diffusive materials invisible in EM, which may play a role in transmitting the movements of the paired plates originated in the bowl complex (**Figure 9**; Henderson and Jensen, 2006; Seybert et al., 2006; Kawamoto et al., 2016). This invisible material is unlikely domain III of P1 adhesin, because the translucent area can be observed in a mutant lacking the P1 adhesin complex (Seto and Miyata, 2003; Kawamoto et al., 2016).

FORMATION AND ORIGIN OF THE ATTACHMENT ORGANELLE

The gene arrangements of many of the component proteins in the genome agree with their suspected protein alignments in the attachment organelle (**Figures 8**, **9**). The gene order, MPN309 (P65) to MPN312 (P24), agrees with the protein order in the core from the front end to the back end. Another gene order MPN453 (P30) to MPN452 (HMW3) also agrees with the protein order. Notably, the proteins of P1 adhesin complex are coded tandemly as MPN141 (P1 adhesin) and MPN142 (P40/P90) and are likely synthesized continuously and assembled into the complexes (Inamine et al., 1988; Krause et al., 1997; Waldo et al., 1999). This assumption is consistent with observations that 5.5% of internal cores have a shape that is branched at the front side (Nakane and Miyata, 2009; Nakane et al., 2015). The proteins featured the most with IDR [i.e., TopJ (MPN119), P65 (MPN309), HMW1 (MPN447), HMW3 (MPN452), P30 (MPN453), and P200 (MPN567)] may be assembled into the existing structures immediately after they are synthesized. The gene clustering of these proteins may also suggest the evolution stages. For example, in the early stage, the sialic acid receptor

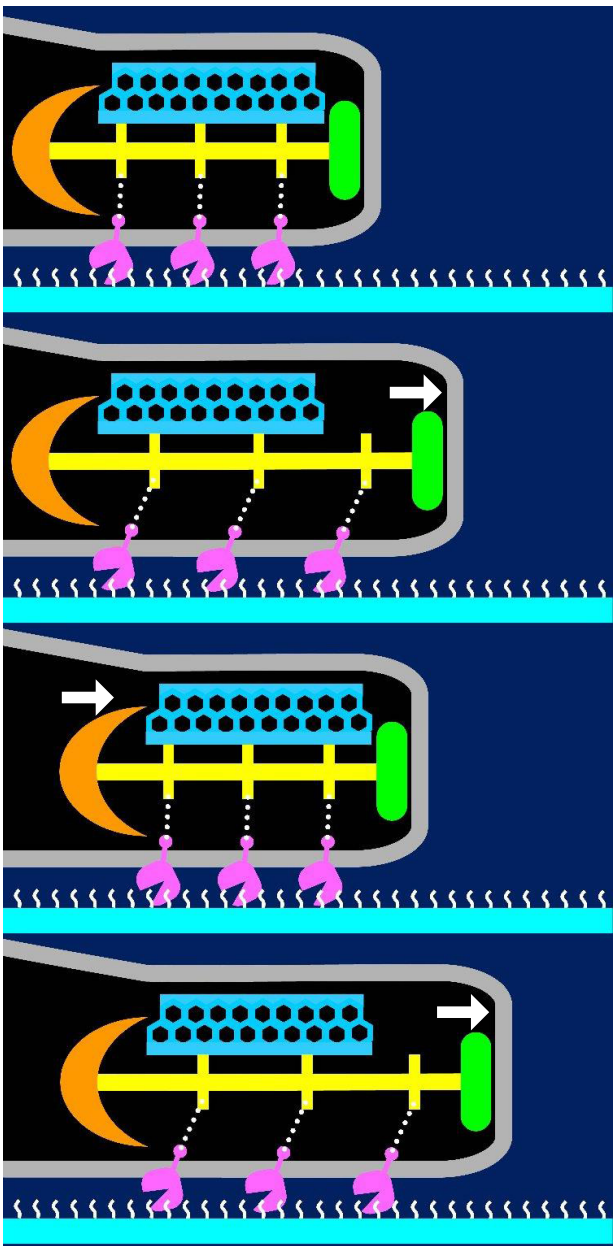

FIGURE 10 | Suggested mechanism underlying gliding. The P1 adhesin, colored pink is responsible for binding to sialylated oligosaccharides on the solid surface colored white. The thin and thick plates, colored blue and yellow, respectively, featured a rigid hexagonal lattice and a striation with a highly variable pitch. The terminal button is colored green. The compression and extension of the thick plate may cause shortening and elongation of the attachment organelle, respectively. The thick plate changes its pitch and induces directed detachment of P1 adhesin from SOs, and leading to cell displacement from left to right. This figure was modified from study (Kawamoto et al., 2016).

of the P1 adhesin complex was supported mechanically by the core composed of P65, HMW2, P41, and P24. In the next stage, the combination of the adhesin and the core was stabilized by P30 and HMW3. In the final stage, other proteins, including a force generator, were integrated into the system. Interestingly, we can find one ATPase, Lon (MPN332), included in the 15 component proteins, and while it has high sequence similarity with Lon proteases, represented by 46% identity with that from *Clostridium spiroforme* (WP_050752792.1), it is possible that this protein functions in the attachment organelle.

ENERGY SOURCE

Information about the energy source is indispensable in clarifying the mechanisms involved in any kinds of motility. Most of bacterial motility systems are based on membrane potential rather than ATP, including bacterial flagella, *Myxococcus* gliding, *Flavobacterium* gliding, and so on, with only pili based motility utilizing ATP energy (Jarrell and McBride, 2008). Probably, this is because the majority of actively moving bacteria at first pool their energy to use, as a membrane potential through respiration. In *M. mobile*, on the contrary, the energy of motility is provided by ATP (Jaffe et al., 2004a; Uenoyama and Miyata, 2005; Kinosita et al., 2014), consistent with the above assumption, because generally *Mollicutes* pool their energy as high energy phosphate compounds, like ATP (Razin et al., 1998). *M. mobile* has been proven to be driven by the energy of ATP through the use of cellular "ghosts" that have damaged membranes, which could be reactivated by the addition of ATP (Uenoyama and Miyata, 2005; Kinosita et al., 2014). We have tried similar experiments with *M. pneumoniae*, but were unable to reactivate the motility of the "ghosts." However, this does not promptly suggest that *M. pneumoniae* is driven by a different energy source. All the essential parts of the gliding machinery are required after the damage is inflicted on the cell envelope by the detergent, so the failure of these experiments may reflect the sensitivity of the gliding mechanism of *M. pneumoniae* to the detergent. The deletion of Ser/Thr protein kinase gene (prkC; MPN248) or its cognate phosphatase gene (prpC; MPN247) also influences the frequency and speed of gliding as well as the phosphorylation levels of the component proteins in the attachment organelle (Dirksen et al., 1994; Schmidl et al., 2010; Page and Krause, 2013), suggesting that changing the local charge coupled with phosphorylation is involved in the gliding mechanism.

These observations, however, do not deny the possibility that membrane potential could be the direct energy source as mycoplasmas also possess membrane potential like other bacterial species, which is likely caused by the F-type ATPase on the membrane using ATP energy (Benyoucef et al., 1981).

SUGGESTION FOR GLIDING MECHANISM

We have been studying the gliding mechanism of *M. mobile* since 1997, and have suggested a "centipede model" mechanism in which *M. mobile* repeatedly catches, pulls, and releases sialylated oligosaccharides on host surfaces, based on the movements generated by an ATPase that are transmitted through the cell membrane (Miyata, 2008, 2010; Miyata and Nakane, 2013; Miyata and Hamaguchi, 2016). Unfortunately, only some common features appear to exist between the gliding mechanisms of *M. mobile* and *M. pneumoniae* (Miyata, 2008; Miyata and Nakane, 2013).

Here, we have suggested a possible model for the gliding mechanism utilized by *M. pneumoniae* that integrates all of the known information (**Figures 9**, **10**; Nakane et al., 2015; Kawamoto et al., 2016). Movements for gliding may be generated in the bowl complex and transmitted efficiently to the paired plates fixed to the cell front through P65 and P30 in the terminal button. Then, extension and retraction of the attachment organelle will occur. This movement is transmitted to the P1 adhesin complexes through distortion of the translucent area and/or the complex array on the surface. The P1 adhesin complexes will then repeat a catch-pull-release cycle with sialylated oligosaccharides on the host surface. The attachment organelle will then pull the other parts of the cell connected to the back end of the bowl complex, resulting in cellular movement. Additional work is warranted to further elucidate the proteins and pathways involved in this process.

AUTHOR CONTRIBUTIONS

TH analyzed protein and sugar structures and prepared **Figures 5**, **8** and a part of **9**. MM prepared other figures and text. Both checked the completed version.

FUNDING

This work was supported by a Grant-in-Aid for Scientific Research on the Innovative Area "Harmonized Supramolecular Motility Machinery and Its Diversity" (MEXT KAKENHI Grant Number 24117002), and by a Grant-in-Aid for Scientific Research (B) (MEXT KAKENHI Grant Number 24390107) to MM.

ACKNOWLEDGMENTS

We are grateful to Duncan C Krause and Jaume Pinyol for helpful discussion, and Tsuyoshi Kenri, Pascal Sirand-Pugnet, Hirofumi Wada, Daisuke Nakane, Akihiro Kawamoto, Taishi Kasai, and Peng Liu for their assistance with the preparation of the figures.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00960

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 26 January 2016
doi: 10.3389/fmicb.2016.00039

Antibody Response to *Mycoplasma pneumoniae*: Protection of Host and Influence on Outbreaks?

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In humans of all ages, the cell wall-less and genome-reduced species *Mycoplasma pneumoniae* can cause infections of the upper and lower respiratory tract. The well-documented occurrence of major peaks in the incidence of community-acquired pneumonia cases reported world-wide, the multifaceted clinical manifestations of infection and the increasing number of resistant strains provide reasons for ongoing interest in the pathogenesis of mycoplasmal disease. The results of recent studies have provided insights into the interaction of the limited virulence factors of the bacterium with its host. In addition, the availability of complete *M. pneumoniae* genomes from patient isolates and the development of proteomic methods for investigation of mycoplasmas have not only allowed characterization of sequence divergences between strains but have also shown the importance of proteins and protein parts for induction of the immune reaction after infection. This review focuses on selected aspects of the humoral host immune response as a factor that might influence the clinical course of infections, subsequent protection in cases of re-infections and changes of epidemiological pattern of infections. The characterization of antibodies directed to defined antigens and approaches to promote their induction in the respiratory mucosa are also preconditions for the development of a vaccine to protect risk populations from severe disease due to *M. pneumoniae*.

Keywords: *Mycoplasma pneumoniae*, community-acquired pneumonia, immune response, adherence, protective antibodies

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *04 December 2015*
Accepted: *11 January 2016*
Published: *26 January 2016*

Citation:
Dumke R and Jacobs E (2016) Antibody Response to Mycoplasma pneumoniae: Protection of Host and Influence on Outbreaks? Front. Microbiol. 7:39. doi: 10.3389/fmicb.2016.00039

From an evolutionary point of view, *Mycoplasma pneumoniae* is a successful minimalized bacterium perfectly adapted to humans as the only known natural host. During the long-term host–pathogen interaction, the genome of these micro-organisms was reduced to only about 816 kb. This includes removal of genes coding for cell wall synthesis as well as a reduction of metabolic capabilities requiring a supply of metabolites from the environment. The adaptation of the bacterium led to distinct tissue tropism using the surface of the respiratory mucosa as the preferred niche, which ensures the survival and parasitic life style of these bacteria. The pathogen possesses a repertoire of factors that cause clinical manifestations after an infection; however, typical virulence determinants of other pathogenic bacteria, such as type III or IV secretion systems, are lacking in *M. pneumoniae*. The species developed an almost unique adherence apparatus. As the main virulence factor (Atkinson et al., 2008; Atkinson and Waites, 2014), this tip-structured protein complex determines not only the adhesion process to the respiratory epithelium as a first step in host colonization but also supports a gliding mechanism which results in bacterial motility

(Miyata, 2010). The close contact of mycoplasmas with epithelial cells reduces the efficiency of mucociliary clearance processes. Damage to epithelial cells of the mucosa causing subsequent symptoms is caused by generation of peroxide and superoxide anion (Hames et al., 2009). In addition, an ADP-ribosylating and vacuolating community-acquired respiratory distress syndrome (CARDS) toxin resembling the pertussis toxin induces ciliastasis and the release of proinflammatory cytokines (Kannan and Baseman, 2006). Further steps in pathogenesis are mainly determined by local inflammation processes and immunomodulation (Meyer Sauteur et al., 2014).

IMMUNODOMINANT ANTIGENS OF *M. pneumoniae* AND HOST RESPONSE

In addition to the role of virulence factors of *M. pneumoniae*, the induction of specific antibodies is not only important for serodiagnosis of infections but also for the colonization process. As result of lacking the classical bacterial cell wall, components of the cell membrane are important for interaction of the pathogen with the host. The importance of an intact immune response is demonstrated by the increased occurrence of severe disease, repeated infections or prolonged persistence of *M. pneumoniae* in patients with deficiencies of humoral immunity (Foy et al., 1973; Taylor-Robinson et al., 1980; Roifman et al., 1986), thus emphasizing the role of specific antibodies for protection. The antigens of *M. pneumoniae* cells determining the host response include glycolipids as well as proteins (Morrison-Plummer et al., 1986) that induce comparable immune reactions in affected individuals (Jacobs et al., 1986; Vu et al., 1987). In comparison with glycolipids, the more specific proteins were mainly characterized as components of the adhesion apparatus of *M. pneumoniae* (Razin and Jacobs, 1992). In particular, antibodies to the P1 protein are regularly found in sera of infected patients. The large membrane protein (168 kDa) was characterized as the main adhesin of the bacteria and is also the most antigenic protein, inducing strong and early production of antibodies (Hu et al., 1983). Using different proteomic approaches such as fractionation of whole proteins (Regula et al., 2001), construction of a whole-genome phage display library (Beghetto et al., 2009) or 2D separation of proteins followed by incubation with sera of infected patients (Nuyttens et al., 2010) resulted in the characterizations of further antigens which are membrane-associated and potentially interact with the host immune system. Besides proteins with a confirmed function in adherence, putative lipoproteins, glycolytic enzymes (e.g., pyruvate dehydrogenase subunit B), chaperones (GroEL, DnaK) and proteins of translation/transcription (e.g., elongation factor Tu) were found. Some of these proteins are surface-localized and involved in interactions with components of the human extracellular matrix (Dallo et al., 2002; Gründel et al., 2015). In addition, CARDS toxin as an important virulence factor of *M. pneumoniae* was characterized as an immune-dominant protein (Kannan and Baseman, 2006). However, the role of antibodies to many of these proteins for the potential to protect the host from re-infections remains to be proved.

With the development of specific tools for investigation of mycoplasmas (Halbedel and Stülke, 2007), such as targeted mutation of TGA triplets coding for tryptophan in *M. pneumoniae* (Inamine et al., 1990), the recombinant production and analysis of proteins of interest for host–pathogen interaction have accelerated. Regarding naturally infected hosts, **Table 1** summarizes defined *M. pneumoniae* proteins that were found in recent years to elicit a specific and strong immune reaction in humans. These studies confirmed that the immune response is dominated by antibodies against the adhesins and adhesion-related proteins of the bacterium that have limited effect on viability (Krause and Baseman, 1983). It can be suggested that the antibody response results mainly in an influence on the gliding process (Seto et al., 2005) and a decrease of adhesion of bacteria to the target cells of the respiratory mucosa. Studies using quantitative methods to measure the adherence of *M. pneumoniae* to human cells *in vitro* showed that specific antisera to total proteins, to adhesins or even to defined regions of adhesins are able to inhibit adhesion to more than 90% in comparison with control sera (Svenstrup et al., 2002; Schurwanz et al., 2009). The importance of the adherence process for further colonization is underlined by the fact that mutants defective in expression of different adhesins and adhesion-related proteins are avirulent (Balish and Krause, 2006).

Beside problems in the sensitivity and specificity of serological assays (Loens et al., 2010; Busson et al., 2013), *M. pneumoniae* infections are complicated by different host-dependent characteristics, such as variable persistence of antibodies, missing IgM response after re-infection and the infrequent production of IgA antibodies in children (Atkinson et al., 2008). IgM antibodies can be detected 7–10 days after infection and IgG immunoglobulins are measurable approximately 14 days later (Atkinson et al., 2008; Atkinson and Waites, 2014).

GENOTYPE-SPECIFIC IMMUNE RESPONSE AND INFLUENCE ON THE EPIDEMIOLOGY OF *M. pneumoniae* INFECTIONS

Genome plasticity and different mechanisms for antigen variation are a typical pattern of different mycoplasma species with pathogenic potential (Citti and Blanchard, 2013). In *M. pneumoniae*, recent studies comparing whole genomes resulted in a remarkable homology between strains of different origin and isolation period (Lluch-Senar et al., 2015; Xiao et al., 2015). However, isolates of *M. pneumoniae* or strains in respiratory tract samples from patients show defined sequence variations which can be used for typing by different methods. Multilocus variable number of tandem repeat analysis (Degrange et al., 2009), multilocus sequence typing (Brown et al., 2015) and SNP minisequencing (Touati et al., 2015) have been developed recently using current molecular tools. These approaches investigate variable regions in the genome which are located mainly in intergenic regions and in genes coding for hypothetical proteins or for house-keeping proteins with an

TABLE 1 | Recombinant *Mycoplasma pneumoniae* proteins tested as antigens for detection of specific antibodies in humans.

Protein (gene)	Function	Remarks	Reference
P1 (*mpn141**)	Adherence	Use of C-terminal protein part	Drasbek et al., 2004
		Full-length characterization using 15 recombinant proteins, construction of a chimeric protein of C-terminal P1 part and P30	Schurwanz et al., 2009
		Construction of a chimeric protein of C-terminal P1 region and parts of P30 and MPN456 (unknown function)	Montagnani et al., 2010
		C-terminal protein part	Nuyttens et al., 2010
		Conserved C-terminal and variable part of repMP4 of P1-types 1 and 2	Dumke et al., 2012
		C-terminal protein part	Xue et al., 2013
		Immunodominant COOH epitope	Wood et al., 2013
		Full-length characterization by using four recombinant proteins	Chourasia et al., 2014
P30 (*mpn453*)	Adherence	Full-length protein	Varshney et al., 2008
		Fragment of P30 (without N-terminus)	Schurwanz et al., 2009
P90 (*mpn142*)	Adherence	Fragment of P90 (aa 751–1088)	Dumke et al., 2012
P200 (*mpn567*)	Adherence	Fragment of P200 (aa 641–678)	Dumke et al., 2012
AtpD (*mpn598*)	Energy metabolism	Full-length protein	Nuyttens et al., 2010
CARDS toxin (*mpn372*)	Cytotoxin	Full-length protein	Wood et al., 2013
P116 (*mpn213*)	Hypothetical (adherence?)	Protein fragment (53 kDa)	Duffy et al., 1999
		Protein fragment (without C-terminus)	Drasbek et al., 2004
		N-terminal protein region (27 kDa)	Tabassum et al., 2010
P400 (*mpn400*)	Hypothetical	Fragment of P400 (aa 407–582)	Dumke et al., 2012

**According to Dandekar et al., 2000.*

intracellular function. Furthermore, the latter two approaches detect small polymorphic sites and even single nucleotide exchanges. Regarding the immune response, an influence of these differences on protective antibodies in infected hosts can hardly be expected. Also, the role in the immune reaction of more different genome regions such as certain genes that contain copies of the repetitive element RepMP1 (Musatovova et al., 2012) is still unclear as the gene products involved and their possible functions have yet to be investigated. Lipoproteins are important antigens in many mycoplasma species showing variations to escape the host immune response (Citti et al., 2010; Szczepanek and Silbart, 2014). In *M. pneumoniae*, a high proportion (nearly 7%) of genes encoding for putative lipoproteins was confirmed and their expression pattern was investigated (Hallamaa et al., 2006, 2008). So far, strain-specific induction of anti-lipoprotein antibodies during infection has not been demonstrated.

The genome region with the greatest discrimination power in combination with well-characterized gene products is the p1 operon coding for P1 adhesin and the adhesion-related proteins P40 and P90, as well as a phosphoesterase (*mpn140*). Copies of repetitive elements RepMP2/3 and RepMP4 (p1 gene, *mpn141*) as well as of RepMP5 (*mpn142*) can be found, which differ between isolates (Su et al., 1990). Analysis of repetitive elements distributed in variable size and sequence over the genome of *M. pneumoniae* strains resulted in the characterization of p1 type 1 and p1 type 2 (Spuesens et al., 2009). Despite a high number of RepMP2/3 ($n = 10$), RepMP4 (8) and RepMP5 (8) copies in the genome of strain M129 (Dandekar et al., 2000), the number of circulating p1 types is relatively low. It can be suggested that the complex functions of the P1 protein limit the possible recombination events that allow effective adhesion, gliding and/or division processes of mycoplasma cells. In addition to the main types 1 and 2, variants differing to a smaller extent from the two p1 types (mainly in the RepMP2/3 copy) were identified after investigation of sequence of p1 genes of clinical strains collected world-wide. According to recent knowledge, these variants cannot be distinguished in the sequence of *mpn142,* indicating lower variability of proteins P40 and P90. Despite the fact that 76% of the p1 sequence can be assigned to both RepMP copies, the divergence between the amino acid sequences of P1 adhesins of types 1 and 2 strains (1,628 and 1,635 aa) is only about 5%. However, these distinct differences in amino acid sequence may influence the immune response of a colonized host. Indeed, it was shown that the variable parts of the P1 adhesins are antigenic resulting in a type-specific response in immunized animals (Dumke et al., 2008). Thus, recombinantly produced protein parts derived from variable regions of the P1 adhesin can be used for determination of type-specific IgG immunoglobulins (Dumke et al., 2012). Furthermore, guinea pigs infected intranasally with a type 2 strain developed protective immunity to type 2 strains after re-infection with a mixture of types 1 and 2 (Dumke et al., 2004). As a consequence of these findings and because of the dominant role of antibodies to the P1 protein in sera of infected patients, it has been suggested that type-specific immunoglobulins have an influence on subsequent re-infections in affected individuals as well as on the epidemiology of infections in greater populations. The results of different studies confirmed that the epidemic peaks of respiratory infections due to *M. pneumoniae* occurring at intervals of 3–7 years were correlated with a change of the predominant p1 type (Kenri et al., 2008; Kogoj et al., 2015; Suzuki et al., 2015; Zhao et al., 2015). In other investigated

populations, significant differences in the proportion of types 1 and 2 strains during endemic and epidemic periods of infections were not detected (Dumke et al., 2015; Jacobs et al., 2015). Therefore, further long-term epidemiological studies are needed to clarify whether type-specific antibodies induced after an outbreak will protect patients from a re-infection with this genotype of *M. pneumoniae*. In a study investigating type-specific antibodies in acute-phase sera (IgA and IgG) from pneumonia patients with known p1 type in the respiratory tract, correlation between the occurrence of genotypes and type-specific immune response was lacking (Dumke et al., 2010). Finally, it cannot be excluded that genotype-specific differences in adhesion-related proteins will influence the interaction with target structures for adherence. Interestingly, recent epidemiological reports describe the replacement of p1 type 2 strains by variants of this type in the investigated human population (Jacobs et al., 2015; Suzuki et al., 2015). The reasons for the shift of genotypes remain unclear since it seems unlikely that the small sequence variations in the p1 gene of type 2 and type 2 variant strains will influence the immune response of infected patients. Confirmed type-specific differences in factors that influence the interaction with the host such as biofilm formation (Simmons et al., 2013) or expression of CARDS toxin (Techasaensiri et al., 2010; Lluch-Senar et al., 2015) were demonstrated between types 1 and 2 strains but not between type 2 and type 2 variants. Further studies should be performed to give more insight into the role of the time-variable occurrence of genotype-specific antibodies (host-dependent) and the consequences of sequence differences in the P1 protein for the adherence process (pathogen-dependent) in the epidemiology of infections.

VACCINE DEVELOPMENT

Despite the benign course of many infections by *M. pneumoniae*, the occurrence of severe manifestations and the existence of risk populations with intensive person-to-person contacts (schools, military camps) or with chronic respiratory disease justify efforts to prevent infections. Recently, a further reason resulted from high rates of macrolide-resistant strains circulating mainly in Asia, limiting the antibiotic treatment options especially in pediatric patients. The availability of safe vaccines seems nowadays the most effective measure for control of *M. pneumoniae* also, and this is supported by the increased knowledge about the virulence factors and by the confirmed genetic homogeneity of this pathogen. Furthermore, the described induction of a strong host immune reaction to defined pathogen factors has encouraged vaccination experiments. However, the results of early studies with volunteers treated with inactivated whole antigen preparations reflect the problems of inducing an immune response that demonstrates effective protection from subsequent infections (summarized in Linchevski et al., 2009). Limited efficacy against pneumonia, adverse reactions and, in some cases, exacerbation of symptoms after infection of immunized individuals have been described and confirmed by recent animal experiments (Sekine et al., 2009; Szczepanek et al., 2012).

Based on the importance of adherence of *M. pneumoniae* cells for initiating host colonization, recent studies have focused on the induction of adhesion-blocking antibodies. Investigation of components of the adhesion complex resulted in defined protein regions which are antigenic and involved in adherence and which have a conserved sequence (Schurwanz et al., 2009; Nakane et al., 2011). The construction of chimeric antigens composed of shorter protein regions with characterized function in adherence provides an opportunity to target different adhesion-related structures (Schurwanz et al., 2009). Immunization of guinea pigs with a hybrid protein consisting of adherence-related parts of the proteins P1 and P30 led to a significant decrease of specific genome copies in respiratory tract samples from vaccinated and subsequently infected animals (Hausner et al., 2013). Since demonstration of the adherence-blocking properties of sera from immunized animals, the induction of potent stimulation of mucosal immunity can be suggested as a crucial aspect for successful vaccination. This included not only (intranasal) administration of the antigen but also combination with biocompatible adjuvants (Zhu et al., 2012; Hausner et al., 2013).

CARRIAGE OF *M. pneumoniae*

As with other respiratory pathogens such as pneumococci (Donkor, 2013), asymptomatic or convalescent individuals have been confirmed as carriers of *M. pneumoniae* cells in the upper respiratory tract (Foy, 1993). This is an important fact, not only for the significance of laboratory test results and for epidemiological aspects of transmission but also for evaluation of the role of protective antibodies. Unfortunately, the results of carriage studies are inconsistent. Gnarpe et al. (1992) showed that culturable mycoplasmas occurred in the throats of a relatively high proportion (13.5 and 4.6%) of subjectively healthy adults in two investigation periods. In a recent study, detection of *M. pneumoniae* infections using ELISA (IgM and IgG) and real-time PCR was similar in groups of asymptomatic children and pediatric patients with respiratory symptoms (Spuesens et al., 2013). In contrast, Jain et al. (2015) reported a low rate (<3%) of PCR-positive results in asymptomatic children in a control group during a period of high incidence of *M. pneumoniae*. This is in accordance with other studies that demonstrated no detection or only low rates of respiratory tract colonization/infection of asymptomatic patients of different ages (Kumar et al., 2008; Nilsson et al., 2008; Chalker et al., 2011). Not only the occurrence of asymptomatic carriers but also the long-term persistence of *M. pneumoniae* in the respiratory tract of immunocompetent patients raises doubts regarding the efficiency of the host immune response for clearance of bacteria. Spuesens et al. (2013) reported that 21% of PCR-positive children (with or without symptoms) showed carriage of bacteria for 1–3 months. In a study of 53 patients, the mean carriage time for *M. pneumoniae* DNA was 7 weeks after disease onset (up to 7 months) after adequate therapy and confirmation of specific antibodies in corresponding sera (Nilsson et al., 2008). Despite the strong immune response after *M. pneumoniae* infection, the

efficiency of specific antibodies to eliminate the bacteria from the upper respiratory tract seems limited in particular individuals. The time-dependent decrease of specific antibodies in combination with the variable, often low production of IgA immunoglobulins in the respiratory tract may contribute to re-infection and long-term carriage.

It should be noted that confirmed persistence and long-term host colonization are typical for bacteria occurring intracellularly. The hypothesis is supported by the aforementioned absence of many metabolic pathways in *M. pneumoniae*, which requires close association with the host cells for growth and reproduction. However, the intracellular localization of *M. pneumoniae* has been described in cell culture assays (Meseguer et al., 2003) but so far not *in vivo*, so the persistence of mycoplasmas in host cells as an aspect of pathogenesis remains hypothetical.

CONCLUSION

The infection of immunocompetent patients with *M. pneumoniae* induces a strong antibody response which is mainly directed to surface-located proteins of the adhesion complex of the mycoplasmas. Thus, an influence of specific immunoglobulins that inhibit the adherence of mycoplasmal cells to the respiratory epithelium of the host can be expected. However, the occurrence of long-term carriage of *M. pneumoniae*, asymptomatic carriers, re-infections as well as a broad spectrum of extra-pulmonary manifestations show that the protective effect of specific serum antibodies is limited as regards the upper respiratory tract. The directed movement of bacteria to the base of the ciliated epithelium of the respiratory mucosa and the specialized adherence to target cells of the host allow the bacteria to reach a protected niche. The role of the local immune response by inhibiting adherence and the immunomodulatory effects of local inflammation on the clinical course have yet to be explained. These factors are also crucial for the development of an effective vaccine. Finally, the confirmed induction of genotype-specific antibodies raises the question of whether this has an influence on re-infections due to different genotypes of *M. pneumoniae*, which might be important for understanding the special epidemiology of a common human respiratory tract pathogen.

AUTHOR CONTRIBUTIONS

RD drafted the manuscript. EJ revised and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 31 March 2016
doi: 10.3389/fmicb.2016.00414

Inflammation-inducing Factors of *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae, which causes mycoplasmal pneumonia in human, mainly causes pneumonia in children, although it occasionally causes disease in infants and geriatrics. Some pathogenic factors produced by *M. pneumoniae*, such as hydrogen peroxide and Community-Acquired Respiratory Distress Syndrome (CARDS) toxin have been well studied. However, these factors alone cannot explain this predilection. The low incidence rate of mycoplasmal pneumonia in infants and geriatrics implies that the strong inflammatory responses induced by *M. pneumoniae* coordinate with the pathogenic factors to induce pneumonia. However, *M. pneumoniae* lacks a cell wall and does not possess an inflammation-inducing endotoxin, such as lipopolysaccharide (LPS). In *M. pneumoniae,* lipoproteins were identified as an inflammation-inducing factor. Lipoproteins induce inflammatory responses through Toll-like receptors (TLR) 2. Because *Mycoplasma* species lack a cell wall and lipoproteins anchored in the membrane are exposed, lipoproteins and TLR2 have been thought to be important for the pathogenesis of *M. pneumoniae*. However, recent reports suggest that *M. pneumoniae* also induces inflammatory responses also in a TLR2-independent manner. TLR4 and autophagy are involved in this TLR2-independent inflammation. In addition, the CARDS toxin or *M. pneumoniae* cytadherence induces inflammatory responses through an intracellular receptor protein complex called the inflammasome. In this review, the inflammation-inducing factors of *M. pneumoniae* are summarized.

Keywords: mycoplasma, lipoprotein, cytadherence, inflammation, pneumonia

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *02 February 2015*
Accepted: *14 March 2016*
Published: *31 March 2016*

Citation:
Shimizu T (2016) Inflammation-inducing Factors of Mycoplasma pneumoniae. Front. Microbiol. 7:414. doi: 10.3389/fmicb.2016.00414

INTRODUCTION

Mycoplasma pneumoniae causes primary atypical pneumonia, tracheobronchitis, pharyngitis, and asthma in humans (Gil et al., 1993; Kraft et al., 1998; Waites and Talkington, 2004). The age distribution of patients with pneumonia caused by *M. pneumoniae* is characteristic. The incidence is highest among school-aged children and young adults and lower in infants and geriatrics (Denny et al., 1971; Foy et al., 1979). Some pathogenic factors of *M. pneumoniae*, such as hydrogen peroxide, Community-Acquired Respiratory Distress Syndrome (CARDS) toxin, and nuclease, have been reported to be associated with the development of pneumonia (Somerson et al., 1965; Cohen and Somerson, 1967; Kannan and Baseman, 2006; Hames et al., 2009; Somarajan et al., 2010). However, these pathogenic factors are insufficient to explain the age distribution of patients with pneumonia caused by *M. pneumoniae*. Generally, the immune system in infants and geriatrics is immature compared with that in young adults. The symptoms of pneumonia caused by *M. pneumoniae* are correlated with the induction of pro-inflammatory cytokines (Tryon and Baseman, 1992; Salvatore et al., 2007). These findings suggest that the excessive immune responses

induced by *M. pneumoniae* play an important role in the development of pneumonia. In this review, the molecular mechanisms of inflammation induced by *M. pneumoniae* are summarized (**Table 1**).

LIPOPROTEINS AND TOLL-LIKE RECEPTORS (TLRs)

Lipoproteins of *Mycoplasma* Species

It has been reported that some *Mycoplasma* species induce pro-inflammatory cytokines and stimulate various immune cells (Atkin et al., 1986; Kirchner et al., 1986; Teh et al., 1988). Because *Mycoplasma* species are devoid of a cell wall and lack immune cell stimulator such as lipopolysaccharide (LPS) or peptidoglycan (Mizel et al., 1978; Staber et al., 1978), the factors responsible for the induction of inflammatory responses have been unclear for a long time. The first report on the inflammation-inducing factor of *Mycoplasma* species was published by Quentmeier et al. (1990). They reported that a high-molecular-weight (HMW) protein of *M. fermentans* known as MDHM possesses interleukin (IL)-6-inducing activity in macrophages. Because the activity of MDHM was resistant to proteinase K, the active component of MDHM was thought to be a low-molecular-weight compound. In 1996, Muhlradt et al. identified the active component of MDHM as *S*-(2,3-dihydroxypropyl) cysteine (Muhlradt et al., 1996). This component was similar to the N-terminal structure of an *Escherichia coli*-derived lipoprotein identified in 1969 by Braun et al. Braun (1975). Muhlradt et al. (1997) also purified the inflammation-inducing factor from *M. fermentans* culture and demonstrated that the active component is the diacylated lipopeptide, *S*-(2,3-bisacyloxypropyl)-CGNNDESNISFKEK. They named it macrophage-activating lipopeptide-2 (MALP-2). After these reports, inflammation-inducing lipoproteins were purified and identified in various *Mycoplasma* species (Jan et al., 1996a; Muhlradt et al., 1997, 1998; Shibata et al., 2000), including *M. pneumoniae* (Shimizu et al., 2005).

TABLE 1 | Summary of the inflammation-inducing factors of *Mycoplasma pneumoniae.*

Gene ID	Original function	Function in inflammation
MPN602	F_0F_1 ATP synthase subunit b	Diacylated lipoprotein
MPN052	Hypothetical	Triacylated lipoprotein
MPN162		
MPN415		
MPN611		
MPN141	Cytadherence, P1 adhesin	Pro-inflammatory cytokine induction
MPN142	Cytadherence, P40, P90	Activation of inflammasome
MPN453	Cytadherence, P30	
MPN447	Cytadherence, HMW1	
MPN372	ADP-ribosylating toxin, CARDS toxin	
MPN333	ABC transporter	Autophagy/TLR4 dependent inflammation
MPN597	F_0F_1 ATP synthase subunit ε	

Structure of Lipoprotein and TLR

Lipoproteins were discovered in 1969 by Braun et al. (Braun, 1975). Lipoproteins are hydrophilic membrane proteins characterized by a conserved N-terminal lipid-modified cysteine residue. Lipoproteins contain *S*-glyceryl cysteine modified with three fatty acids (*N*-acyl-*S*-diacylglyceryl cysteine) at their N-terminal. This triacylated structure is also called Braun's lipoprotein. Braun's Lipoproteins are synthesized by the following three steps (**Figure 1**): (1) Transfer of the diacylglyceryl moiety from a membrane phospholipid to a cysteine residue of a protein through the recognition of the lipobox (L-[A/S/T]-[G/A]-C) by prolipoprotein diacylglyceryl transferase (Lgt); (2) Digestion of the signal sequence at the amino-terminal side of the cysteine by prolipoprotein signal peptidase (Lsp); and (3) Linkage of an acyl chain to the amino group of the amino-terminal cysteine (*N*-acylation) by prolipoprotein *N*-acyl-transferase (Lnt). Because genes orthologous to Lnt gene are not found in some bacterial species (Firmicutes and and Tenericutes), including *Mycoplasma* species, lipoproteins from these bacterial species have been assumed to be of the diacylated form (Nakayama et al., 2012).

Toll-like receptors are a type of pattern-recognition receptors that play critical roles in early innate recognition and host inflammatory responses against invading microbes (Kopp and Medzhitov, 1999; Akira and Takeda, 2004). Among the 11 reported TLR family members, TLR2 plays a central role in the recognition of lipoproteins. TLR2 recognizes the *S*-diacylglyceryl cysteine portions of both diacylated and triacylated lipoproteins (Botos et al., 2011). TLR1 coordinate with TLR2 to recognize triacylated lipoproteins through the recognition of the third acyl chain amide-linked to the cysteine using a hydrophobic pocket within TLR1 (Jin et al., 2007). However, TLR2 alone is not able to recognize diacylated lipoproteins. TLR6 assists in the recognition of diacylated lipoproteins by associating with the amino acid portion of diacylated lipoproteins (Kang et al., 2009). Although there are some exceptions (Buwitt-Beckmann et al., 2005; Kurokawa et al., 2009), diacylated and triacylated lipoproteins are generally recognaized by TLR2/6 and TLR2/1 heterodimers, respectively.

The presence of triacylated lipoproteins in *Mycoplasma* species has been controversial. The lipoproteins from *M. fermentans*, *M. hyorhinis*, *M. salivarium*, and *M. gallisepticum* have been shown to be diacylated lipoproteins and not *N*-acylated (Jan et al., 1996a; Muhlradt et al., 1997, 1998; Shibata et al., 2000). The Lnt gene, which encodes the enzyme responsible for N-acylation has not been found in any mycoplasma genomes (Fraser et al., 1995; Himmelreich et al., 1996; Sasaki et al., 2002). However, a study on the ratio of *N*-amide and *O*-ester bonds in *M. gallisepticum* and *M. mycoides* suggested the presence of triacylated lipoproteins (Jan et al., 1996b). Furthermore, the

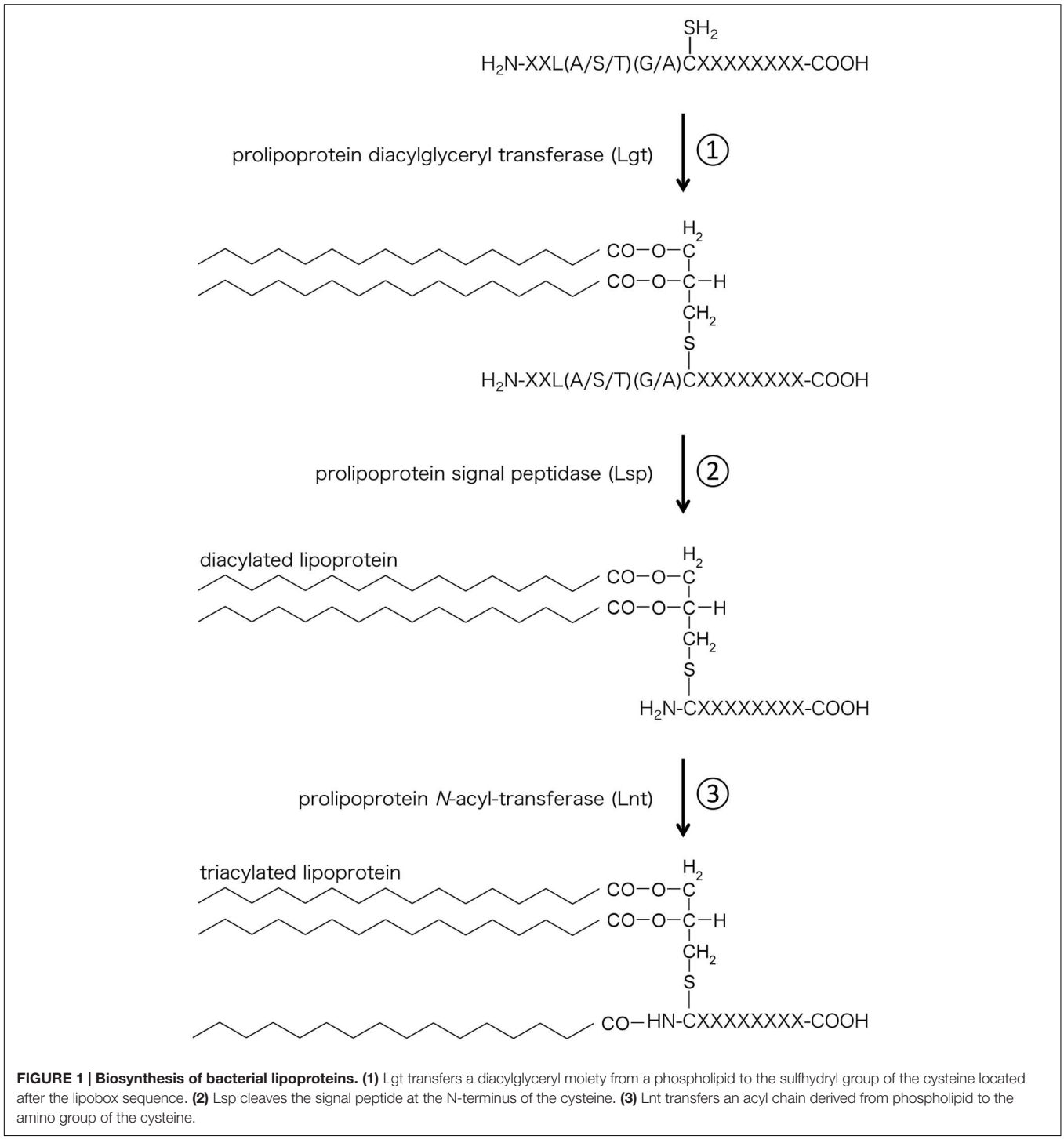

FIGURE 1 | Biosynthesis of bacterial lipoproteins. (1) Lgt transfers a diacylglyceryl moiety from a phospholipid to the sulfhydryl group of the cysteine located after the lipobox sequence. **(2)** Lsp cleaves the signal peptide at the N-terminus of the cysteine. **(3)** Lnt transfers an acyl chain derived from phospholipid to the amino group of the cysteine.

resistance to Edoman degradation of proteins from *M. mycoides* also indicated the presence of *N*-acylation (Chambaud et al., 1999). These data imply the presence of triacylated lipoproteins in *Mycoplasma* species.

Lipoproteins of *M. pneumoniae*

Lipoproteins of *M. pneumonie* are summarized in **Table 2**. In *M. pneumoniae*, Shimizu et al. (2005) reported that the subunit b of the F_0F_1 ATP synthase (MPN602) is a diacylated lipoprotein that induces inflammatory responses through TLR2. Into et al. (2007) also reported that *M. pneumoniae* has 48 lipoproteins and that the common N-terminal structure of these lipoproteins induces inflammatory responses. Interestingly, Some of these lipoproteins (MPN162, MPN611) were recognized by TLR1 and TLR2, suggesting that *M. pneumoniae* contains triacylted lipoproteins (Shimizu et al., 2007). Kurokawa et al.

TABLE 2 | Summary of lipoproteins of *M. pneumoniae*.

Gene ID	Gene symbol	Original function	Usage of TLR	Number of acyl chain
MPN011		Hypothetical		
MPN052		Hypothetical		3[a]
MPN054		Hypothetical		
MPN058		Hypothetical		
MPN083		Hypothetical		
MPN097		Pseudo		
MPN133		Hypothetical		
MPN152		Hypothetical		
MPN162		Hypothetical	1, 2	3[b]
MPN199		Hypothetical		
MPN200		Hypothetical		
MPN271		Hypothetical		
MPN281		Pseudo		
MPN284		Hypothetical		
MPN288		Hypothetical		
MPN363		Hypothetical		
MPN369		Hypothetical		
MPN408		Hypothetical		
MPN411		Hypothetical		
MPN415		High affinity transport system protein P37		3[a]
MPN436		Hypothetical		
MPN439		Pseudo		
MPN442		Hypothetical		
MPN456		Hypothetical		
MPN459		Hypothetical		
MPN467		Hypothetical		
MPN489		Hypothetical		
MPN506		Hypothetical		
MPN523		Hypothetical		
MPN582		Hypothetical		
MPN585		Hypothetical		
MPN587		Hypothetical		
MPN588		Hypothetical		
MPN590		Hypothetical		
MPN592		Hypothetical		
MPN602	atpF	F_0F_1 ATP synthase subunit b	2, 6	2[b]
MPN611		Phosphate ABC transporter substrate-binding protein	1, 2	3[b]
MPN639		Hypothetical		
MPN640		Hypothetical		
MPN641		Hypothetical		
MPN642		Hypothetical		
MPN643		Hypothetical		
MPN644		Hypothetical		
MPN645		Hypothetical		
MPN646		Hypothetical		
MPN647		Hypothetical		
MPN650		Hypothetical		
MPN654		Hypothetical		

[a]*Determined by lipase-based mass spectrometry analysis.*
[b]*Estimated from TLR usage.*

(2012) analyzed the detailed structure of *M. pneumoniae* lipoproteins using lipoprotein lipase-based mass spectrometry analysis, and demonstrated that some of *M. pneumoniae* lipoproteins (MPN052, MPN415) are triacylated. In this study, triacylated lipoproteins were also found in *M. genitalium*. These findings led to the conclusion that *Mycoplasma* species possess triacylated lipoproteins and indicated that a new enzyme with Lnt activity exists in *Mycoplasma* species. Although the modification of other 43 lipoproteins of *M. penoumoniae* is still unclear, the lipoproteins of *M. pneumoniae* seem mixture of diacylated and triacylated lipoproteins. Induction of inflammatory responses through both TLR2/6 and TLR2/1 by diacylated and triacylated lipoproteins may affect the strong inflammation in *M. pneumoniae* infection.

TLR2-INDEPENDENT INFLAMMATION

M. pneumoniae and Autophagy

Because *Mycoplasma* species lack cell walls, they do not contain immunostimulants such as LPS, peptidoglycan, or lipoteichoic acid. Therefore, lipoproteins seem to be key factors in *M. pneumoniae*-induced inflammatory responses and to facilitate the development of pneumonia in humans. However, the existence of lipoproteins in non-pathogenic *Mycoplasma* species suggests the presence of an alternative mechanism by which *M. pneumoniae* induce inflammatory responses.

Autophagy is a cellular response that involves the sequestration of regions within the cytosol with double membrane compartments. Autophagy has been shown to play important roles in the cellular response to starvation, cell death, removal of damaged organelles, and neurodegenerative diseases (Levine, 2005). It has recently been recognized that autophagy is involved in both innate and adaptive immunity against various microorganisms (Schmid and Munz, 2007; Deretic et al., 2013; Ma et al., 2013).

Recently, Shimizu et al. demonstrated that *M. pneumoniae* induces strong inflammatory responses, even in macrophages derived from TLR2 knockout (KO) mice (Shimizu et al., 2014). *M. pneumoniae* internalized into macrophages through phagocytosis were co-localized with the autophagosome, and autophagy inhibitors decreased the induction of pro-inflammatory cytokines, suggesting the autophagy-mediated induction of inflammatory responses. Because this TLR2-independent induction was inhibited in macrophages derived from TLR2/4 double KO mouse, TLR4 is also involved. In this study, they also reported that the ABC-transporter (MPN333), and F_0F_1 ATP synthase subunit ε (MPN597) of *M. pneumoniae* are essential for the activation of the autophagy/TLR4-mediated pathway.

M. pneumoniae and the Inflammasome

Inflammasomes are intracellular receptors (Martinon et al., 2009), that respond to various signals, including intracellular bacterial toxins, pathogen-associated molecular patterns

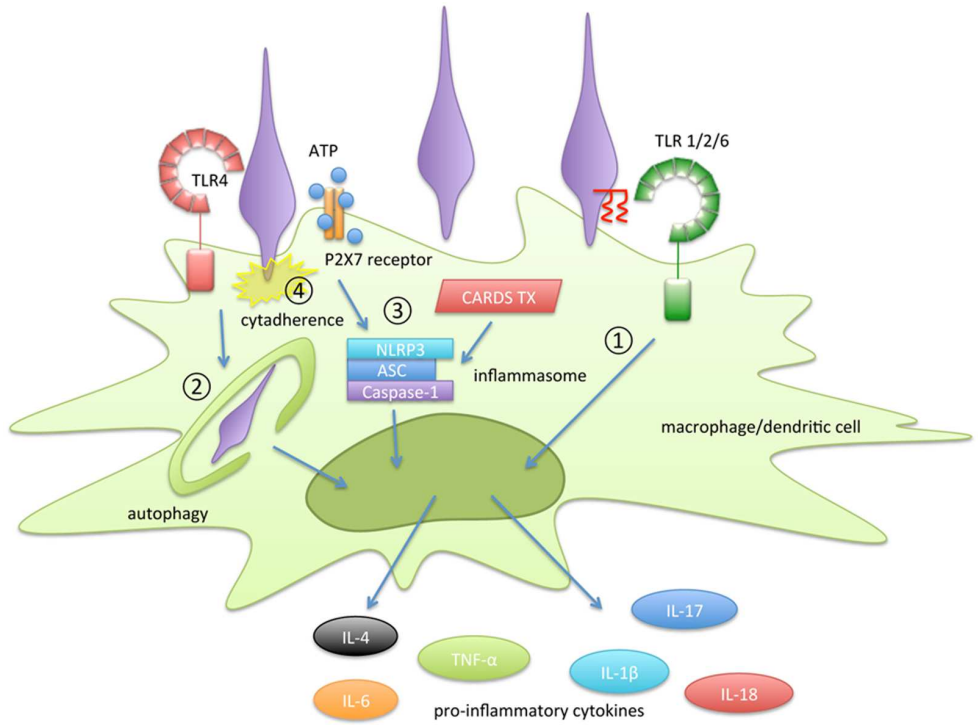

FIGURE 2 | Summary of the inflammation-inducing pathways in *Mycoplasma pneumoniae* infection. The following four pathways are involved in the induction of inflammatory responses: **(1)** recognition of lipoprotein by TLR2, **(2)** autophagy-mediated signaling, **(3)** activation of inflammasomes, and **(4)** cytadherence property.

(PAMPs) (Martinon et al., 2004; Miao et al., 2007), damage-associated molecular patterns (DAMPs) (Kanneganti et al., 2006; Mariathasan et al., 2006; Sutterwala et al., 2006), and reactive oxygen species (Dostert et al., 2008; Allen et al., 2009). Activated inflammasomes cleave the precursors of pro-inflammatory cytokines, such as IL-1β and IL-18 through caspase-1 or caspase-11, and release them (Boyden and Dietrich, 2006).

Shimizu et al. (2011) reported that *M. pneumoniae* induces efflux of ATP from host cells. The efflux of ATP activated inflammasomes via the P2X7 receptor, which is followed by the secretion of IL-1β. A recent report by Sugiyama et al. (2015) also demonstrated that *M. pnumoniae* induces IL-1β through the NLRP3 inflammasome in a dendritic cell line.

Interestingly, Bose et al. (2014) showed that CARDS toxin (MPN372) regulates NLRP3 inflammasome activity. CARDs toxin is a vacuolating cytotoxin produced by some *Mycoplasma* species, including *M. pneumoniae*. Its C-terminal region is responsible for its vacuolating activity (Kannan and Baseman, 2006; Kannan et al., 2014). Its N-terminal region shares sequence similarity with pertussis toxin and is essential for its ADP-ribosylating activity. In this study, they demonstrated that CARDS toxin activates inflammasomes through the ADP-ribosylation of NLRP3 and enhances the secretion of IL-1β.

Taken together, these findings suggest that inflammasomes play an important role in the inflammation induced by *M. pneumoniae*.

Cytadherence of *M. pneumoniae* and Inflammation

Cytadherence property is one of the unique characteristics of *M. pneumoniae*. Cytadherence in the respiratory tract, the initial event in *M. pneumoniae* infection, is mediated by P1 (MPN141) adhesin and other accessory proteins, such as P30 and HMW proteins (Krause and Balish, 2001; Balish and Krause, 2002; Miyata, 2008a,b). The relationship between cytoadherence and the induction of inflammatory responses was first reported in Yang et al. (2002). They demonstrated that protease treatment or anti-P1 antibody treatment decreases the induction of pro-inflammatory cytokines, including IL-1β. Hoek et al. (2005) reported that culturing *M. pneumoniae* in polypropylene bottles reduces the expression of P1 adhesin. Under these conditions, the induction of IL-4 from mast cells was significantly decreased. As described above, Shimizu et al. reported that *M. pneumoniae* induces the efflux of ATP from host cells, followed by the activation of inflammasomes and secretion of IL-1β. In this study, they also reported that cytadherence-deficient mutants lacking P90 and P40 (MPN142, 130 kDa precursor) or HMW1 and P30 (MPN447 and MPN453, respectively) fail to induce IL-1β through ATP efflux. Cytadherence was also associated with autophagy/TLR4-mediated induction of inflammatory responses. Mutation in ABC-transporter (MPN333), and F_0F_1 ATP synthase subunit ε (MPN597) failed to induce inflammatory responses, and these

mutants showed a deficiency in cytadherence (Shimizu et al., 2014). Taken together, these findings indicate that cytadherence of *M. pneumoniae* is strongly associated with the induction of inflammatory responses.

CONCLUSION

In this review, the molecular mechanisms of inflammatory responses induced by *M. pneumoniae* were reviewed (**Figure 2**). The following four pathways are important for the induction of inflammatory responses in *M. pneumoniae* infection: 1) recognition of lipoprotein by TLR2, 2) autophagy-mediated signaling; 3) activation of inflammasomes, and 4) cytadherence property. Lipoproteins, which were the first immunostimulants discovered in *Mycoplasma* species, have been well studied. However, the structures of the lipoproteins in *Mycoplasma* species are identical to those of lipoproteins from other bacteria, including normal microflora. Therefore, lipoproteins alone are insufficient to explain the inflammatory responses induced by *M. pneumoniae*. *M. pneumoniae* also has the ability to induce inflammatory responses through a TLR2-independent pathway. Autophagy and TLR4 are involved in this induction. Some pro-inflammatory cytokines, such as IL-1β and IL-18, are matured and released through inflammasome activation. Inflammasome activation is necessary to release these cytokines during *M. pneumoniae* infection. It is noteworthy that CARDS toxin enhances inflammasome activation. The distribution of CARDS toxin in *Mycoplasma* species is limited to a small number of *Mycoplasma* species. In addition, cytadherent property of *M. pneumoniae* is strongly associated with the autophagy/TLR4- and inflammasome-mediated induction of inflammatory responses. Although some *Mycoplasma* species, such as *M. genitalium* and *M. gallisepticum*, have partially similar adhesin, cytadherence mediated by P1 adhesin is unique in *M. pneumoniae*. These characteristics may contribute to the greater ability of *M. pneumoniae* to induce inflammatory responses than non-pathogenic *Mycoplasma* species.

In addition to inflammation-inducing factors, *M. pneumoniae* shows cytotoxicity through CARDS toxin, nuclease, and hydrogen peroxide produced during glycerol metabolism. The symptoms of mycoplasmal pneumonia, such as fever and severe cough, are thought to appear as a result of a combination of inflammation and cytotoxicity induced by *M. pneumoniae*. Mycoplasmal pneumonia is still an important issue in the field of pediatric medicine. Although measures to prevent mycoplasmal pneumonia are desired worldwide, preventive measures, including vaccines, have not been developed. Therefore, the inflammation-inducing factors of *M. pneumoniae* described here may be suitable targets for the development of new preventive measures.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 12 April 2016
doi: 10.3389/fmicb.2016.00513

A Compendium for *Mycoplasma pneumoniae*

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Historically, atypical pneumonia was a term used to describe an unusual presentation of pneumonia. Currently, it is used to describe the multitude of symptoms juxtaposing the classic symptoms found in cases of pneumococcal pneumonia. Specifically, atypical pneumonia is a syndrome resulting from a relatively common group of pathogens including *Chlamydophila* sp., and *Mycoplasma pneumoniae*. The incidence of *M. pneumoniae* pneumonia in adults is less than the burden experienced by children. Transmission rates among families indicate children may act as a reservoir and maintain contagiousness over a long period of time ranging from months to years. In adults, *M. pneumoniae* typically produces a mild, "walking" pneumonia and is considered to be one of the causes of persistent cough in patients. *M. pneumoniae* has also been shown to trigger the exacerbation of other lung diseases. It has been repeatedly detected in patients with bronchitis, asthma, chronic obstructive pulmonary disorder, and cystic fibrosis. Recent advances in technology allow for the rapid diagnosis of *M. pneumoniae* through the use of polymerase chain reaction or rapid antigen tests. With this, more effort has been afforded to identify the causative etiologic agent in all cases of pneumonia. However, previous practices, including the overprescribing of macrolide treatment in China and Japan, have created increased incidence of macrolide-resistant *M. pneumoniae*. Reports from these countries indicate that >85% of *M. pneumoniae* pneumonia pediatric cases are macrolide-resistant. Despite its extensively studied past, the smallest bacterial species still inspires some of the largest questions. The developments in microbiology, diagnostic features and techniques, epidemiology, treatment and vaccines, and upper respiratory conditions associated with *M. pneumoniae* in adult populations are included within this review.

Keywords: *Mycoplasma pneumoniae*, pneumonia, atypical pneumonia, community-acquired pneumonia, walking pneumonia, CARDS toxin

OPEN ACCESS

Edited by:
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Tsuyoshi Kenri, National Institute of Infectious Diseases, Japan

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *04 December 2015*
Accepted: *29 March 2016*
Published: *12 April 2016*

Citation:
Parrott GL, Kinjo T and Fujita J (2016) A Compendium for Mycoplasma pneumoniae. Front. Microbiol. 7:513. doi: 10.3389/fmicb.2016.00513

INTRODUCTION

Mycoplasma pneumoniae, was first discovered in Eaton et al. (1944). It was originally known as the Eaton agent. The tiny pathogenic agent could pass through a sterile filter, but could not be grown on standard bacteriologic media. Thus, during that time, it was originally thought to be a virus. Volunteer and field studies during the 1950s and early 1960s provided evidence verifying the Eaton agent was a cause of lower respiratory tract infections in humans (Chanock et al., 1960, 1961; Mufson et al., 1961). Chanock et al. (1962) was able to culture the Eaton agent on a cell-free

medium and proposed both the taxonomic designation as well as the name of the organism as we know it today.

Currently, as one of the most studied mycoplasmas, a large collective knowledge on *M. pneumoniae* has accumulated. Recent discoveries in microbiology and improvements in diagnostic techniques and treatments have led to dramatic advances. At the same time knowledge regarding *M. pneumoniae* epidemiology, associated upper respiratory conditions, and vaccine candidates has also expanded. These topics and how they affect adult populations will be briefly covered in this review.

MICROBIOLOGY

With a small cell size and volume, just 1–2 μm long and 0.1–0.2 μm wide, mycoplasmas cannot individually be detected by light microscopy (Waites and Talkington, 2004) and colonies rarely exceed 100 μm in diameter. Sequenced in Himmelreich et al. (1996), the genome of *M. pneumoniae* was shown to consist of only 816,394 bp and 687 genes. Because of this small genome, the organism is limited in its capabilities and unable to synthesize rigid peptidoglycan cell walls. Alternatively, sterols provide the structural support in the triple-layer cell membrane. As a result, these organisms are insensitive to β-lactam antimicrobial agents, pleomorphic, and are unaffected by the gram staining method.

Mycoplasma pneumoniae reproduces via binary fission with well-organized chromosome segregation. Preceding binary cell fission, the attachment organelle, a specialized cellular structure that is responsible for cytadherence of this bacterium, duplicates (Krause and Balish, 2004; Balish, 2006; Balish and Krause, 2006). The resulting daughter organelle will migrate to the opposite pole of the cell before the completion of chromosomal separation. After duplication of chromosomal and cellular material, the now polar opposite attachment organelles will simultaneously bind to a surface and initiate gliding motility, pulling away from the central point; thus, creating daughter cells (Bredt, 1968; Miyata and Ogaki, 2006).

The attachment organelle is also crucial for host cellular interactions. Cytoskeletal proteins found within and around the attachment organelle facilitate adherence and motility. Of note, P1 and supporting proteins P30, P90, and P40 promote adhesion and binding to host cells via sialic acid receptors (Krivan et al., 1989; Roberts et al., 1989; Razin and Jacobs, 1992; Seto and Miyata, 2003; Waldo and Krause, 2006; Chaudhry et al., 2007), while P1, P30, P41, and P200 promote and regulate gliding motility (Kenri et al., 2004; Krause and Balish, 2004; Seto et al., 2005; Hasselbring and Krause, 2007; Jordan et al., 2007). Many authors allude to a cellular construction based on electron micrograph observations of cryosections (Hegermann et al., 2002; Henderson and Jensen, 2006; Seybert et al., 2006; Nakane et al., 2015). Integration and interpretation of these studies propose that the attachment organelle is connected to a rod consisting of two paired plates (rods), one thick and one thin, which is attached, at the proximal end, to a wheel-like protein complex (bowl-complex), the wheel (bowl) further connects to other cytoskeleton filaments in the peripheral cell. A schematic, assembled from literature interpretation, is depicted in **Figure 1** (Meng and Pfister, 1980; Razin et al., 1998; Trachtenberg, 1998; Krause and Balish, 2001, 2004; Hegermann et al., 2002; Henderson and Jensen, 2006; Miyata and Ogaki, 2006; Seybert et al., 2006; Nakane et al., 2015). However, the exact mechanism by which the attachment organelle and associated structures and proteins initiate motility is still relatively unknown, although numerous authors support inchworm-like movement (Henderson and Jensen, 2006; Hatchel and Balish, 2008; Nakane et al., 2015).

Mycoplasma pneumoniae has not been found freely living in nature. It is predominantly considered a mucosal pathogen existing parasitically on the epithelial surface of its host. Interactions between *M. pneumoniae* and host tissue, continuously show the attachment organelle bound to the host cell surface (Wilson and Collier, 1976; Razin and Jacobs, 1992; Rottem, 2003). However, reports of internalization by lung epithelial cells also exist (Yavlovich et al., 2004). It is assumed cytadherence protects mycoplasma from clearance via the mucociliary apparatus and this attachment is ultimately considered the initiating event of disease.

PATHOGENESIS

Once bound to host tissue, pathogenic processes begin to occur. Hydrogen peroxide and superoxide, produced by *M. pneumoniae* through the metabolism of glycerol, have been shown to cause injury to epithelial cells and their associated cilia (Somerson et al., 1965; Low, 1971; Tryon and Baseman, 1987; Minion and Jarvill-Taylor, 1994). The effects of hydrogen peroxide on host cells such as erythrocytes include denaturation of hemoglobin,

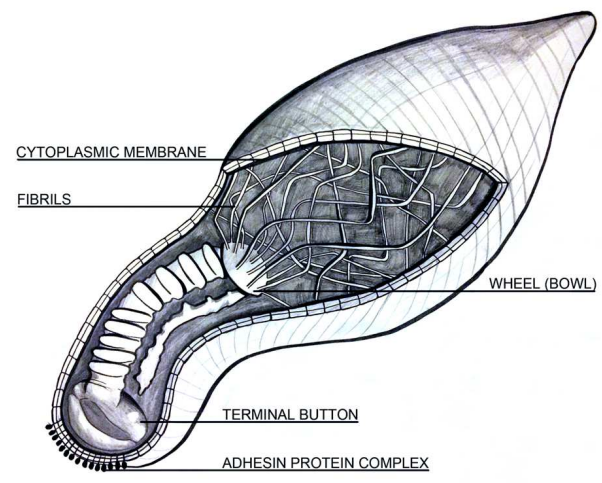

FIGURE 1 | A longitudinal schematic depicting the cellular architecture of *Mycoplasma pneumoniae*. It contains known structural features including cell shape, lack of flagella, a terminal organelle including the "rod" composed of two segmented plates, one thick and one thin, and a wheel (bowl) complex with fibrils extending throughout the cytoplasm. The outer cell membrane is integrated with membrane proteins while the inner lining encloses the cytoplasm. Image is not to scale.

peroxidation of lipids, and eventual cell lysis. The same oxidative stress in the respiratory epithelium can result in both structural and functional deterioration of cilia (Waites and Talkington, 2004).

Recently discovered, the community-acquired respiratory distress syndrome (CARDS) toxin has also been shown to facilitate localized disruption and cytotoxicity (Kannan et al., 2005, 2010; Kannan and Baseman, 2006; Johnson et al., 2011; Becker et al., 2015). Cells exposed to this 68-kDa proteinaceous toxin, with homologies to the pertussis toxin, exhibited distinct vacuolization and cell rounding (Kannan and Baseman, 2006). Other cellular effects of CARDS could include the loss of cilia, reduced oxygen consumption, glucose utilization, and amino acid uptake, as well as detachment and ultimate shedding of the infected cells, documented elsewhere (Clyde, 1971, 1983; Collier, 1983; Johnson et al., 2011). The dry, hacking cough commonly associated with early infection, is most likely the clinical manifestation of the aforementioned cellular damages endured by the upper respiratory tract (Waites and Talkington, 2004).

The CARDS toxin has also been shown to activate its own pathogenic response in animal models. A direct relationship between the number of *M. pneumoniae* organisms, amounts of the CARDS toxin, and severity of lung histopathology was observed in murine models (Techasaensiri et al., 2010; Kannan et al., 2011). Moreover, mice and baboons, which received recombinant CARDS toxin alone, elicited cellular inflammation similar to *M. pneumoniae* infection. This suggests the CARDS toxin, plays a major role in pathogenesis (Hardy et al., 2009).

Further, research has shown the CARDS toxin can localize with the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome and catalyze the ADP-ribosylation of NLRP3 (Bose et al., 2014). As a result, the CARDS toxin is the first example of a toxin exhibiting both ADP-ribosylating and vacuolating properties (Krueger and Barbieri, 1995; Kannan and Baseman, 2006). In **Figure 2**, we propose a potential cell signaling model based on information collected from previously published reports (Fan et al., 2003; Yang et al., 2004; Chu et al., 2005; Xiang and Fan, 2010; Johnson et al., 2011; Kannan et al., 2011; Franchi and Nunez, 2012; Franchi et al., 2012; Bose et al., 2014; Saraya et al., 2014; Shimizu et al., 2014; Vanaja et al., 2015).

Interaction between respiratory epithelial cells and surface lipoproteins of *M. pneumoniae* is likely to induce the host immune system via Toll-like receptor (TLR)-2 (Chu et al., 2005; Kraft et al., 2008) or TLR-4 (Shimizu et al., 2014) stimulating the synthesis of intracellular adhesion molecule (ICAM) receptors. Stimulation and crosstalk of the TLRs can trigger and amplify the production of chemokines promoting lymphocyte and neutrophil trafficking and inflammation in the lung (Fan et al., 2003; Yang et al., 2004; Saraya et al., 2011). In addition to respiratory epithelial cells, *M. pneumoniae* has been shown to directly activate and induce the production of cytokines from unsorted peripheral blood leukocytes (Kita et al., 1992), lymphocytes (Simecka et al., 1993; Medina et al., 2012), and monocytes and macrophages (Yang et al., 2003; Broaders et al., 2006). Opsonization may also occur but the evidence is minimal since re-infection is common.

Macrophages, including alveolar macrophages likely play a central role as an innate immune defense mechanism through phagocytosis. Alveolar macrophages, in particular, can also secrete pro-inflammatory cytokines, such as RANTES, which is a known chemo-attractant for neutrophils and basophils (Bischoff et al., 1993; Saraya et al., 2011; Tani et al., 2011). According to some reports, the most distinguishing pathological feature resulting from this organism in human pneumonia is an increase of plasma cell-rich lymphocytic infiltration in the peri-bronchovascular areas (PBVAs), with accumulation of macrophages, neutrophils, and lymphocytes in alveolar spaces (Coultas et al., 1986; Hayashi et al., 1986; Rollins et al., 1986; Saraya et al., 2014).

The underlying cell-mediated immunity of the host also plays an important role in the progression and development of *M. pneumoniae* related diseases. Furthermore, cell-mediated immunity level or predominant response is potentially correlated to the variable pulmonary patterns seen in chest images (Tanaka et al., 1996; Saraya et al., 2011, 2014). Multiple studies have acknowledged the importance of IL-12, interferon-γ, and Th1 type T-cell responses during the course of *M. pneumoniae* infections (Fonseca-Aten et al., 2005; Tagliabue et al., 2008; Hardy et al., 2009; Techasaensiri et al., 2010). However, a recombinant CARDS toxin has resulted in potent allergic-type pulmonary inflammation characterized by T-cell dependence, airway hyperreactivity, and production of Th2 type cytokines (Medina et al., 2012). In sensitized mice, *M. pneumoniae* can lead to Th2 type T-cell allergic inflammation (Chu et al., 2003, 2005). While, other previous animal studies have shown the histopathological score of *M. pneumoniae* pneumonia is significantly higher in BALB/c mice (Th2 predominant) than in C57BL/6 mice (Th1 predominant; Fonseca-Aten et al., 2005). The collective interpretation of all these findings suggest an attractive link to the differences observed in human host cell-mediated immunity response against the organism and the resulting variable pathogenic patterns seen in chest images and serological responses (Kraft et al., 1998; Nisar et al., 2007; Atkinson et al., 2009; Saraya et al., 2011).

EPIDEMIOLOGY

Mycoplasma pneumoniae is able to infect both the upper and lower respiratory tracts and it can create both endemic and epidemic situations among children and adults worldwide. From 2001 to 2006, *M. pneumoniae* was the most common atypical pathogen identified in 39 hospitals across 11 countries (Arnold et al., 2007). However, most available data regarding *M. pneumoniae* infections comes from studies performed in Japan, Europe, and the United States. Within Europe, *M. pneumoniae* is frequently included in regular surveillance, but studies from arctic and tropical zones point to the many diverse populations with infections due to this organism (Suhs and Feldman, 1966; Golubjatnikov et al., 1975; Joosting et al., 1976; Campos et al., 1993).

Mycoplasma pneumoniae has a diminutive size, which allows it to spread from person to person through droplet infection

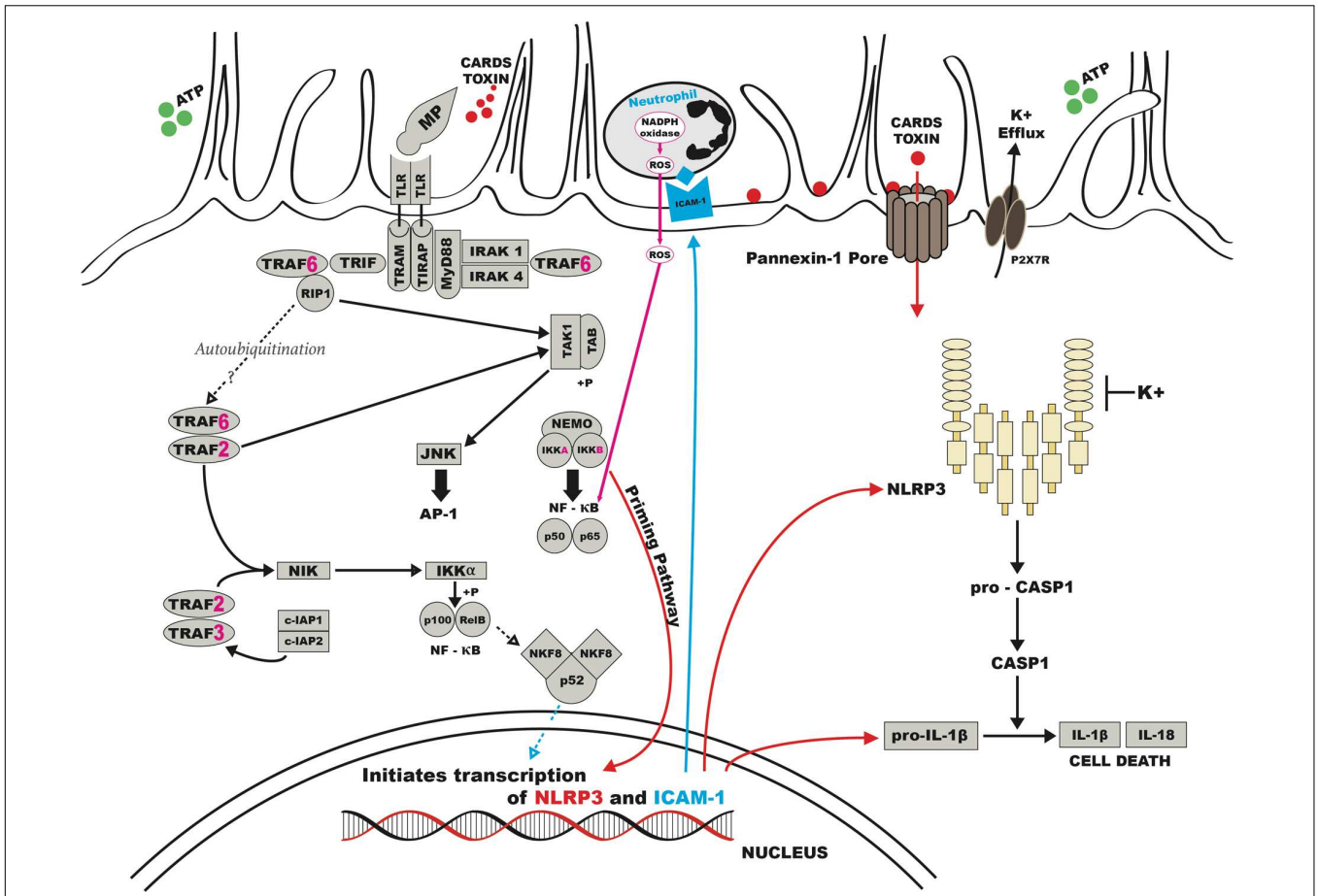

FIGURE 2 | Possible schematic for pathogenesis of human M. *pneumoniae*. CARDS, Community Acquired Respiratory Distress Syndrome; ATP, adenosine tri-phosphate; K, potassium; ICAM, intracellular adhesion molecule; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; TLR, toll like receptor.

during close contact. Following a coughing event, contaminated droplets disperse through the air. The eventual cytadherence by the bacteria in an alternate host describes successful transmission. Some patients may remain infectious for prolonged periods despite the disappearance of many symptoms, other than cough (Hallander et al., 1999; Wadowsky et al., 2002; Ishida et al., 2010; Wang et al., 2011).

Immunity is not long lasting; the bacteria and its associated disease can relapse in patients even after adherence to an effective antibiotic regimen (Watson and Storch, 2008). The genomic variation of the P1 adhesin may contribute to this complex and recurring epidemiology. There are two main subtypes of *M. pneumoniae* frequently isolated from clinical specimens (Su et al., 1990), though other variants have been reported (Dorigo-Zetsma et al., 2000; Dumke et al., 2010b; Pereyre et al., 2012). Japan has reported the cycling of prevalent subtypes. Between 1995 and 2001, subtype 2 was accountable for the majority of infections, but between 2002 and 2005, subtype 1 became more prevalent (Kenri et al., 2008). It is not yet understood if recurrence within one individual is caused by reactivated infection or exposure to different genetic subtypes.

Interestingly, epidemic and endemic settings also report a polyclonal spread of the bacteria (Chalker et al., 2011; Pereyre et al., 2012, 2013), with multiple types or strains propagating within the human population simultaneously. This observation indicates point source infection is not the probable cause of countrywide epidemics; rather, a more likely cause is some environmental factor. Rates of *M. pneumoniae* vary annually, yet cyclic epidemic patterns have been observed every three to five years in long-term studies and geographic surveillance (Lind et al., 1997; Nir-Paz et al., 2012). It has been suggested most epidemics occur in either late summer or autumn within North America (Alexander et al., 1966; Feikin et al., 1999). However, other geographic regions report maintained epidemics through all seasons (Foy et al., 1979; Blystad et al., 2012; Nir-Paz et al., 2012; Polkowska et al., 2012; Uldum et al., 2012). A recent study from Fukuoka, Japan has reported climactic events related to the El Niño Southern Oscillation and Indian Ocean Dipole were significantly associated with monthly incidence of *M. pneumoniae* in both 2005–2007 and 2010–2011 (Onozuka and Chaves, 2014). These weather events may be responsible for both the cyclic 3–5 years

resurgence pattern as well as the differing seasonality across continents.

In adults, *M. pneumoniae* is potentially responsible for more than 35% of hospitalized community-acquired pneumonia (CAP) cases (Marston et al., 1997; Dey et al., 2000; Cunha and Pherez, 2009). However, a recent report from the Centers for Disease Control and Prevention, estimated only 2% of detectable pathogens in hospitalized CAP patients were due to *M. pneumoniae* (Jain et al., 2015). Moreover, a report from the Atypical Pathogens Reference Laboratory Database attributes 12% (range 11–15%) of global CAP incidence to *M. pneumoniae* (Arnold et al., 2007). This last estimate does take into account the fluctuation of both seasonal and outbreak years, covering almost five winter seasons in its data analysis. However, patients with CAP typically have mild symptoms and are treated as outpatients, if at all. Therefore, the number of *M. pneumoniae* cases reported may be an underestimation of actual burden.

Prevalence, documented in other studies, can have a wide range Marston et al. (1997), reported 5.4% of CAP in hospitalized adults in the United States was due to *M. pneumoniae* by serology. Also using serology in Porath et al. (1997), from Israel, reported 29.2% of hospitalized CAP adults were infected with *M. pneumoniae*. Wattanathum et al. (2003), Thailand reported similarly high rates by serology, with 29.6% of outpatients with symptoms due to *M. pneumoniae*. Whereas, von Baum et al. (2009), polymerase chain reaction (PCR) and serological analysis from Germany showed 6.8% of CAP cases were due to *M. pneumoniae*, of which 55% were treated as outpatients. Prevalence reporting for most countries, however, is difficult due to the non-availability of reliable, rapid diagnostic techniques and an organized reporting system (Kashyap and Sarkar, 2010).

We calculated an estimated number of *M. pneumoniae* cases per 100,000 people by linear interpolation based on in country reports collected during our review process (Bii et al., 2002; Chaoprasong et al., 2002; Accomando et al., 2004; Nagalingam et al., 2004; Obeidat et al., 2005; Matute et al., 2006; Petitjean Lecherbonnier et al., 2006; Shankar et al., 2006; Somer et al., 2006; Kung, 2007; Huang et al., 2008; Boettcher et al., 2010; Prodromidou et al., 2010; Touati et al., 2010; Eick et al., 2011; Song et al., 2011; Wang et al., 2011; Bajraktarevic et al., 2012; Blystad et al., 2012; Feikin et al., 2012; Hoffmann et al., 2012; Lenglet et al., 2012; Polkowska et al., 2012; Uldum et al., 2012; Wellinghausen et al., 2012; Chen et al., 2013, 2015; Hong et al., 2013; Kawai et al., 2013; Luchsinger et al., 2013; Wu et al., 2013; Carrim et al., 2014; Grassi et al., 2014; Moore et al., 2014; Neocleous et al., 2014; Zhao et al., 2014; Kogoj et al., 2015; Liu et al., 2015). The resulting data is shown in **Figure 3**, with elevated rates possibly found throughout China, Russia, Mexico, and Brazil.

The majority of outbreaks have occurred within a community or in closed or semi-closed settings such as military bases or universities (Mogabgab, 1968; Edwards et al., 1976; Gray et al., 1997, 1999; Feikin et al., 1999; Crum et al., 2005; Walter et al., 2008; Centers for Disease Control and Prevention [CDC], 2012, 2013; Waller et al., 2014), hospitals (Fischman et al., 1978; Kashiwagi et al., 1985; Kleemola and Jokinen, 1992; Hyde et al., 2001; Shangguan et al., 2014), and facilities for the developmentally disabled or elderly (Marrie, 1993; Klausner et al., 1998; Hastings et al., 2015). Although, these outbreaks can disrupt and consume significant resources in the workforce, long-term morbidity is uncommon (Waites and Talkington, 2004). Controlling an outbreak often includes simple strategies such as, cohorting infected patients and the correct use of antibiotics as treatment.

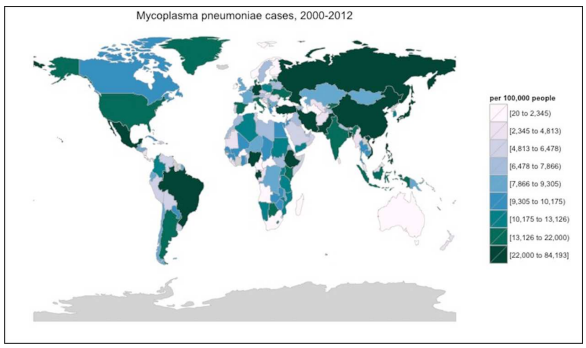

FIGURE 3 | Interpolated *M. pneumoniae* incidence from 2000 to 2012. These statistics were calculated using linear interpolation from reported incidence found within the literature. Gray countries were incalculable. This interpolation process did not take into account any genetic, cultural, environmental, social, or other differences across the various countries and regions. Thus, interpolations may have very limited relevance to the actual incidence of *M. pneumoniae* in any region. Image created using R v.3.2.2 with the package choroplethr.

CLINICAL MANIFESTATIONS

In the acute phase of infection, a dry cough develops which may progress to a wet cough in 3–4 days. Coughing represents progressing tracheobronchitis, the most common form of infection. Chest auscultation may be unhelpful for diagnosis in most situations, but scattered rhonchi and expiratory wheezes may sometimes present (Norisue et al., 2008). If pneumonia develops, atypical pneumonia is the predominant syndrome observed in adult patients. The syndrome is portrayed by the gradual onset of pharyngitis, sinus congestion, infrequent otitis media, and eventually prolonged lower respiratory involvement up to and including pneumonia with low-grade fever and bibasilar pulmonary infiltrates. The incubation period prior to symptom emergence may be short or as long as 3 weeks. In severe pneumonia cases, dry rales and frank consolidation may be observed, but this is fairly uncommon, and may be due to co-infection with *Streptococcus pneumoniae* or *Chlamydophila pneumoniae* (Nambu et al., 2006; Norisue et al., 2008).

There may be mild leukocytosis, but the total white blood cell count does not often exceed 15,000/μL. Expectorated sputum is not viscous. If sputum is sufficient, gram staining shows nothing discernable due to the small size of *M. pneumoniae* and its lack of cell wall. Severe cough and chest images depicting bronchopneumonia are common, as inflammation occurs in response to ciliated cell damage. Respiratory symptoms in severe pneumonia cases may necessitate admission to the hospital due

to decreased blood oxygen and increasingly labored breathing. The most common radiological and high resolution computed tomography (CT) images of *M. pneumoniae* pneumonia include air-space opacification, bronchovascular thickening, atelectasis, nodular infiltration, and linear opacities outward from the hilum, but are indistinguishable from other bacterial or viral pneumonia patterns (Reittner et al., 2000; Miyashita et al., 2009). Putman et al. (1975) proposed three possible reasons for alternate chest images: the existence of an underlying or co-existing pulmonary disease, previous exposure to *M. pneumoniae*, or the varied immune response and host susceptibility. To date, many researchers favor prior exposure (Saraya et al., 2011; Medina et al., 2012) and host immune differences (Tanaka et al., 1996; Saraya et al., 2014). Still, others report alternate host differences such as age (Lee et al., 2006), health conditions (Lambert, 1968), or environmental factors (Putman et al., 1975; Yang et al., 2004). Severity of disease has also been shown to be strain- and toxin concentration-dependent (Techasaensiri et al., 2010).

As mentioned previously, the cell-mediated immunity of patients may have a strong impact on the course of disease development following *M. pneumoniae* infection (Putman et al., 1975; Tanaka et al., 1996; Yang et al., 2002; Saraya et al., 2011, 2014). Some studies report detection of *M. pneumoniae* in seemingly healthy individuals. One such study reports peak incidence of 13.5% of 758 healthy volunteers (Gnarpe et al., 1992), while another study detected positive throat cultures 4 months after illness (Foy et al., 1966). It is possible these asymptomatic or mild infections still allow for shedding of the pathogen. These patients may act as a reservoir from which further spreading can occur. Similar to other respiratory infections, the duration of signs and symptoms will be shorter if antibiotics are initiated early in the course of infection.

Other Respiratory Manifestations

It has been suggested *M. pneumoniae* infection contributes to the development of chronic respiratory diseases, including persistent cough and asthma. While medical care for a persistent cough is frequently sought out, this symptom is commonly associated with *M. pneumoniae* in children (Hallander et al., 1999; Wang et al., 2011) and older adults (Miyashita et al., 2008; Takahashi et al., 2009). The organism may be found frequently in school-aged children with persistent cough particularly during active epidemics of *M. pneumoniae* (Wang et al., 2011) or concurrently with *Bordetella pertussis* (Hallander et al., 1999). Similar outbreaks of persistent cough are likely to occur in other ideal settings, such as dormitories, among military recruits, and in hospitals or nursing homes. In Japan, *M. pneumoniae* was confirmed by serology in 5.5% of adult patients with persistent cough (Ishida et al., 2010). Despite this evidence, Wadowsky et al. (2002), concludes *M. pneumoniae* is infrequently the active agent of cough illnesses longer than 5 days in adolescents and adults. However, cough may persist after acute infection in adults due to the continued presence of mycoplasma cell products or the CARDS toxin (Kannan et al., 2011).

Mycoplasma pneumoniae has for a long time been implicated in the exacerbation of asthma (Biscardi et al., 2004; Nisar et al., 2007; Hong, 2012; Wood et al., 2013). Moreover, some studies have isolated the bacteria in higher prevalence among asthmatics (Kraft et al., 1998; Smith-Norowitz et al., 2013). Sutherland et al. (2004), evaluated a questionnaire given to patients with a history of CAP after an episode of pneumonia. There, patients with a history of atypical pneumonia were more likely to be asthmatics. Other studies go on to document *M. pneumoniae* infection preceding an initial asthmatic event (Mok et al., 1979; Yano et al., 1994; Biscardi et al., 2004). It is possible mycoplasma infection leads to the destruction of respiratory cells and facilitates mucosal penetration by other antigens. Thereby, allowing the patient to become atopic to *M. pneumoniae* and other allergens (Nisar et al., 2007). However, at this time, none of these studies have distinguished increased susceptibility or exposure to *M. pneumoniae* from genetic predisposition for asthma (Mok et al., 1979).

Although, the *M. pneumoniae* connection with asthma is well-established, the mechanism behind development of the disease is still relatively unknown. One clue to pathogenesis in this regard may be immunoglobin (Ig) E responses. Some studies have reported control patients, may be capable of mounting a higher antibody response than those with asthma (Kraft et al., 2002; Atkinson et al., 2009; Wood et al., 2013). The role of T lymphocytes in the pathogenesis of asthma has been well-documented. The release of type 2 cytokines, including interleukin (IL)-4 and 5, is also increased in the serum of patients with *M. pneumoniae* (Esposito et al., 2002; Jeong et al., 2012). These cytokines in turn promote IgE production, which also plays a part in asthma. Also, antigenic mycoplasmas may initiate an antibody response resulting in IgE attaching to mast cells interacting with *M. pneumoniae*, which ultimately stimulates histamine release (Gil et al., 1993). However, additional studies are needed to fully understand the role of *M. pneumoniae* plays in the initial onset and exacerbation of asthma.

Extrapulmonary Manifestations

Extrapulmonary manifestations, although less common, have also been described. Patients with compromised immunity, including humoral immunodeficiences may be at higher risk for developing these complications. Extrapulmonary complications may occur in no more than 10% of patients with *M. pneumoniae*. Central nervous system (CNS) complications comprise the bulk of commonly seen extrapulmonary manifestations (Guleria et al., 2005). Such complications include, encephalitis, meningitis, optic neuritis, and Guillain–Barré syndrome among others. The mechanism of action behind these ominous manifestations remains unknown. In most patients, respiratory illness precedes, 2–14 days before, CNS findings (Tsiodras et al., 2005). CNS complications may result from direct invasion of *M. pneumoniae* in the brain (Tsiodras et al., 2005) or through extreme immune-mediated damages (Lee et al., 2013). Immune-mediated responses could be the result of cross-reacting antibodies and antigens shared by *M. pneumoniae* and the brain, depression of T-lymphocyte function, immune complex deposition, or intravascular clotting (Guleria et al., 2005; Tsiodras et al., 2005; Johnson, 2006).

Dermatological conditions such as erythematous maculopapular, vesicular rashes and Stevens-Johnson syndrome,

are also somewhat common as extrapulmonary manifestations (Walicka et al., 2008; Kashyap and Sarkar, 2010; Kunimi et al., 2011; Shimizu et al., 2012). Whereas, hematological, gastrointestinal, musculoskeletal, renal and other inflammatory manifestations have occurred in rare cases (Cameron et al., 1992; Perez et al., 1997; Parisi and Filice, 2001; Perez and Artola, 2001; Waites and Talkington, 2004; Johnson et al., 2007; Atkinson et al., 2008; Maia et al., 2009; Kashyap and Sarkar, 2010; Bayram et al., 2011). Further, information regarding the details of extrapulmonary diseases as a result of *M. pneumoniae* can be found in numerous case reports. However, because *M. pneumoniae* is quite common, there is the possibility some of these instances are only coincidental.

DIAGNOSIS

As a common cause of illness for both children and adults, *M. pneumoniae* should regularly be considered as a possible etiology in any upper respiratory infection, especially in immunocompromised patients or patients who have not responded to β-lactam antibiotics. Recently, there are many new techniques adapted to detecting the presence of *M. pneumoniae* suitable for both research and diagnostic purposes. These techniques were described in length by Daxboeck et al. (2003). Here, we will include a brief review and updated techniques.

The Japanese Respiratory Society (JRS) developed a scoring system to differentiate between typical and atypical pneumonia (Ishida et al., 2007) using clinical findings. The differential items include: (1) patient under 60 years of age; (2) no or minor underlying diseases; (3) stubborn cough; (4) poor chest auscultatory findings; (5) no sputum or etiologic agent identified by gram staining; and (6) a peripheral white blood cell below 10,000/μL. In cases where a patient presents with four or more of the six items, JRS guidelines recommend the use of macrolides or tetracyclines because of a suspected atypical pneumonia.

Many countries consider the most reliable diagnosis for acute pneumonia infection would come from a combination of two or more separate laboratory methods, such as serology and PCR (Petitjean et al., 2002; Daxboeck et al., 2003; Martinez et al., 2008; Nilsson et al., 2008; Chaudhry et al., 2013), or a clinical prediction rule, such as the JRS scoring system, with a rapid laboratory test (Ishida et al., 2007; Miyashita et al., 2011, 2015). The use of the laboratory tests listed below along with clinical prediction rules can more easily distinguish among acute, persistent infection and asymptomatic patients.

Historically, the use of cold agglutinins and detection of *M. pneumoniae* by culture methods were widespread diagnostic techniques. Cold agglutinin testing was once considered a valuable tool, but it is not a highly specific indicator of *M. pneumoniae,* as autoantibodies in the blood can be elevated from other diseases or syndromes (Jacobs, 1993; Beersma et al., 2005). A decade ago, the complement fixation (CF) method that detects the human body's early responses to *M. pneumoniae* was common around the world. A single 1:64 CF titer was considered an indication of recent *M. pneumoniae* infection. However, the CF test has a well-documented lack of sensitivity and specificity (Ponka et al., 1981; Waites and Talkington, 2004).

Rapid Diagnostics

Currently, a more advanced test using a similar method to CF is the microparticle agglutination assay (MAG), wherein specific antibodies to *M. pneumoniae* create hemagglutination and erythrocytes are replaced by latex particles to avoid non-specific reactions (Barker et al., 1990). Additionally, in August 2013, two rapid antigen kits for the detection of *M. pneumoniae* in nasopharyngeal samples became available in Japan (Miyashita et al., 2015; Yamazaki et al., 2015). These rapid antigen kits detect two different targets L7/L12 ribosomal protein or P1 adhesion protein. Two studies have compared the ribosomal protein rapid antigen kit to real-time PCR and the resulting theoretical diagnostic sensitivities were approximately 60% (Miyashita et al., 2015) and 74% (Yamazaki et al., 2015) in these samples. Hatano et al. (2013), reported the use of this rapid P1 adhesion protein detecting kit in 462 patients with a resulting sensitivity and specificity of 90 and 89.5%, respectively. However, further prospective studies are needed to evaluate the sensitivity of these rapid tests more thoroughly.

Rapid diagnostic tests are most useful in the early stages of CAP to assist decisions related to patient therapy. Despite the development of these two tests, however, the majority of *M. pneumoniae* cases continue to be diagnosed using serological methods or through the detection of nucleic acids. A variety of tests have been developed in this regard, each with their own advantages and disadvantages. However, numerous confounding variables inherent to the pathogenesis of *M. pneumoniae* contribute to obscuring the diagnostic accuracy of laboratory methods.

Serology

Serology remains as relevant now as it was in the past for the diagnosis of *M. pneumoniae.* The ease of sample collection coupled with further regard to conclusive evidence of *M. pneumoniae* as the causative agent, maintain serology's presence within diagnostics. There are currently several commercially available serological tests utilizing a variety of methods to detect the presence of *M. pneumoniae.*

Enzyme immunoassays (EIAs) use whole-cell lysates, containing glycolipid antigens, or protein extracts without glycolipid antigens. IgG seroconversion in *M. pneumoniae* infected patients is estimated to occur from 3 to 8 weeks following infection. EIAs are more sensitive than both the CF and MAG tests (Moule et al., 1987; Aubert et al., 1992; Nir-Paz et al., 2006) for detecting acute infection. Most EIAs implement a 96 well-microtiter plate. However, rapid membrane based procedures are available for the detection of a single specimen (Alexander et al., 1996; Matas et al., 1998).

For diagnosis during acute infection, a separate detection of IgM or IgA is useful. IgM antibodies appear in the first week of illness and reach their highest titers during the third week (Jacobs, 1993). IgA antibodies are also produced in early stages of the disease (Watkins-Riedel et al., 2001). Assays for

IgM and IgA detection are primarily based on the enzyme-linked immunosorbent assay (ELISA) principle. Rapid assays, like those previously mentioned are also available and generally use direct immunoflourescence, counter immunoelectrophoresis, immunoblotting or antigen capture EIs (Kashyap and Sarkar, 2010). Talkington et al. (2004) and Beersma et al. (2005), more thoroughly evaluated commercially available EIAs.

Despite its many strengths and versatility, serology lacks sensitivity. Many ELISA tests have the possibility of false positive results by cross-reactions with other mycoplasma species (Morrison-Plummer et al., 1987). Additionally, antibodies to *M. pneumoniae* may not appear until 2 weeks following initial infection and onset of symptoms (Vikerfors et al., 1988). Other studies report substantially longer times until positive serology results (Nir-Paz et al., 2006; Nilsson et al., 2008; Zhang et al., 2011). Further still, physicians must also consider the status of a patient's immune system. Particularly, in adults the response to IgM may be non-specific or absent (Uldum et al., 1992); while other underlying conditions may indicate an immunocompromised patient. However, serology as a diagnostic approach remains a convenient alternative for the detection of *M. pneumoniae* in respiratory secretions.

Nucleic Acid Amplification

Polymerase chain reaction amplification from respiratory secretions, such as nasopharyngeal, oropharyngeal, or sputum samples can provide more sensitive detection. Many studies have shown amplification methods can detect *M. pneumoniae* even in seemingly healthy individuals (Leng et al., 1994; Tjhie et al., 1997). PCR tests have been designed around the 16S rDNA, P1 adhesion protein, and the ATPase operon genes of *M. pneumoniae*. Sensitivity can be further increased by nested PCR, which involves reamplification of a PCR product with a different set of primers for the same target.

Quantitative real-time PCR (qRT-PCR) may also provide an attractive alternative to serology and conventional PCR (Pitcher et al., 2006). It is as sensitive as conventional PCR with the additional possibility of quantitative capabilities, which may indicate acute infections. The qRT-PCR method has also been successful for rapidly and reliably distinguishing between the two dominant *M. pneumoniae* types (Schwartz et al., 2009). Furthermore, a qRT-PCR assay, designed to target the CARDS toxin gene, proved to be the most sensitive assay to identify positive specimens in an outbreak investigation and again in other specimens (respiratory and cerebrospinal fluid) in sporadic cases (Winchell et al., 2008). Currently, a commercially available kit, implementing detection of the CARDS toxin gene does not exist. A publication authored by Chaudhry et al. (2013) indicates in-house qRT-PCR designed methods have a small but significant increase in sensitivity over traditional PCR methods, but primer design and standardization could be problematic for less experienced laboratories.

The use of conventional PCR and qRT-PCR has standardized the detection of *M. pneumoniae* along with other pneumonia and atypical pneumonia inducing bacterial pathogens (Mustafa et al., 2011). A recent study comparing four commercially available multiplex PCR assays found performance across different manufacturers remains relatively high and stable, with 93–100% agreement for all comparisons (Anderson et al., 2013). Khanna et al. (2005) developed a multiplex PCR assay for detection of five pneumonia-causing bacteria; it is now available commercially. The use of multiplex PCR or multiplex qRT-PCR technology enables the detection of multiple pathogens simultaneously with excellent sensitivity and specificity. Multiplex technology is particularly useful for the diagnosis of CAP patients, in etiological studies, or when broad-spectrum antibiotics fail to improve patient conditions.

Developed in Japan, a new amplification technique called loop-mediated isothermal amplification (LAMP) has also been applied to rapid diagnosis of *M. pneumoniae* (Saito et al., 2005; Yoshino et al., 2008; Kakuya et al., 2014). This molecular amplification method occurs in a single tube at constant temperature, eliminating the necessity of a thermocycler. Endpoint analysis can be performed rapidly by visual confirmation of turbidity and precipitates or can be integrated into more advanced photometrics for more accurate quantification. The sensitivity of the LAMP assay was 88.5% compared to a validated qRT-PCR test on samples collected in the United States, and no cross reactivity was observed against 17 other mycoplasma species, human DNA, nor other common respiratory pathogens (Petrone et al., 2015). Unfortunately, due to the limits inherent within primer design, multiplexing of this assay is not possible. In the near future LAMP assays could enable rapid, low cost detection of *M. pneumoniae* cases and earlier recognition of outbreaks by medical providers (Petrone et al., 2015), particularly in resource limited settings.

Culture Techniques

Although still seen as the "gold standard," bacterial culture for *M. pneumoniae* from oropharyngeal samples can be time consuming due to the nutritive requirements. Specificity is 100%, when protocols are successful. Isolation of the pathogen has advanced the knowledge surrounding extrapulmonary manifestations, because successful isolation provides evidence of direct invasion by living bacteria (Daxboeck et al., 2003). Similar conclusions cannot be made from all positive PCR results, because target DNA may still be detected in patients beyond the death of the bacteria. However, the sensitivity of culture for diagnosis can be low and dependent both on the skill of the laboratory as well as the quality of the specimen. Thus, *M. pneumoniae* is cultured with cell-free media formulations, primarily for research purposes.

CHEMOTHERAPY AND VACCINES

These organisms lack a peptidoglycan cell wall, therefore therapy, which interferes with DNA synthesis, i.e., quinolones, or protein synthesis such as, macrolides and tetracyclines, are used more frequently than beta-lactams and glycopeptides and generally have a greater influence on disease (Niederman, 2001; Watkins and Lemonovich, 2011). For young children, macrolides should be considered first, due to potential severe side effects of tetracyclines and quinolones (Suzuki et al., 2006).

Macrolides are well-known for their antibiotic capabilities. However, considerable data has also been gathered confirming macrolides also possess anti-inflammatory properties, which can also contribute to patient improvement (Gotfried, 2004; Tamaoki, 2004). Macrolides seem to modify or regulate the immune system by inhibiting inflammatory cell chemotaxis, cytokine synthesis, adhesion molecule expression, reactive oxygen species production and intracellular signaling pathways (Kanoh and Rubin, 2010). A study conducted by Kraft et al. (2002) focused on the effect of clarithromycin on lung function of *M. pneumoniae* infected and uninfected asthmatic patients. They determined clarithromycin treatment caused improvement in forced expiratory volume and reduced airway expression of IL-5, but only in *M. pneumoniae* positive patients. These findings suggest, in cases of *M. pneumoniae*, macrolides may act primarily as antibiotics as well as an anti-inflammatory agent. The optimal dosage and duration of therapy is not clear; however, 10–14 days is generally recommended. The use of steroids in combination with macrolides has also been recommended in severe cases of *M. pneumoniae* pneumonia.

Macrolide-resistant *M. pneumoniae* was first reported in Japan in Okazaki et al. (2001) and has since been continuously reported in increasing percentages among the population (Matsuoka et al., 2004; Miyashita et al., 2012, 2013; Kawai et al., 2013; Hanada et al., 2014). However, the prevalence of macrolide-resistant *M. pneumoniae* varies among countries. Cao et al. (2010), China documented a 69% prevalence of resistance as well as treatment failure, but no cases of macrolide-resistant *M. pneumoniae* were found from 1997 to 2008 in the Netherlands (Spuesens et al., 2012). It is well-established, point mutations, leading to A-to-G transitions in the peptidyl transferase loop domain V of the 23S rRNA gene at positions 2063 and 2064, reduce the affinity of macrolide for the ribosome (Suzuki et al., 2006; Dumke et al., 2010a). Identification of these resistant strains currently relies on restriction fragment length polymorphism or gene sequence analysis. The creation of a laboratory technique, such as qRT-PCR to rapidly detect macrolide resistant strains may be useful for surveillance and outbreak situation.

Of the major bacterial respiratory pathogens including *Streptococcus pneumoniae* and *Haemophilus influenzae*, *M. pneumoniae* is the only one without an available vaccine (Nir-Paz et al., 2012). During the 1960 and 1970s a number of studies were carried out testing immunogenicity and the protective efficacy of several different vaccines. A recent meta-analysis by Linchevski et al. (2009), has shown the overall pneumonia prevention efficacy of those studies was 41% (54% for *M. pneumoniae* specific pneumonia), when diagnosis was based on culture or serology. No serious adverse effects were reported and only mild local reactions were suffered.

Schurwanz et al. (2009), P1 and P30 adhesions showed strong reactivity with human and animal sera, proving as promising candidates for further vaccine formulation and optimization. However, in 2011 study reported vaccination with only an avirulent P30 mutant resulted in disease exacerbation in mice (Szczepanek et al., 2012). Most recently though the subcutaneous administration of a P1–P30 chimeric recombinant protein, followed by two intranasal booster administrations induced high, consistent, and long lasting IgA levels in guinea pigs (Hausner et al., 2013). The CARDS toxin could also serve as an effective vaccine candidate (Kannan et al., 2011).

Mycoplasma pneumoniae is increasingly the cause of upper and lower respiratory tract infections for adults and children. It is associated with prolonged carriage and lacks natural protective immunity following primary infections. Continued development of a vaccine for high-risk individuals such as school children, military recruits, and elderly people in nursing homes or long term hospital care, may help to reduce morbidity from pneumonia and secondary complications. A vaccine may also slow the development of other macrolide resistant strains and reduce the impact of macrolide-resistant strains in communities and epidemics.

CONCLUSION

Mycoplasma pneumoniae is a commonly found pathogen within adults around the world. It is a common cause of pneumonia but can also initiate other extrapulmonary manifestations. The simplicity of the genome and the small size of mycoplasmas have led many to conclude these organisms are uncomplicated. In reality, much regarding the microbiology and pathogenesis of this organism remains unknown. A variety of respiratory diseases portray similar clinical symptoms. Recent and continued advances in multiplex PCR and qRT-PCR may prove useful in distinguishing the pathogen causing disease. In many situations, the mild symptoms of *M. pneumoniae* pneumonia may be ignored by the patient and remain undiagnosed.

Much energy has been devoted to the CARDS toxin. Information regarding its structure and involvement in pathogenesis may prove useful to the continued development of more effective options for both diagnostics and therapies. Currently, there is limited availability of rapid and accurate testing methods for *M. pneumoniae*. Further development of rapid tests specifically to distinguish macrolide-resistant strains may also be useful within Asia. Physicians and patients around the world must continue to monitor macrolide-resistance strains, preventing dissemination and increased incidence. However, the development of an effective vaccine could prove useful at reducing the burden among the elderly and within the workforce around the world.

AUTHOR CONTRIBUTIONS

GP and TK made substantial contributions to the conception and design of the work. GP primarily drafted the work and TK and JF revised it critically for important intellectual content. All had final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

We thank Matthew T. Roseler and Stephanie Untz for their expertise in creating the figures included in this document.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 23 May 2016
doi: 10.3389/fmicb.2016.00693

Epidemiology of *Mycoplasma pneumoniae* Infections in Japan and Therapeutic Strategies for Macrolide-Resistant *M. pneumoniae*

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OPEN ACCESS

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *31 December 2015*
Accepted: *26 April 2016*
Published: *23 May 2016*

Citation:
Yamazaki T and Kenri T (2016) Epidemiology of Mycoplasma pneumoniae Infections in Japan and Therapeutic Strategies for Macrolide-Resistant M. pneumoniae. Front. Microbiol. 7:693. doi: 10.3389/fmicb.2016.00693

Pneumonia caused by *Mycoplasma pneumoniae* (*M. pneumoniae* pneumonia) is a major cause of community-acquired pneumonia worldwide. The surveillance of *M. pneumoniae* pneumonia is important for etiological and epidemiological studies of acute respiratory infections. In Japan, nation-wide surveillance of *M. pneumoniae* pneumonia has been conducted as a part of the National Epidemiological Surveillance of Infectious Diseases (NESID) program. This surveillance started in 1981, and significant increases in the numbers of *M. pneumoniae* pneumonia patients were noted in 1984, 1988, 2006, 2010, 2011, 2012, and 2015. The epidemics in 2011 and 2012 were particularly widespread and motivated researchers to conduct detailed epidemiological studies, including genotyping and drug resistance analyses of *M. pneumoniae* isolates. The genotyping studies based on the *p1* gene sequence suggested that the *p1* gene type 1 lineage has been dominant in Japan since 2003, including the epidemic period during 2011–2012. However, more detailed *p1* typing analysis is required to determine whether the type 2 lineages become more relevant after the dominance of the type 1 lineage. There has been extensive research interest in implications of the *p1* gene types on the epidemiology of *M. pneumoniae* infections. Serological characterizations of sera from patients have provided a glimpse into these associations, showing the presence of type specific antibody in the patient sera. Another important epidemiological issue of *M. pneumoniae* pneumonia is the emergence of macrolide-resistant *M. pneumoniae* (MRMP). MRMPs were noted among clinical isolates in Japan after 2000. At present, the isolation rate of MRMPs from pediatric patients is estimated at 50–90% in Japan, depending on the specific location. In view of the situation, Japanese societies have issued guiding principles for treating *M. pneumoniae* pneumonia. In these guiding principles, macrolides are still recommended as the first-line drug, however, if the fever does not subside in 48–72 h from first-line drug administration, a change of antibiotics to second-line drugs is recommended.

Keywords: *Mycoplasma pneumoniae*, community-acquired pneumonia, infectious diseases surveillance, periodic epidemics, P1 cytadhesin, P1 typing, hemadsorption, macrolide resistance

SURVEILLANCE OF *M. pneumoniae* PNEUMONIA IN JAPAN

In Japan, the National Epidemiological Surveillance of Infectious Diseases (NESID) program is conducted under the Infectious Diseases Control Law (Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections), which includes nationwide surveillance of pneumonia cases caused by *Mycoplasma pneumoniae*. *M. pneumoniae* pneumonia is classified as a category V infectious disease in the NESID, and the numbers of affected patients (total of outpatients and inpatients) are reported weekly from sentinel hospitals. Approximately 500 hospitals across Japan that have departments of pediatrics and internal medicine and more than 300 beds are currently selected as the sentinels for surveillance of *M. pneumoniae* pneumonia in Japan. For notification of each new *M. pneumoniae* pneumonia patient, confirmation is required using one of the tests listed in **Table 1** in addition to clinical symptoms observed by a clinician. Previously, culture isolation of *M. pneumoniae* and detection of serum antibodies against *M. pneumoniae* were employed as the tests for notification. However, detection of *M. pneumoniae* genomic DNA by polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) and detection of *M. pneumoniae* antigens by immuno-chromatographic methods have been recently included in the tests for notification[1]. The data from sentinels are integrated at the Infectious Disease Surveillance Center (IDSC), National Institute of Infectious Diseases (NIID) and published weekly[2]. Since the NESID program was initiated in July 1981, the surveillance of primary atypical pneumonia (PAP) was continuously performed until March 1999. The criteria of PAP include pneumonia other than *M. pneumoniae* pneumonia, such as that caused by *Chlamydophila pneumoniae*, *Legionella pneumophila*, or several viruses. However, as the major cause of PAP is *M. pneumoniae*, this surveillance largely represented the general trend of mycoplasma epidemics. As of April 1999, *M. pneumoniae* pneumonia-specific surveillance was initiated by NESID under the revised Infectious Diseases Control Law. **Figure 1** shows the most recent *M. pneumoniae* pneumonia surveillance data collected by the NESID. In the early period of data collection, there were large increases of PAP patients observed in 1984 and 1988. Before the NESID surveillance was started in Japan, an extensive epidemiological study of *M. pneumoniae* pneumonia in school children was performed in the 1960s and 1970s in Sendai city (Niitu, 1984). In this study, an increase of *M. pneumoniae* pneumonia patients was observed every 4 years (i.e., 1964, 1968, 1972, and 1976), suggesting periodicity in the epidemics of this disease. Epidemics observed by the NESID in 1984 and 1988 (**Figure 1**) are compatible with this 4-year periodicity pattern observed in Sendai city. Given that these 4-year-cycle epidemics occurred in Olympic years, *M. pneumoniae* pneumonia has often been referred to as "Olympic disease" in Japan. However, after this period, 4-year-cycle epidemics were no longer observed in the NESID surveillance, although slight increases in the number of patients were observed in 1992 and 1996. The reason for disappearance of periodic epidemic is unknown, however, it is noteworthy that clarithromycin has been introduced for treatment of PAP since 1991. After 2000, *M. pneumoniae* pneumonia epidemics were observed in 2006, 2010, 2011, and 2012. The epidemics in 2011 and 2012 were particularly widespread and attracted public attention. Although the reason for these large epidemics in 2011 and 2012 is unknown, large increases in the numbers of *M. pneumoniae* pneumonia patients were also reported in Europe and other countries during this period (Chalker et al., 2011; Blystad et al., 2012; Nir-Paz et al., 2012; Pereyre et al., 2013; Kim et al., 2015). After these large epidemics in 2011 and 2012, the number of *M. pneumoniae* pneumonia patients decreased rapidly, and was quite low in 2014. However, the number of patients increased again since the summer of 2015 and reached a higher level during the winter (**Figures 1** and **2**). An increase of patient number was also reported in China in 2015 (Yan et al., 2016).

Such period occurrences of *M. pneumoniae* pneumonia epidemics in 3–7-year intervals has been observed in surveillances of *M. pneumoniae* pneumonia in many areas of the world (Lind and Bentzon, 1976; Foy et al., 1979; Lind et al., 1997; Rastawicki et al., 1998; Ito et al., 2001; Eun et al., 2008; Chalker et al., 2011; Blystad et al., 2012; Youn and Lee, 2012; Kim et al., 2015). This phenomenon is considered to be one of the characteristic features of this disease. One potential reason for the pattern of periodic epidemics of *M. pneumoniae* pneumonia may be related to interactions between the pathogen and the immunological status of the human population (Fernald and Clyde, 1970; Mogabgab et al., 1975; Hayatsu et al., 1981; Leith et al., 1983; Barile et al., 1994). Once *M. pneumoniae* pneumonia epidemics occur, protective immunity may arise in the human population. However, this protective immunity may not last long, and the next generation will be dominated by younger individuals who had not been exposed, and thus do not have the protective immunity. In this situation, *M. pneumoniae*

[1]http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou11/01-05-38.html
[2]http://www.nih.go.jp/niid/ja/10/2096-weeklygraph/1659-18myco.html

TABLE 1 | Tests required for notification of *Mycoplasma pneumoniae* pneumonia from sentinel clinics.

Test (Method)	Specimen
Isolation of *M. pneumoniae* (Culture method)	Specimens derived from the patient's respiratory tract
Detection of *M. pneumoniae* antigen (Immuno-chromatographic method)[a]	
Detection of *M. pneumoniae* DNA (PCR, LAMP, etc.)[b]	
Detection of antibody (serological diagnosis)	Serum

[a]*Four commercial immuno-chromatographic diagnosis kits for M. pneumoniae have been approved and used in Japan since 2013: Prime check Mycoplasma™ (Alfresa Parma), Ribotest Mycoplasma™ (Asahi Kasei), Prorast Myco™ (LSI Medience), and Immuno Ace Mycoplasma™ (Tauns Laboratories). The detection targets of these four kits are P1 adhesin, ribosomal L7/L12, DnaK and P30 proteins, respectively.* [b]*Genomic DNA detection tests have been introduced to the surveillance since 2011.*

become active and may cause the next epidemic. A mathematical model of this process was recently reported (Omori et al., 2015). There are also reports of weather factors that might affect *M. pneumoniae* infections (Onozuka et al., 2009; Onozuka and Chaves, 2014). Examination of the NESID data from a seasonal perspective shows that the number of *M. pneumoniae* pneumonia patients generally increases in autumn and winter every year. However, depending on the year, small increases are sometimes also observed in early summer (**Figure 2**). From a regional view, the number of patients varies according to

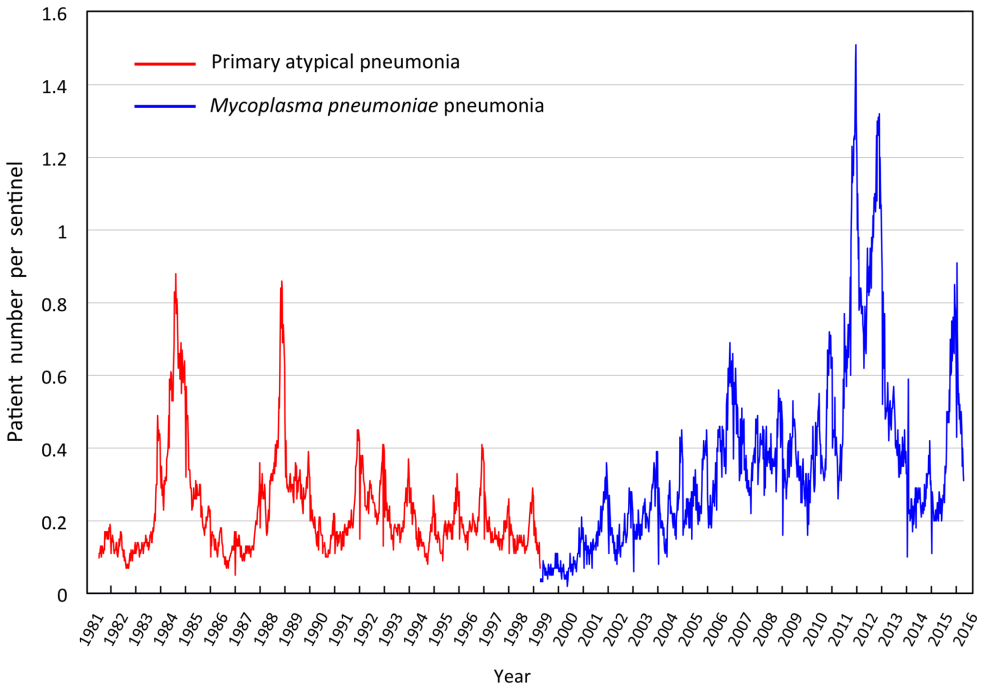

FIGURE 1 | Weekly cases of primary atypical pneumonia (from April 1981 to March 1999, red line) and *Mycoplasma pneumoniae* pneumonia (from April 1999 to present, blue line) in Japan reported by The National Epidemiological Surveillance of Infectious Diseases (NESID).

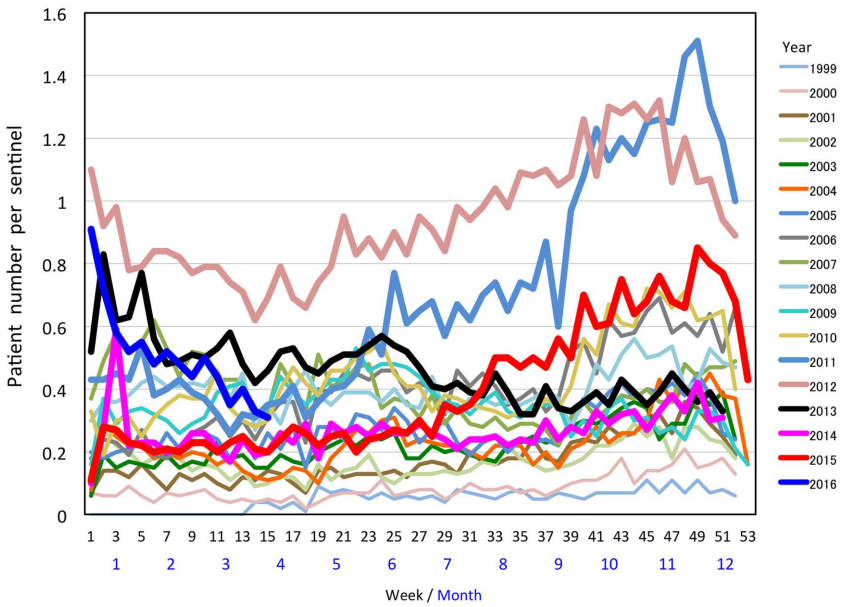

FIGURE 2 | Weekly cases of *M. pneumoniae* pneumonia in Japan between 1999 and 2015 reported by the NESID. http://www.nih.go.jp/niid/ja/10/2096-weeklygraph/1659-18myco.html.

prefectures in Japan. In the 2011 and 2012 epidemic period, the numbers of patients increased in most of the prefectures compared to those in previous years (**Figure 3**). Approximately 80% of *M. pneumoniae* pneumonia patients in Japan are in the 1–14-year-old age group, although *M. pneumoniae* pneumonia occurs in all age groups.

The national surveillance of *M. pneumoniae* pneumonia in the NESID program is based on reports from sentinel hospitals, and is thus not a survey of the total number of patients with this disease. Since the sentinels are large hospitals, information of *M. pneumoniae* pneumonia patients who are diagnosed and treated in small outpatient clinics is not included in the NESID data. The NESID also does not provide molecular epidemiological data or drug resistance information of *M. pneumoniae* clinical isolates. Although these issues need to be addressed, the weekly surveillance data provided by the NESID is nevertheless useful and functions as an alert for public health workers, medical institutions, and researchers. The NESID data allow for researchers to conduct detailed epidemiological studies to grasp the actual situation of *M. pneumoniae* infections, including molecular epidemiological and drug resistance aspects of this infectious disease, especially when the signs of epidemics are observed in the surveillance data.

MOLECULAR EPIDEMIOLOGY

In general, *M. pneumoniae* clinical isolates can be classified into two distinct genetic lineages (type 1 and 2 lineages) based on their genomic background (Simmons et al., 2013; Brown et al., 2015; Lluch-Senar et al., 2015; Touati et al., 2015; Xiao et al., 2015). The *p1* gene, which encodes the major cytadhesin P1 protein, exhibits sequence polymorphism between the type 1 and 2 lineages (Su et al., 1990; Kenri et al., 1999; Spuesens et al., 2010; Zhao et al., 2011; Dumke et al., 2015). The *p1* types were also referred as the *p1* subtypes or groups in previous studies. A number of typing analyses of *M. pneumoniae* isolates based on *p1* gene polymorphism have been reported to date (Jacobs et al., 1996; Sasaki et al., 1996; Cousin-Allery et al., 2000; Kenri et al., 2008; Dumke et al., 2010b; Liu et al., 2010; Martinez et al., 2010). In our previous study, we genotyped *M. pneumoniae* isolated mainly in Kanagawa prefecture, Japan, and found that the rate of *p1* types 1 and 2 detection was not constant but rather varied year by year (**Figure 4**) (Sasaki et al., 1996; Kenri et al., 2008). In brief, type 2 was dominant in the early 1980s, 1990s, and at the beginning of the 2000s, while type 1 was dominant in the late 1980s and after 2003. This study demonstrated an alternative type-shift phenomenon of *M. pneumoniae p1* types with intervals of about 10 years. The time dependency of the

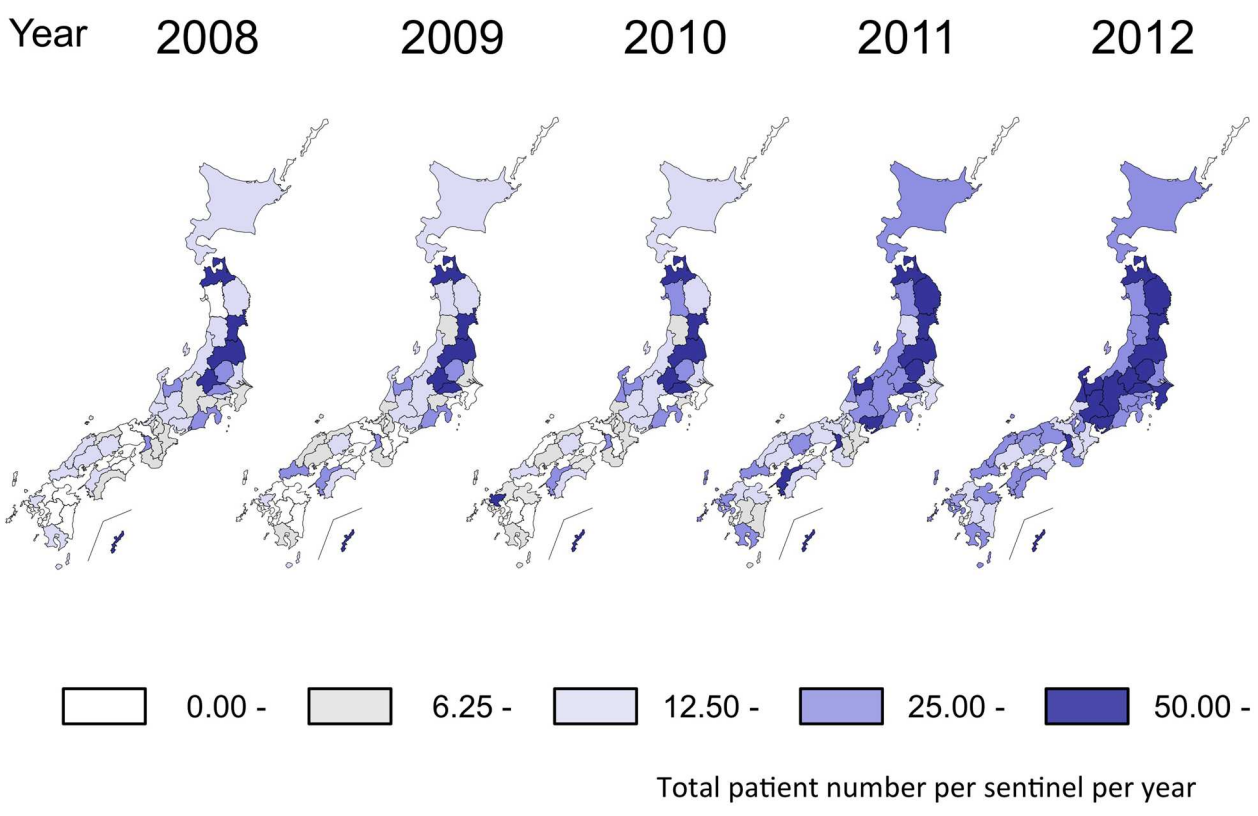

FIGURE 3 | Incidence of *M. pneumoniae* pneumonia in Japan by prefecture reported by the NESID. The ranges of the number of cases in each prefecture (annual cases per sentinel) are shown in different colors. The figure is adapted from Infectious Agents Surveillance Report (IASR), 33 (10), 2012. http://www.nih.go.jp/niid/en/iasr-vol33-e/865-iasr/2738-tpc392.html.

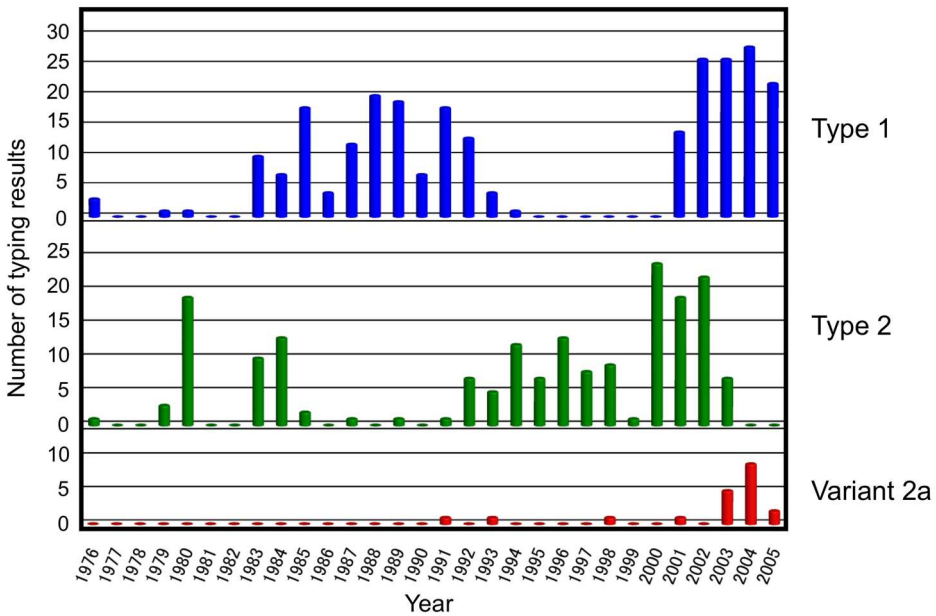

FIGURE 4 | Typing analysis of the *M. pneumoniae p1* gene from isolates detected in Japan between 1976 and 2005. The majority of *M. pneumoniae* strains and clinical specimens included in this analysis were collected in the Kanagawa prefecture. Figure adapted from Kenri et al. (2008).

rate of types 1 and 2 of the *p1* gene on the study period has also been observed in isolates from other parts of the world; however, no reports have shown a clear type-shift pattern similar to that observed in Kanagawa prefecture (Cousin-Allery et al., 2000; Pereyre et al., 2007; Martinez et al., 2010; Spuesens et al., 2012; Diaz et al., 2015; Kogoj et al., 2015). In typing analyses of isolates in Germany, the rate of types 1 and 2 detection was found to be relatively constant during the research period of about 10 years including the epidemics in 2011–2013 (Dumke et al., 2010b, 2015; Jacobs et al., 2015). The relationship between *M. pneumoniae* pneumonia epidemics and two type lineages of *M. pneumoniae* remains unclear (Jacobs et al., 1996, 2015; Kenri et al., 2008).

According to several *p1* typing studies, the dominant *M. pneumoniae* in Japan has been considered to be type 1 strains since 2003 (Fujii et al., 2012; Horino, 2012; Ohya et al., 2012; Ishiguro et al., 2015; Kubota et al., 2015; Suzuki et al., 2015). Apparent *p1* type shift of *M. pneumoniae* clinical strains was not observed during the large epidemic period in 2011 and 2012. For example, 126 *M. pneumoniae* strains were isolated at the Kanagawa Prefectural Institute of Public Health between 2003 and 2011. Of these, 101 (80%) were type 1, 7 (6%) were type 2, and 18 (14%) were type 2 variants (Ohya et al., 2012). At the Yamagata Prefectural Institute of Public Health, 358 isolates were genotyped between 2004 and 2013. Of these, 278 (77.7%) were type 1, 10 (2.8%) were variant 2a, 5 (1.4%) were variant 2b, and 65 (18.2%) were variant 2c. No type 2 strain was detected in Yamagata. In the case of Yamagata, type 1 accounted for 85–100% of the annual isolates recorded between 2004 and 2011. However, the isolation rate of type 1 reduced to 73.5% (83/113) and 33.9% (21/62) in 2012 and 2013, respectively. The proportion of variant 2c isolates increased in 2012 and 2013, as a counterpart of type 1 (Suzuki et al., 2015). At this point, it is not clear whether this is the sign of *p1* type change in this area. Although type 1 is considered to be dominant across a wide area of Japan at present, there is a report of a local area where type 2 is prevalent (Ishiguro et al., 2015). Most of type 2 lineage strains isolated in Japan in recent years are variants 2a and 2c while variant 2b and type 2 strains are rare. Type 2 was frequently found among the clinical isolates before 2000 (**Figure 4**), but type 2 was replaced by its variants (2a and 2c) almost completely during the last decade. The dominance of type 1 was also reported in recent clinical isolates in China and France (Liu et al., 2010; Wang et al., 2012; Pereyre et al., 2013; Tian et al., 2013; Zhao et al., 2013a; Xiao et al., 2014; Xue et al., 2014; Zhou et al., 2015). The results of a recent study conducted in Beijing, China indicate an increasing trend of type 2 lineage (Zhao et al., 2015), although it is not yet clear whether the type-shift phenomenon from type 1 to 2 is occurring. Further *p1* typing analysis is needed to explore whether type 2 lineage strains might become prevalent in the future in areas where type 1 are currently dominant.

Multilocus variable-number tandem-repeat analysis (MLVA)-based typing is a newly developed strategy for molecular epidemiological analyses of *M. pneumoniae* (Degrange et al., 2009; Chalker et al., 2015). Several reports demonstrated that Japanese clinical isolates can be also separated into multiple MLVA types as same as the clinical strains isolated in the other areas (Degrange et al., 2009; Kubota et al., 2015; Touati et al., 2015). However, more MLVA typing studies are needed to discuss the characteristic profiles of Japanese strains in terms of this typing method.

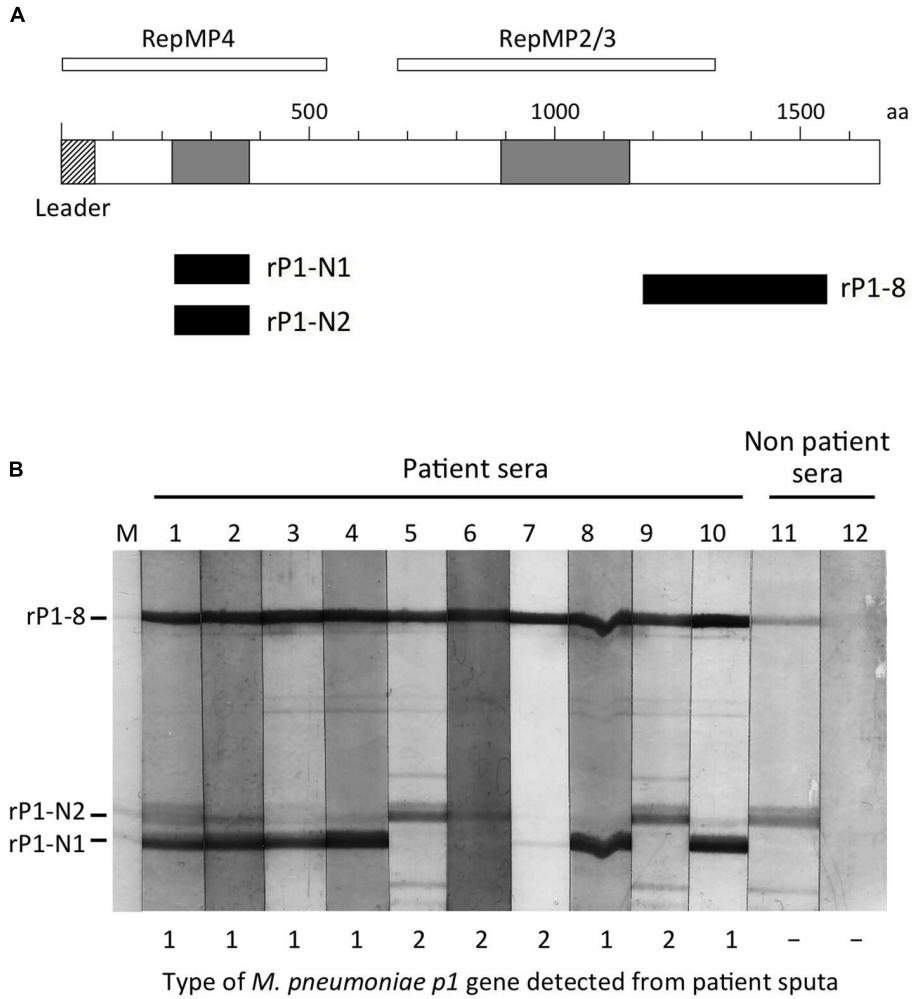

FIGURE 5 | Western blotting of the sera from *M. pneumoniae* pneumonia patients against recombinant P1 antigens. (A) Schematic illustration of the P1 protein structure. P1 is a 170-kDa membrane protein consisting of about 1,630 amino acids (aa) depending on the strains. The first 59 aa of this protein (hatched box) is a leader peptide, which is removed during maturation. The two gray boxes indicate polymorphic regions of P1 protein that exhibit amino acid sequence variation between type 1 and 2 strains. RepMP4 and RepMP2/3 indicate repetitive regions of the *p1* gene. Multiple copies of DNA sequences similar to the RepMP4 and RepMP2/3 regions are present throughout the *M. pneumoniae* genome. The three filled boxes indicate the positions of recombinant P1 protein fragments produced in *E. coli*. The rP1-N1 region corresponds to aa 218 to 352 of the P1 protein of M129 strain (type 1). The rP1-N2 region corresponds to aa 218 to 357 of the P1 protein of FH strain (type 2). The rP1-8 region corresponds to aa 1160 to 1518 of M129 P1 (Kenri et al., 2006b). **(B)** Western blotting of patient sera against rP1-N1, rP1-N2, and rP1-8 recombinant proteins. Purified rP1-N1, rP1-N2, and rP1-8 proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were blotted on a nitrocellulose membrane. Diluted (100-fold) serum samples from nine patients and two healthy subjects were reacted with the membrane. IgGs bound to the membrane were detected by alkaline phosphatase-conjugated anti-human IgG secondary antibody. The positions of recombinant P1 protein fragments are shown on the left. Lane M: Coomassie Brilliant Blue stain of the blotted membrane; lanes 1–10: patient sera (serum samples of lanes 5 and 9 were obtained from the same patient in an interval of 1 week); lanes 11 and 12: serum from healthy subjects. Types of *p1* genes detected by the nested PCR method (Kenri et al., 2008) from the sputum of same patient are shown on the bottom. The analyses of serum and sputum samples were performed as a part of previous studies (Yamazaki et al., 2006; Kenri et al., 2008).

SEROLOGICAL CHARACTERIZATION OF *M. pneumoniae* PNEUMONIA PATIENT SERA AGAINST TYPE 1 AND 2 P1 PROTEINS

P1 cytadhesin is one of the major antigens of *M. pneumoniae* that induce antibody production. Indeed, the anti-P1 antibody is frequently detected in the sera of *M. pneumoniae* pneumonia patients (Hirschberg et al., 1991; Razin and Jacobs, 1992; Rastawicki et al., 1996; Tuuminen et al., 2001). Since the P1 protein exhibits amino acid sequence polymorphism between the type 1 and 2 lineages, there was a possibility that P1 proteins of types 1 and 2 have different immunogenicity and induce specific antibodies during infection. In support of this idea, there are reports of the production of a monoclonal antibody that specifically recognizes type 1 P1 protein (Gerstenecker and

Jacobs, 1990; Jacobs et al., 1996), and induction of type-specific immunity was achieved with P1 fragment antigens in guinea pigs (Dumke et al., 2008). To obtain more evidence for specific immunological responses to the two P1 types, we engineered and produced three recombinant P1 protein fragments, shown in **Figure 5A**. The amino acid sequences of the rP1-N1 and rP1-N2 regions are derived from P1 protein of the strains M129 (type 1) and FH (type 2), respectively (Kenri et al., 2006a,b). These regions exhibit the highest differences between type 1 and 2 P1 proteins, whereas the amino acid sequence of rP1-8 region is identical between type 1 and 2 P1 proteins. Using these three P1 fragments as antigens, we performed a western blotting analysis of the sera of nine *M. pneumoniae* pneumonia patients to detect anti-P1 IgG (**Figure 5B**). The patient sera chosen for this western blot were those that exhibited high antibody titers in the serological diagnosis for *M. pneumoniae* pneumonia (see **Table 2**). Furthermore, *p1* genes were detected from sputum samples of the same patients and were genotyped by PCR (**Figure 5B** and **Table 2**). This information indicates the most probable type of *M. pneumoniae* that infected the patients. The western blot result is shown in **Figure 5B**. All of the patient sera exhibited similar reactivity against the rP1-8 fragment, whereas the reactivity against rP1-N1 and rP1-N2 varied depending on the serum sample (**Figure 5B**). The sera from patients whose sputa were type 1 *p1*-positive in PCR showed stronger reactivity against rP1-N1 (**Figure 5B**, lanes 1–4, 8, and 10). On the other hand, the sera from patients with type 2 *p1*-positive sputa exhibited stronger reactivity against rP1-N2 (**Figure 5B**, lanes 5, 6, and 9) except for one serum (**Figure 5B**, lane 7). One of the serum samples from a healthy subject, used as a negative control, also showed weak reactivity against rP1-8 and rP1-N2 (**Figure 5B**, lane 11). This individual most likely had a previous, and perhaps unnoticed, infection with type 2 *M. pneumoniae*. These results demonstrated that induction of type-specific anti-P1 antibodies occurs in humans during *M. pneumoniae* infection (Kenri et al., 2006a,b).

HEMADSORPTION (HA) INHIBITORY ACTIVITY OF *M. pneumoniae* PNEUMONIA PATIENT SERA

It has been reported that *M. pneumoniae* pneumonia patient sera exhibit inhibitory activity toward the adsorption of red blood cells to *M. pneumoniae* colonies [i.e., hemadsorption (HA) inhibitory activity; Hirschberg et al., 1991; Razin and Jacobs, 1992; Rastawicki et al., 1996; Tuuminen et al., 2001; Schurwanz et al., 2009]. Thus, we examined the HA inhibitory activity of the patient sera analyzed by western blotting in **Figure 5B**, and the results are shown in **Table 2**. The sera from type 1-infected patients (No. 1–4, 8, and 10) tended to show stronger HA inhibitory activity against M129 strain (type 1). On the other hand, the sera from type 2-infected patients (No. 5, 6, and 9) exhibited stronger HA inhibitory activity against FH strain (type 2) compared to M129. This result indicated that *M. pneumoniae* pneumonia patient sera with a high antibody titer possessed type-specific HA inhibitory activities (Kenri et al.,

TABLE 2 | Hemadsorption inhibitory activity of patient and non-patient sera.

Serum No.[a]	Antibody titer[b]	*p1* gene types detected from patient sputa[c]	Hemadsorption (HA) inhibitory activity[d]			
			M129 (Type 1)		FH (Type 2)	
			1/5	1/10	1/5	1/10
1	1280	1	+	±	−	−
2	2560	1	+	±	±	−
3	1280	1	+	+	+	±
4	>2560	1	+	+	−	−
5	>2560	2	±	−	+	±
6	>2560	2	±	−	+	±
7	1280	2	±	−	±	−
8	>2560	1	+	+	−	−
9	1280	2	±	−	+	±
10	>2560	1	+	−	−	−
11	ND	NT	−	−	−	−
12	ND	NT	−	−	−	−

[a]*Serum number corresponds to the lane number of* ***Figure 5B****.* [b]*Antibody titer was measured with Serodia Myco II kit (Fujirebio, Tokyo, Japan); ND, not determined.* [c]*Detection and typing of the p1 gene was performed by the nested PCR method as a part of previous study (Kenri et al., 2008); NT, not tested.* [d]*Five to ten colonies of M. pneumoniae M129 (type 1) or FH (type 2) strains were formed on PPLO agar cast in a 24-well microplate (1.5 ml PPLO agar per well). Colony-forming wells were soaked with 1 ml of phosphate-buffered saline (PBS) for 5 min at room temperature. After removal of PBS, 0.1 ml of diluted patient serum (fivefold or 10-fold dilution by PBS) was added to the well and incubated for 20 min at 37°C. After removal of the patient serum, 0.5 ml of a diluted sheep red blood cell (RBC) suspension (100-fold dilution by PBS) was added to the well and incubated for 20 min at 37°C. After incubation, excess RBCs were removed by washing with 1 ml of PBS three times. The state of hemadsorption of M. pneumoniae colonies was evaluated by microscopic observation. +: complete hemadsorption inhibition (no adsorption of RBCs was observed); ±: partial inhibition (partial adsorption of RBCs was observed); –: no inhibition (colonies were fully covered by RBCs; Kenri et al., 2006b).*

2006a,b). Indeed, some anti-P1 antibodies have been reported to show HA inhibitory activity (Krause and Baseman, 1983; Jacobs et al., 1989; Gerstenecker and Jacobs, 1990); however, it is not clear whether the type-specific anti-P1 antibodies detected in the western blotting in **Figure 5B** play a role in determining the type-specific HA inhibitory activity. It is possible that antibodies against another type-specific antigen are responsible for the observed type-specific HA inhibitory activity. If these type-specific HA inhibitory activities of patient sera serve as protective immunity for *M. pneumoniae* infection, this might explain the type shift phenomenon of *M. pneumoniae* isolates.

EMERGENCE OF MACROLIDE-RESISTANT *M. pneumoniae* (MRMP)

It has been known at least since the 1970s that *M. pneumoniae* can acquire resistance against macrolides relatively easily in laboratory culture (Niitu et al., 1970; Nitu et al., 1974; Lucier et al., 1995; Pereyre et al., 2004). There are also some early reports of MRMP clinical isolates (Stopler et al., 1980; Clara et al., 1989). However, MRMPs rapidly and broadly spread after 2000, especially in eastern Asian countries such as China, Korea, and Japan (Okazaki et al., 2001; Matsuoka et al., 2004; Morozumi et al., 2005, 2008; Cao et al., 2010; Bebear et al., 2011; Hong et al., 2013; Zhao et al., 2013b, 2014). The frequencies of MRMP detection are now increasing in other areas of the world (Pereyre et al., 2007; Li et al., 2009; Peuchant et al., 2009; Dumke et al., 2010a; Averbuch et al., 2011; Chironna et al., 2011; Yamada et al., 2012; Eshaghi et al., 2013; Tsai et al., 2013; Wu et al., 2013; Caballero Jde et al., 2014; Saraya et al., 2014; Diaz et al., 2015; Zheng et al., 2015). MRMPs are isolated more frequently from adolescent and pediatric patients than from adults, which is likely related to the frequent use of macrolides for treatments of mycoplasmal infections at younger ages. However, isolation of MRMPs from adult patients is also on the rise (Isozumi et al., 2009; Miyashita et al., 2012; Yoo et al., 2012; Hanada et al., 2014; Zhou et al., 2015). Most of the MRMPs isolated in Japan carry the A2063G mutation in domain V of the 23S rRNA gene that confers strong resistance to 14- and 15-membered macrolides and lincosamides. However, there is a report of a local outbreak caused by MRMPs carrying the A2063T mutation, which exhibits only moderate resistance to macrolides (Suzuki et al., 2013). The most recent estimate of the isolation rate of MRMPs from adolescent and pediatric patients in Japan is 50–90%, depending on the area (Morozumi et al., 2008; Akaike et al., 2012; Miyashita et al., 2012; Kawai et al., 2013b; Ishiguro et al., 2015). Given this situation, the Japan Pediatric Society[3] and The Japanese Society for Mycoplasmology[4] have issued guiding principles for treating *M. pneumoniae* pneumonia (The committee on the guiding principle for treatment of *Mycoplasma pneumoniae* pneumonia, 2014).

[3]http://www.jpeds.or.jp/uploads/files/saisin_130219_2.pdf

[4]http://square.umin.ac.jp/jsm/shisin.pdf

THERAPEUTIC STRATEGIES FOR *M. pneumoniae* PNEUMONIA

Mycoplasma pneumoniae is generally susceptible to macrolides, tetracyclines, and the new quinolone antibiotics. However, as stated above, the emergence of MRMP since 2000 has made the treatment of *M. pneumoniae* pneumonia challenging. Although MRMP strains have been reported in European countries and in the United States, the detection rates from these countries are lower than those in East Asia, including Japan. Caution in monitoring and treating MRMP strains and the necessity of continuous surveillance for these strains are partially described in the guidelines of the Infectious Diseases Society of America (IDSA), American Thoracic Society (ATS; Mandell et al., 2007), European Respiratory Society, European Society for Clinical Microbiology and Infectious Diseases for adults (Woodhead et al., 2011), and in the Pediatric Infectious Diseases Society (PIDS) and IDSA for children (Bradley et al., 2011). However, the necessity and strategies of alternative antibiotic treatment for MRMP strains have not been described in detail. The detection rates of MRNPs are associated with age (Miyashita et al., 2013): detection rates are higher in children aged $\leq$15 years than in adults. In addition, the detection rates among adults are higher in adolescents aged 16–19 years than in those aged $\geq$20 years.

The guidelines of the IDSA and ATS recommend macrolides or tetracyclines as the first-line drugs for *M. pneumoniae* pneumonia, and fluoroquinolones as the second-line drugs in adults. The guidelines of the PIDS and IDSA for children recommend azithromycin as the first-line oral drug, and clarithromycin, erythromycin, or doxycycline (for patients aged $\geq$8 years) along with levofloxacin or moxifloxacin (for adolescent patients) as the second-line oral drugs, for mild cases. For treatment via injection, azithromycin is recommended as the first-line drug (although this is not indicated for children in Japan), and erythromycin and levofloxacin (also not indicated for children in Japan) are recommended as the second-line drugs.

Thus, the applicability of antibiotics differs between adults and children; in addition, the indication for antibiotics for children is different between Japan and other countries. In view of these points, the therapeutic guiding principles[3,4] for *M. pneumoniae* pneumonia issued in Japan are reviewed in the following sections.

Treatment in Adults: Recommendations by the Japanese Society of Mycoplasmology

The first-line drugs for *M. pneumoniae* pneumonia in adults are macrolide antibiotics. Oral administration of clarithromycin (400 mg/day administered in two divided doses) or erythromycin (800–1,200 mg/day administered in 4–6 divided doses for

7–10 days) is recommended for patients on an outpatient basis. Oral azithromycin administered at 500 mg once a day for 3 days or at 2 g once a day for 1 day is also indicated (**Table 3**). The minimum inhibitory concentration (MIC) values of macrolides for *M. pneumoniae* without macrolide resistance genes are extremely low, while those of fluoroquinolones and tetracyclines are higher (Akaike et al., 2012). Minocycline, levofloxacin, garenoxacin, moxifloxacin, sitafloxacin, and tosufloxacin are recommended as second-line drugs (**Table 3**). Intravenous administration is indicated for inpatients: minocycline, azithromycin, and erythromycin are recommended as first-line drugs, and levofloxacin, ciprofloxacin, and pazufloxacin are recommended as the second-line drugs (**Table 4**).

There is concern surrounding the emergence of quinolone resistance in *Streptococcus pneumoniae* and other respiratory pathogenic bacteria owing to the use of quinolones as the initial treatment for pneumonia. Quinolones have been shown capable of inducing resistance in *M. pneumoniae* strains *in vitro* (Gruson et al., 2005). Therefore, quinolones should be avoided during the initial treatment in young patients with suspected *M. pneumoniae* pneumonia. *M. pneumoniae* pneumonia is less common in the elderly than in the younger generation. Quinolones are prescribed for elderly patients with pneumonia, because microorganisms other than *M. pneumoniae* are typically involved. On the other hand, tetracyclines are not likely to induce resistance in *M. pneumoniae*. Intravenous administration of tetracycline is recommended as the first-line treatment for hospitalized patients (**Table 4**). Although there is no sufficient evidence regarding the duration of antibiotic administration for the treatment of *M. pneumoniae* pneumonia in adults, the Japanese Society of Mycoplasmology recommends a duration of 7–10 days.

When the fever does not subside within 48–72 h after the administration of macrolides, the antibiotics should be changed considering the possibility of MRMP involvement. At present, MRMP is susceptible to both tetracyclines and fluoroquinolones. A study of the treatment for infection with MRMP in children demonstrated that minocycline showed a higher rate of bacterial elimination than tosufloxacin, and the fever subsided faster upon treatment with minocycline than with tosufloxacin (Kawai et al., 2013a).

Considering the emergence of quinolone resistance, the first-line drug recommended for MRMP pneumonia is minocycline (**Table 5**). Oral minocycline administered at 200 mg/day in two divided doses on an outpatient basis, and intravenous minocycline administered at 100 mg twice a day for inpatients is recommended. Quinolones are recommended as second-line drugs for both outpatients and inpatients (**Tables 5** and **6**). For outpatient treatment, levofloxacin, garenoxacin, moxifloxacin, sitafloxacin, or tosufloxacin is orally administered (**Tables 3** and **5**). For inpatient treatment, levofloxacin, ciprofloxacin, or pazufloxacin is intravenously administered (**Tables 4** and **6**). The duration of antibiotic treatment is 7–10 days, similar to that for infection with macrolide-sensitive *M. pneumoniae*.

In severe cases of *M. pneumoniae* pneumonia involving respiratory failure, methylprednisolone is administered at 500–1,000 mg/day for 3–5 days concomitantly with an appropriate antimycoplasmal drug. It should be noted that the relationship between MRMP and severe *M. pneumoniae* infection remains unclear.

TABLE 3 | Recommended treatments for adult outpatients of *M. pneumoniae* pneumonia.

	Drug	Route of administration	mg/dose	Dose/day
First-line drug	Clarithromycin (CAM)	Oral	200	2
	Azithromycin (AZM) (Slow-release formulation)	Oral	2000	1 (1 day)
	Azithromycin (AZM)	Oral	500	1 (3 days)
	Erythromycin (EM)	Oral	200	4–6
Second-line drug	Minocycline (MINO)	Oral	100	2
	Levofloxacin (LVFX)	Oral	500	1
	Garenoxacin (GRNX)	Oral	400	1
	Moxifloxacin (MFLX)	Oral	400	1
	Sitafloxacin (STFX)	Oral	100	2
		Oral	200	1
	Tosufloxacin (TFLX)	Oral	150	2–3

The table is adapted from the guiding principle (Chiryo–Shishin) issued by the Japanese Society of Mycoplasmology (JSM). http://square.umin.ac.jp/jsm/shisin.pdf.

TABLE 4 | Recommended treatments for adult inpatients of *M. pneumoniae* pneumonia.

	Drug	Route of administration	mg/dose	Dose/day
First-line drug	Minocycline (MINO)	Intravenous (drip infusion)	100	2
	Azithromycin (AZM)	Intravenous (drip infusion)	500	1
	Erythromycin (EM)	Intravenous (drip infusion)	300–500	2–3
Second-line drug	Levofloxacin (LVFX)	Intravenous (drip infusion)	500	1
	Ciprofloxacin (CPFX)	Intravenous (drip infusion)	300	2
	Pazufloxacin (PZFX)	Intravenous (drip infusion)	500–1000	2

The table is adapted from the guiding principle of JSM (http://square.umin.ac.jp/jsm/shisin.pdf).

Treatment in Children: Recommendations by the Japanese Society of Pediatric Pulmonology, Japanese Society for Pediatric Infectious Diseases, Japan Pediatric Society, and Japanese Society of Mycoplasmology

Macrolides are also recommended as the first-line drugs for *M. pneumoniae* pneumonia in children. The incidence of MRMP isolates from pediatric cases varies both regionally and epidemically in Japan, ranging from <50% in patients without a history of macrolide treatment to >90% in patients that were treated by macrolides without clinical effectiveness. The MIC

TABLE 5 | Recommended treatments for adult outpatients of macrolide-resistant *M. pneumoniae* pneumonia.

	Drug	Route of administration	mg/dose	Dose/day
First-line drug	Minocycline (MINO)	Oral	100	2
Second-line drug	Levofloxacin (LVFX)	Oral	500	1
	Garenoxacin (GRNX)	Oral	400	1
	Moxifloxacin (MFLX)	Oral	400	1
	Sitafloxacin (STFX)	Oral	100	2
			200	1
	Tosufloxacin (TFLX)	Oral	150	2–3

The table is adapted from the guiding principle of JSM (http://square.umin.ac.jp/jsm/shisin.pdf).

TABLE 6 | Recommended treatments for adult inpatients of macrolide-resistant *M. pneumoniae* pneumonia.

	Drug	Route of administration	mg/dose	Dose/day
First-line drug	Minocycline (MINO)	Intravenous (drip infusion)	100	2
Second-line drug	Levofloxacin (LVFX)	Intravenous (drip infusion)	500	1
	Ciprofloxacin (CPFX)	Intravenous (drip infusion)	300	2
	Pazufloxacin (PZFX)	Intravenous (drip infusion)	500–1000	2

The table is adapted from the guiding principle of JSM (http://square.umin.ac.jp/jsm/shisin.pdf).

values of macrolides for macrolide-sensitive *M. pneumoniae* are extremely low, and the rates of bacterial elimination are high. The MIC values of tetracyclines and tosufloxacin for macrolide-sensitive *M. pneumoniae* are relatively higher than those of macrolides. In addition, *M. pneumoniae* may not be eliminated after treatment with these drugs. Tosufloxacin is the only fluoroquinolone approved for children in Japan (**Table 7**).

In children with pneumonia caused by macrolide-sensitive *M. pneumoniae*, treatment with macrolides has been shown to alleviate the fever within 48 h in more than 80% of cases. On the other hand, fever persists in approximately 70% of children with MRMP infection. These data indicate that the clinical effectiveness of macrolides should be evaluated on the basis of the presence of fever at 48–72 h after treatment. Persistent fever suggests the possibility of infection with macrolide-resistant strains or mixed infection with microorganisms other than *M. pneumoniae*. For cases of *M. pneumoniae* pneumonia with failure of macrolide treatment, either tosufloxacin or tetracyclines are applicable. Quinolones might induce drug resistance in the microflora other than *M. pneumoniae*, and should be administered with caution. Tetracyclines may cause side effects such as transient anostosis, staining of the teeth, and enamel hypoplasia. Tetracyclines are contraindicated in children aged under 8 years. The recommended duration of treatment for *M. pneumoniae* pneumonia caused by macrolide-sensitive strains is 14 days with erythromycin, 10 days with clarithromycin, and 3 days with azithromycin. Treatment with tosufloxacin or tetracyclines for pneumonia caused by

TABLE 7 | Recommended treatments for pediatric patients of *M. pneumoniae* pneumonia.

Drug	Route of administration	Drug dose (mg/kg/day)	Divided dose/day	Treatment period (days)
Erythromycin (EM)	Oral	25–50	4–6	14
Clarithromycin (CAM)	Oral	10–15	2–3	10
Azithromycin (AZM)	Oral	10	1	3
Tosufloxacin (TFLX)	Oral	12	2	7–14
Minocycline (MINO)	Oral or intravenous drip infusion	2–4	2	7–14

The table is adapted from the guiding principle of JSM (http://square.umin.ac.jp/jsm/shisin.pdf).

MRMPs should be administered for 7–14 days. Systemic administration of steroids for severe pneumonia should be considered; however, the indications of systemic steroid use are not yet determined. One study showed that steroid therapy is effective in patients with fever lasting >7 days, with a serum lactate dehydrogenase level of ≥480 IU/L (Oishi et al., 2011).

CONCLUSION

The nationwide surveillance of *M. pneumoniae* pneumonia in Japan is based on reports collected from approximately 500 sentinel hospitals, and is thus not reflective of the total number of patients. However, this weekly monitoring can detect previous epidemics and patterns, and has played a significant role as an alert system for medical and public health workers as well as researchers. Therefore, the effort for this surveillance program should be continued.

There are two distinct genetic lineages of *M. pneumoniae* that exhibit polymorphism in the cytadhesin P1 protein sequence. However, the involvements of these two lineages in pneumonia epidemics and differences in their pathogenicity are not yet fully understood. Nevertheless, cytadhesin P1 is an important factor that plays a critical role in the infection mechanism of this pathogen and in the interactions with host cells. We believe that *p1* gene typing is an important aspect for molecular epidemiological studies of *M. pneumoniae* and should be performed by combining modern genotyping methods based on MLVA, multi-locus sequence typing, and/or whole-genome SNP strategies.

Emergence of MRMP is serious problem for the treatment of *M. pneumoniae* pneumonia. Given this situation, several Japanese academic and medical societies have issued specific guiding principles for treatment of *M. pneumoniae* pneumonia. Macrolides are still recommended as the first-line drug in children and adults. However, if the fever does not subside in 48–72 h from first-line drug administration, a change of antibiotics to second-line drugs (i.e., fluoroquinolones and tetracyclines) is recommended.

AUTHOR CONTRIBUTIONS

TY wrote the therapeutic strategy section of the paper. TK wrote the surveillance and epidemiology sections of the paper.

FUNDING

This work was supported by a grant (H26-Kokui-Shitei-001) from the Ministry of Health, Labor, and Welfare of Japan and a Grant-in Aid for Scientific Research on Innovative Areas (grant 25117530 and 15H01337) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

ACKNOWLEDGMENT

The authors are grateful to Dr. Mitsuo Narita of Sapporo Tokushukai Hospital for reading the manuscript and helpful suggestions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 February 2016
doi: 10.3389/fmicb.2016.00157

Mycoplasma pneumoniae Epidemiology in England and Wales: A National Perspective

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Investigations of patients with suspected *Mycoplasma pneumoniae* infection have been undertaken in England since the early 1970s. *M. pneumoniae* is a respiratory pathogen that is a common cause of pneumonia and may cause serious sequelae such as encephalitis and has been documented in children with persistent cough. The pathogen is found in all age groups, with higher prevalence in children aged 5–14 years. In England, recurrent epidemic periods have occurred at ∼4-yearly intervals. In addition, low-level sporadic infection occurs with seasonal peaks from December to February. Voluntarily reports from regional laboratories and hospitals in England from 1975 to 2015 were collated by Public Health England for epidemiological analysis. Further data pertaining cases of note and specimens submitted to Public Health England from 2005 to 2015 for confirmation, molecular typing is included.

Keywords: *Mycoplasma pneumoniae*, epidemiology, England, wales, microbiology

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *04 December 2015*
Accepted: *29 January 2016*
Published: *16 February 2016*

Citation:
Brown RJ, Nguipdop-Djomo P, Zhao H, Stanford E, Spiller OB and Chalker VJ (2016) Mycoplasma pneumoniae Epidemiology in England and Wales: A National Perspective. Front. Microbiol. 7:157. doi: 10.3389/fmicb.2016.00157

INTRODUCTION

Mycoplasma pneumoniae is a respiratory bacterial pathogen causing upper and lower respiratory disease in humans of all ages. It is a major cause of community-acquired pneumonia (CAP) and is considered to be responsible for 15–20% of CAP cases in adults and up to 40% of cases in children, especially in children of school age (Foy, 1993; Korppi et al., 2004; Dumke et al., 2012). Up to 25% of *M. pneumoniae* infections may manifest as extra-pulmonary sequelae after the onset of or in some cases in the absence of respiratory illness (Cassell and Cole, 1981; Narita, 2010). Encephalitis is one of the most severe complications (Narita, 2009; Meyer Sauteur et al., 2014b) estimated in 5–10% of pediatric encephalitis patients (Bitnun et al., 2001; Christie et al., 2007) of which up to 60% of have additional neurologic sequelae (Bitnun et al., 2001, 2003). *M. pneumoniae* infection can result in dermatological manifestations including Stevens-Johnson syndrome (Olson et al., 2015). Hemolytic anemia is a rare but serious complication of *M. pneumoniae* infection and is more frequent children than in adults (Gu et al., 2014). *M. pneumoniae* infections occur both endemically and epidemically worldwide, with epidemic peaks every 4–7 years (Chalker et al., 2011a, 2012a; Jacobs, 2012). Typical outbreaks of *M. pneumoniae* infection occur in areas of close personal contact for example, schools and military barracks. Both symptomatic and asymptomatic individuals with *M. pneumoniae* carry the organism in the respiratory tract and it can be transmitted from person to person via aerosols and cough (Clyde, 1979; Waites and Talkington, 2004; Meyer Sauteur et al., 2014c). Long-term morbidity due to *M. pneumoniae* infection is uncommon however; the acute illness is often disruptive and can consume significant resources (Waites and Talkington, 2004).

In England and Wales (EW), seasonal peaks of infection are detected from December to February each year with epidemics at ∼4-yearly intervals (Chalker et al., 2011b, 2015). Cyclical patterns, as observed in EW, are also seen in Denmark, Sweden, Norway, Finland, Korea, and Japan (Ito et al., 2001; Rasmussen et al., 2010; Blystad et al., 2012; Linde et al., 2012; Polkowska et al., 2012; Kim et al., 2015). It has recently been suggested that minor variations in the duration of immunity may be essential to the cyclic epidemic peaks (Omori et al., 2015). A con-current increase in reported *M. pneumoniae* cases was documented in several European countries in 2011 (Lenglet et al., 2012) and in EW the most recent increase has been noted in 2015 (this study).

The recent global increase in macrolide resistance observed in cases of *M. pneumoniae* infection is of increasing concern and importance to the international community (Bébéar, 2012). In China resistance has been documents in over 90% of clinical isolates of *M. pneumoniae* studied (Zhao et al., 2013) however resistance is lower in European counties including France, Germany, Switzerland, and Sweden (Peuchant et al., 2009; Meyer Sauteur et al., 2014a; Nilsson et al., 2014; Dumke et al., 2015). Macrolides are currently recommended as the first-line treatment for *M. pneumoniae* infection in the UK (Harris et al., 2011). The 2011 British Thoracic Society guidelines for the management of CAP in children and adults suggest empirical macrolide treatment at any age if there is no response to first-line β-lactam antibiotics (which are ineffective against cell wall-less bacteria such as *M. pneumoniae*) or in the case of very severe disease (Lim et al., 2009; Harris et al., 2011). Macrolide-resistance in EW has recently been documented at 9.3% and is therefore not included in this article (Brown et al., 2015a). This is considerably lower than in Scotland (19%) (Ferguson et al., 2013).

This study aims to provide up to date overview of the number of laboratory reports and incidence of *M. pneumoniae* infection in EW, molecular typing data, and briefly highlight cases of note in recent years.

MATERIALS AND METHODS

A total of 16,878 serological, culture, genomic, and unspecified laboratory diagnostic methodology *M. pneumoniae* positive cases reported to Public Health England, via the Communicable Disease Report Network comprising ∼250 laboratories from January 1989 to June 2015 were aggregated into 3 weekly periods. These report the organisms identified from specimens (e.g., throat swabs, serum, or sputum) submitted by general practitioners and hospitals with the patient's age and sex, the reporting laboratory, and date of the first sample; the system has changed little over time. Duplicate specimens were removed and reports plotted to examine the general pattern (3-weekly moving average). National reporting categories include antibody detection and antibody-detection rising titre. A rising titre is defined as a four-fold increase in detectable anti-*M. pneumoniae* antibody level. Rising titres are not demonstrated for all patients as it is not always possible to obtain a second specimen. It is possible that a fraction of cases reported as antibody detection only include some cases of rising titre that have not been appropriately coded. A distinction between IgA, IgG and IgM cannot be made when collating figures, however the number of cases with rising titre demonstrating active infection mirror the overall total case pattern of epidemic periodicity. A total of 39,758 laboratory reports of *M. pneumoniae* infections in England and Wales from January 1975 to June 2009 previously examined indicated that cyclic epidemics occurred every 4 years, were synchronous across all regions in the country, and occurred during the winter (Nguipdop-Djomo et al., 2013). Epidemic periods were defined as a clear increase in cases resulting in more than 20 cases in a 3 weekly average rolling period (**Figure 1**). From this dataset, we computed average age specific incidence for epidemic and non-epidemic periods using the England and Wales (EW) population censuses of 1981, and 2001 for the denominator for the periods 1975–1988 and 1998–2009 respectively. Data from 1989 to 1997 were excluded from age-specific analyses because age was missing in ∼90% records during that period. Age distribution incidence rates from 2010 to June 2015 were calculated using the Office for National Statistics (ONS) mid-year population estimates for EW.

Diagnostic methodology of choice has altered with time in EW, with the decline in culture and use of the complement fixation test being superseded by enzyme immunosorbent assays and the introduction of molecular testing. To ascertain the proportion of reports now obtained using molecular methods, differing methodologies in use with time was examined from 1989 to 2015 (**Figure 2**). Molecular typing of *M. pneumoniae* positive clinical specimens and isolates was undertaken using MLST (50) (Brown et al., 2015b), MLVA (156) (Chalker et al., 2015), and P1 type (84) determinations (Dumke et al., 2006) from 1977 to 2011 (**Figures 3–5**). Referred cases to the Bacteriology Reference Department, Public Health England from 2005 to 2015 were examined for cases with unusual or severe presentation.

RESULTS

From January 1989 to June 2015 seven epidemics of *M. pneumoniae* were noted of declining amplitude with recent peak in 2015 (**Figure 1**). For some epidemic periods clear annual fluctuations can also be seen apparent as a double peak over two winter seasons. The clarity of epidemic periods have in recent years declined with less reported cases overall. From 1975 to 2009 incidence was found to be similar by gender, both during epidemic and inter-epidemic periods. The annual notification rate in 2010–2015 was consistently highest in those aged 15–44 years, detailed in **Table 1**. The use of culture has declined in recent years and despite serology being the most commonly used methodology the implementation and increased use of molecular methods has resulted in a proportional increase in reports based on molecular tests from 0.32% (3/936 95%CI 0.06–0.98) in 2010 to 28.5% (95/333 95%CI 24.0–33.6) in the first 6 months of 2015 (**Figure 2**). Molecular typing data were grouped into 4-yearly

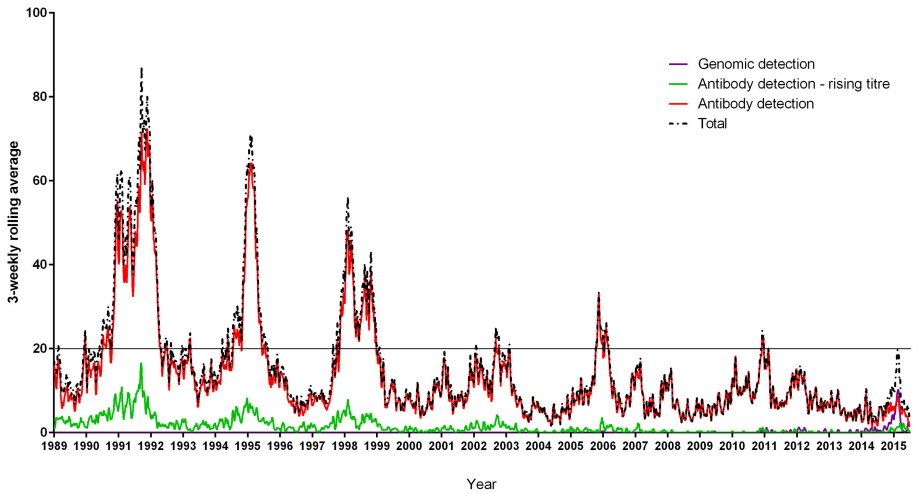

FIGURE 1 | Laboratory reports of *Mycoplasma pneumoniae* infection detection by genomic and serological methods in England and Wales from January 1989 to June 2015. The line at 20 cases per 3 weekly average rolling period defines seven epidemic periods of declining magnitude and clarity, lasting up to 2 years (1991–1992, 1994–1995, 1998–1999, 2001–2003, 2005–2006, 2011, 2015). National reporting categories include antibody detection and antibody-detection rising titre. A rising titre is defined as a four-fold increase in detectable anti-*Mycoplasma pneumoniae* antibody level.

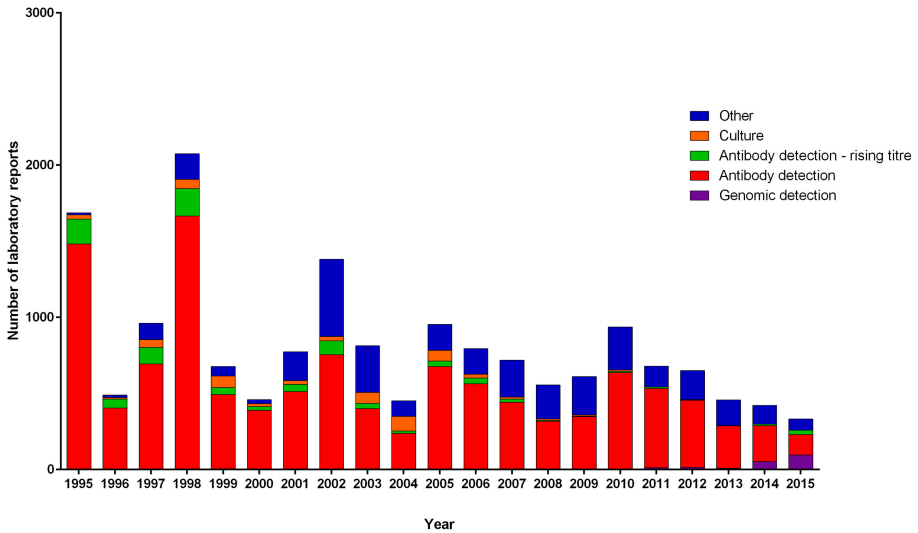

FIGURE 2 | Number of laboratory reports per year from January 1995 to June 2015 separated by detection methodology. National reporting categories included are: antibody detection and antibody-detection rising titre. A rising titre is defined as a four-fold increase in detectable anti-*Mycoplasma pneumoniae* antibody level (methods not specified). Other indicates specimens for which *M. pneumoniae* infection was determined using antigen detection (method not specified), microscopy and unknown categories. Culture indicates cases from which specimens yielded isolates of *M. pneumoniae* and genomic detection those for which DNA of *M. pneumoniae* was detected by PCR.

intervals, representing the epidemic cycles observed in the UK. Multiple MLST, MLVA, and P1 types were observed in each 4-yearly interval (3–5) however a predominance of P1 type 1 can be seen for all intervals except 1981–1984 where equal numbers of P1 type 1 and type 2 strains were observed. This data is limited by low sample number in 4-yearly intervals, therefore the variation in P1 types observed is likely to be an underestimate of the actual *M. pneumoniae* population present.

Cases of Note: 2005–2015

From January 2005 to June 2015 eleven cases were referred to the Bacterial Reference Department, Public Health England that were identified as positive for *M. pneumoniae* that were of particular note. The majority of cases were patients with lower respiratory tract infection. Stevens-Johnson syndrome is an immune-mediated hypersensitivity complex typically involving the skin and mucous membranes. Two cases of Stevens-Johnsons

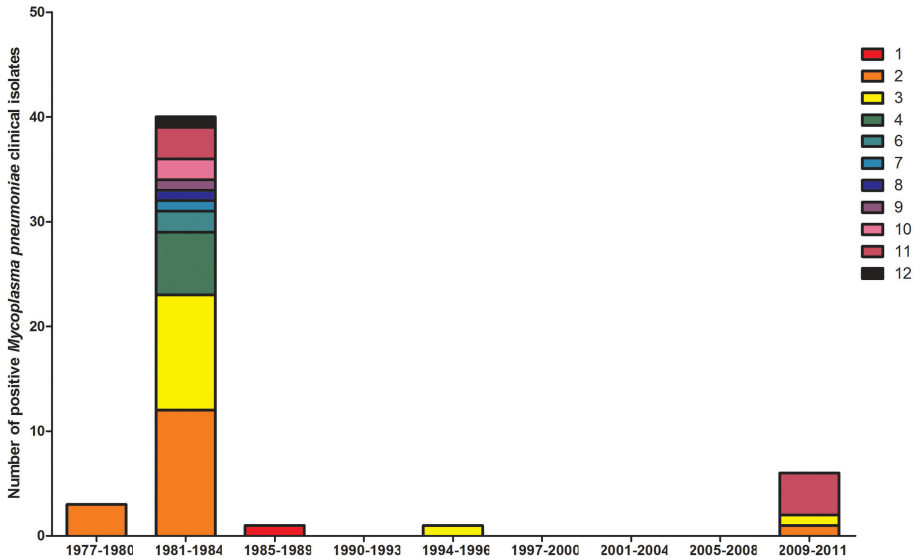

FIGURE 3 | Distribution of MLST sequence types for 57 *M. pneumoniae* clinical isolates in the 4-yearly epidemic cycles observed in the UK. Year groups indicative of epidemic periods are listed on the *x*-axis. Sequence types (ST) 1–12 are listed in the key and indicated with differing colors. Allelic profiles are available on http://pubmlst.org/mpneumoniae.

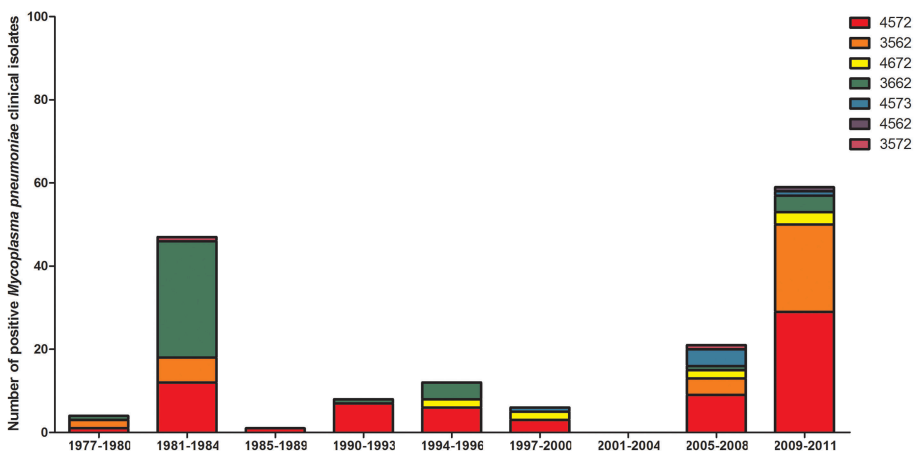

FIGURE 4 | Distribution of MLVA types in positive *M. pneumoniae* clinical specimens/isolates collated into the 4-yearly epidemic cycles observed in the UK. Year groups indicative of epidemic periods are listed on the *x*-axis. MLVA profiles are listed in the key and indicated with differing colors.

syndrome were noted in 2009 and 2010 in male children aged 8 and 6 respectively. Two cases were noted in respiratory specimens in immunocompromised patients following extra-pulmonary organ transplantation (2013 and 2015). Infection in donor transplant patient respiratory secretions was also noted in 2015. *M. pneumoniae* was detected by qPCR in the nasopharyngeal aspirate but not the cerebral spinal fluid (CSF) of a patient with pneumonia and reactive transverse myelitis in a child in 2005, and in the bronchoalveolar lavage of a child with encephalitis and seizures in 2011. In 2011 a young adult patient presented post respiratory tract infection with encephalitis and transverse myelitis that progressed to tetraplegia with ventilator dependency. *M. pneumoniae* was confirmed by qPCR on throat swab specimens taken 19 and 21 days post onset but was not detected in concurrent CSF specimens (Chalker et al., 2012b). Detection of *M. pneumoniae* in CSF is unusual and it is postulated that neurological manifestation of *M. pneumoniae* infection is antibody mediated rather than by direct presence of the bacteria itself (Waites and Talkington, 2004). Of 68 CSF specimens referred only 1 positive case was detected in 2010, in a child with a ventriculoperitoneal shunt, in which contamination of the CSF during sampling could not be excluded. In 2012 *M. pneumoniae* was detected by qPCR in the lung tissue of *two* co-habiting adults that both suffered sudden fatal collapse. This was presumed a secondary infection as one of the two patients also had confirmed *Staphylococcus aureus* infection.

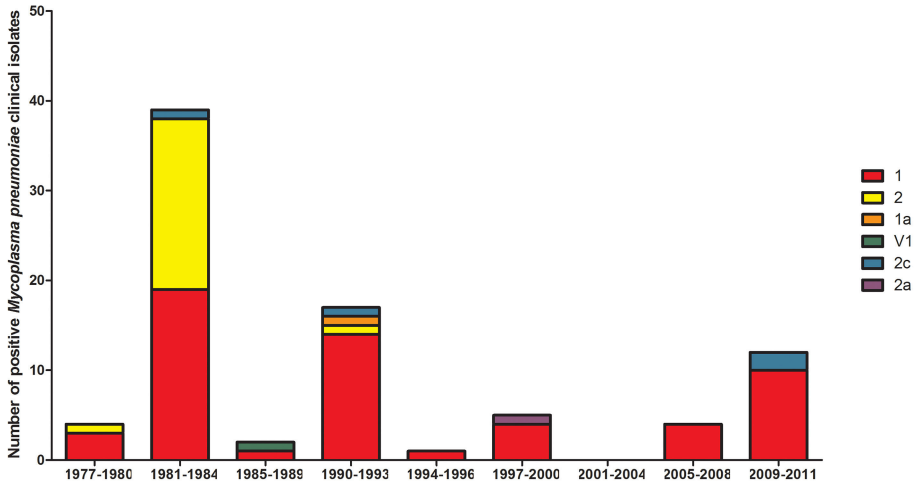

FIGURE 5 | Distribution of P1 type in 4-yearly epidemic cycles observed in the UK for positive *M. pneumoniae* clinical specimens/isolates. Year groups indicative of epidemic periods are listed on the *x*-axis. P1 types are listed in the key and indicated with differing colors.

TABLE 1 | Incidence of *M. pneumoniae* positive laboratory reports per million persons.

	Year	Incidence (per million persons in overall population)								
		0–4 years	5–9 years	10–14 years	15–24 years	25–44 years	45–64 years	65+ years	Unknown	Total
Serology + PCR	2010	1.17	1.35	0.99	1.40	2.09	1.44	0.61	0.00	9.05
	2011	1.14	1.83	1.16	1.51	2.40	1.28	0.87	0.02	10.21
	2012	1.45	1.47	1.15	1.45	2.84	2.05	1.22	0.00	11.63
	2013	0.61	0.95	0.74	0.93	1.77	1.82	1.42	0.00	8.25
	2014	0.82	0.78	0.47	0.89	2.09	1.10	1.31	0.02	7.47
	2015*	1.78	1.22	0.70	1.15	3.07	1.88	1.32	0.00	11.11
	Average	1.16	1.27	0.87	1.22	2.38	1.60	1.13	0.01	9.62

**Data for 2015 from January to June and rates adjusted for half-year data were collected.*

DISCUSSION

Laboratory reports show that cyclic epidemics of *M. pneumoniae* infections in EW recur every 4 years on average, concurrent with annual seasonal fluctuations with incidence peaking and dipping in the winter and summer respectively. The reduction in clarity and magnitude of epidemic periods in recent years could be indicative of a genuine reduction in cases, increasing population pulmonary health, or reflect the changing nature of testing strategies moving away from techniques such as complement fixation. Overall incidence, although declining over the period 1989–2015, has remained static since 2010 and age-specific differences in epidemic period incidence were noted for the limited periods studied. Annual notification rate in 2010–2015 was highest in 15–44 year olds perhaps reflecting reliance on serological confirmation or infection. Globally, epidemics of *M. pneumoniae* are considered to occur every 3–7 years, however recent epidemiological studies have documented varying trends in epidemic patterns. Serological studies performed in Denmark showed a pattern of *M. pneumoniae* infections over a 50 year period from 1946 through 1995 with endemic disease transmission punctuated with cyclic epidemics every 3–5 years (Lind et al., 1997). In Jerusalem, historically, epidemics were observed every 3–5 years with seasonal peaks in October and early spring; however, since autumn 2014 a constant rate of infection has been observed, diverging from the historical pattern (Nir-Paz et al., 2012). Indeed, similar to the data for EW; 3-yearly cyclic epidemic periods with declining magnitude have been documented in Japan from 1979 to 1999 (Ito et al., 2001).

Speculations regarding the mechanisms driving fluctuations in population incidence of *M. pneumoniae* infections have included decline in immunity or increase of the immunologically naive population level (Chalker et al., 2011a) or shifts in the proportion of individual strains with specific P1 type or concurrent increased incidence of several strains. Additionally, it is believed that the genotype of *M. pneumoniae* may be changing, generating diverse genetic material in each epidemic with a study reporting the detection of polyclonal strains in a single epidemic (Pereyre et al., 2012). Recent modeling of epidemic

peaks has suggested that fluctuations may be attributable to minor variations in the duration of immunity at the population level (Omori et al., 2015). Speculation that a shift in P1 adhesin type may be the cause of epidemics has been disputed with evidence indicating the presence of multiple P1 adhesin types in observed increases of infection (Sasaki et al., 1996; Dégrange et al., 2009; Pereyre et al., 2012). It was hypothesized that a decline in immunity or an increase of the immunologically naïve population may result in the 4-year cycle of epidemic periods (Chalker et al., 2011a). In other geographical locations, it has also been observed that multiple P1 types can be detected during outbreaks, and it has been suggested that although immunological pressure may favor shifts of P1 type, a co-circulation of P1 types appears to be common (Nilsson et al., 2010; Dumke et al., 2015). This is further supported by the presence of multiple MLST types within specimens in EW, reflecting the concurrent presence of strains of varied genetic lineage. As expected an increase in molecular detection of infection is noted, with declining use of culture. Macrolide resistance has recently been documented at 9.3% found in adult patients only (Brown et al., 2015a) and is also of concern in children in other countries (Meyer Sauteur et al., 2014a). However, this was derived from results of specimens submitted to the reference laboratory which may be biased toward those developing resistance during treatment and one patient was documented to have received macrolides prior to sampling therefore this level may be an over-representation to the actual level in the community.

Extrapulmonary complications of *M. pneumoniae* infection can arise involving the skin and the nervous, cardiovascular, renal, gastrointestinal, musculoskeletal, and hematologic systems. The presence of *M. pneumoniae* in these extrapulmonary sites has been confirmed by PCR as well as culture (Koletsky and Weinstein, 1980; Kasahara et al., 1985; Narita et al., 1996; Saïd et al., 1999; Bar Meir et al., 2000). Complications that occur within the central nervous system (CNS) are recognized as the most common extrapulmonary manifestations of *M. pneumoniae* infections. A recent study of 1988 children with encephalitis showed *M. pneumoniae* as the most common causative agent (Bebear and Robertson, 1996). It is thought that the host immune response that develops after *M. pneumoniae* infection contributes to these complications as well as contributing to autoimmunity (Waites and Talkington, 2004). The mechanisms that result in these neurological manifestations of *M. pneumoniae* infection are not completely understood however, immune-mediated mechanism are suspected due to the development of cross-reactive antibodies to the brain and other neurological structures (Waites et al., 2008). PCR testing of 68 CSF specimens in EW resulted in detection of *M. pneumoniae* DNA in a single specimen in which contamination during sampling could not be excluded. Consideration to testing of CSF specimens in tandem with paired respiratory specimens should be given as in practice detection of *M. pneumoniae* in CSF is extremely rare and positive patients may be detected using pulmonary specimens.

In summary, epidemics of *M pneumoniae* infections recur every 4 years on average in EW affecting all age groups, predominantly children and adults <44 years of age. Macrolide resistance has recently been documented at 9.3% and extrapulmonary complications can be severe.

AUTHOR CONTRIBUTIONS

RB worte the manuscript, PN, HZ, and ES undertook epidemiological data, OS and VC oversaw the study and wrote the manuscript.

FUNDING

RB studentship was funded by Cardiff University and Public Health England, all other works was funded by Public Health England.

ACKNOWLEDGMENTS

The authors are grateful to P E M Fine and E Vynnycky Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine and G Dabrera and K Halsby Public Health England for epidemiological expertise and T Harrison for critical reading of the manuscript, S Chalker for assistance with IT, previous members of the Mollicute reference laboratory and all colleagues and patients for referring specimens.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 23 March 2016
doi: 10.3389/fmicb.2016.00329

Infection with and Carriage of *Mycoplasma pneumoniae* in Children

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"Atypical" pneumonia was described as a distinct and mild form of community-acquired pneumonia (CAP) already before *Mycoplasma pneumoniae* had been discovered and recognized as its cause. *M. pneumoniae* is detected in CAP patients most frequently among school-aged children from 5 to 15 years of age, with a decline after adolescence and tapering off in adulthood. Detection rates by polymerase chain reaction (PCR) or serology in children with CAP admitted to the hospital amount 4–39%. Although the infection is generally mild and self-limiting, patients of every age can develop severe or extrapulmonary disease. Recent studies indicate that high rates of healthy children carry *M. pneumoniae* in the upper respiratory tract and that current diagnostic PCR or serology cannot discriminate between *M. pneumoniae* infection and carriage. Further, symptoms and radiologic features are not specific for *M. pneumoniae* infection. Thus, patients may be unnecessarily treated with antimicrobials against *M. pneumoniae*. Macrolides are the first-line antibiotics for this entity in children younger than 8 years of age. Overall macrolides are extensively used worldwide, and this has led to the emergence of macrolide-resistant *M. pneumoniae*, which may be associated with severe clinical features and more extrapulmonary complications. This review focuses on the characteristics of *M. pneumoniae* infections in children, and exemplifies that simple clinical decision rules may help identifying children at high risk for CAP due to *M. pneumoniae*. This may aid physicians in prescribing appropriate first-line antibiotics, since current diagnostic tests for *M. pneumoniae* infection are not reliably predictive.

Keywords: ***Mycoplasma pneumoniae*, pneumonia, carriage, children, diagnosis**

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *15 January 2016*
Accepted: *02 March 2016*
Published: *23 March 2016*

Citation:
Meyer Sauteur PM, Unger WWJ, Nadal D, Berger C, Vink C and van Rossum AMC (2016) Infection with and Carriage of Mycoplasma pneumoniae in Children. Front. Microbiol. 7:329. doi: 10.3389/fmicb.2016.00329

INTRODUCTION

The clinical entity of "atypical" pneumonia was recognized in the 1930s many years before the etiological agent was established (McCoy, 1946). The term separated this entity of pneumonia from classical pneumococcal pneumonia due to its lack of response to available antibiotics and the distinct clinical presentation without typical lobar pneumonia and a less severe disease course. That is why the term "walking pneumonia" has been introduced to denote this mild form of pneumonia.

It was in a patient with "atypical" pneumonia in 1944, where *Mycoplasma pneumoniae* was first isolated from sputum in tissue culture by Eaton et al. (1944). At that time, it was believed to

be a virus because it was resistant to penicillin and sulfonamides and passed through bacteria-retaining filters. Experiments with Marine recruits and adult prisoners demonstrated that the so-called Eaton agent caused lower respiratory tract infections in humans (Chanock et al., 1961a,b). In 1963, it was first cultured on cell-free medium and classified as *M. pneumoniae* (Chanock et al., 1962; Chanock, 1963). Today we know that mycoplasmas are prokaryotes that lack a cell wall and represent the smallest self-replicating organisms (**Figure 1**). With a size of 816,394 base pairs, the genome of *M. pneumoniae* is at least five times smaller than that of *Escherichia coli* (Himmelreich et al., 1996). The absence of a cell wall and the specialized attachment organelle facilitate close contact with the host respiratory epithelium, which supplies the bacterium with the necessary nutrients for its growth and proliferation.

Mycoplasma pneumoniae causes both upper and lower respiratory tract infections, with community-acquired pneumonia (CAP) as the major burden of disease. Although *M. pneumoniae* infections are generally mild and self-limiting, patients of every age can develop severe and fulminant disease (Kannan et al., 2012). *M. pneumoniae* can also cause extrapulmonary manifestations that affect almost every organ (Narita, 2010).

In children, *M. pneumoniae* infections were first reported in 1960 when 16% of 110 children with lower respiratory tract disease were tested positive by a fourfold rise in antibody titers against the Eaton agent (Chanock et al., 1960). To date, it is known that the incidence of *M. pneumoniae* infections is generally higher in children than in adults (Foy et al., 1979). This review focuses on the characteristics of *M. pneumoniae* infections in children, and discusses simple clinical decision rules that may further aid clinicians in identifying patients at high risk for *M. pneumoniae* CAP.

EPIDEMIOLOGY

Mycoplasma pneumoniae is transmitted by respiratory droplets through close contact. The incubation period can be long from 1 up to 3 weeks. Outbreaks have been reported within families, schools, universities, institutions, camps, and military bases. Family members of index patients with acute respiratory infection and detection of *M. pneumoniae* in the upper respiratory tract were found positive in 15% by polymerase chain reaction (PCR) (Dorigo-Zetsma et al., 2001). Thereof, 75% were <16 years of age and 44% did not develop any respiratory symptoms. At universities, the largest outbreak within 35 years in the U.S. was observed during September 1–December 4, 2012, where a total of 83 CAP cases were identified among students, and 12 out of 19 tested cases (63%) were positive for *M. pneumoniae* by quantitative real-time PCR (Centers for Disease Control and Prevention [CDC], 2013).

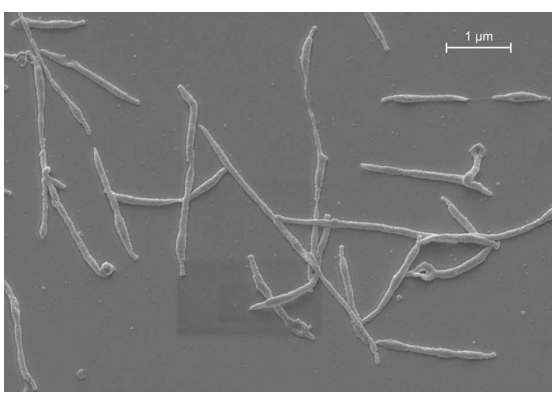

FIGURE 1 | *M. pneumoniae* morphology *in vitro*. Scanning electron micrograph of *M. pneumoniae* strain Mac (subtype 2).

Outbreaks appear mainly during *M. pneumoniae* epidemics that occur in 3–7 years cycles, in addition to a background endemic pattern (Jacobs, 2012). The most recent epidemic in Europe occurred in 2010–2012 with a peak incidence in Finland of 145/100,000 cases in 2011 (Polkowska et al., 2012; Jacobs et al., 2015). The cyclic occurrence of epidemics may be facilitated by a decreasing herd immunity and different *M. pneumoniae* genotypes circulating in the human population (Jacobs, 2012). The two major circulating genotypes, or subtypes, of *M. pneumoniae* are indicated as subtype 1 and 2. Differences between these subtypes in the amino acid sequence of the major adhesion protein P1 are believed to play a role in the epidemiology of infections with *M. pneumoniae* (Su et al., 1990; Vink et al., 2012). The differences between the 169-kDa P1 proteins of subtype 1 and 2 isolates were found to be concentrated in two specific amino acid stretches within the protein. These regions are encoded by two DNA elements within the P1 gene, i.e., repetitive elements RepMP2/3 and RepMP4. The RepMP2/3 and RepMP4 are not unique to the P1 gene, but are also found at other sites within the bacterial genome (Spuesens et al., 2009). Homologous recombination events between these repetitive elements, which are similar to each other, but not identical, may form the basis of antigenic variation of the P1 protein of *M. pneumoniae* (Vink et al., 2012). While such recombination events may induce antigenic variation within subtype 1 or subtype 2 strains, *M. pneumoniae* strains cannot switch from one subtype to the other, as the entire set of RepMP elements found in one subtype differs significantly from those found in the other subtype. Moreover, changes in the proportion of the two subtypes of *M. pneumoniae* were not observed between 2003 and 2012 in Europe (Jacobs et al., 2015).

INFECTION

Respiratory Disease

Although CAP is the major burden of disease, milder clinical presentations of *M. pneumoniae* respiratory infections may be much more common than CAP. These include acute bronchitis and upper respiratory tract infections (Esposito et al., 2000, 2002). *M. pneumoniae* could be detected by PCR and/or serology in 24% of non-streptococcal pharyngitis cases (Esposito et al., 2002).

It is estimated that 3–10% of children with *M. pneumoniae* respiratory infection develop CAP and that <5% of CAP cases are

severe enough to require hospitalization (Waites and Talkington, 2004). Between 1963 and 1975, *M. pneumoniae* was detected by culture of respiratory specimens and/or a fourfold titer rise in complement fixation test (CFT) in 15–20% of radiologically confirmed CAP cases in Seattle, U.S. (Foy et al., 1979). In subsequent etiological studies, *M. pneumoniae* accounted for 4–39% of the isolates identified by PCR and/or serology in children with CAP admitted to the hospital (Juven et al., 2000; Principi et al., 2001; Baer et al., 2003; Michelow et al., 2004). *M. pneumoniae* was first reported as the most common bacterial cause of CAP in children requiring hospitalization in a U.S. multicenter study from 2011 to 2012 in Nashville and Salt Lake City (Jain et al., 2015). In this study, *M. pneumoniae* could be detected by PCR in 178 (8%) out of 2179 cases with CAP, whereas *Streptococcus pneumoniae* was found in 79 cases (4%).

Manifest upper and/or lower respiratory tract infections with *M. pneumoniae* occur at all ages (Foy et al., 1979). Recent observations have indicated that *M. pneumoniae* has also a relatively high prevalence in the respiratory tract of children <5 years (Principi et al., 2001; Gadsby et al., 2012). *M. pneumoniae* CAP, however, was reported to be most frequent among school-aged children from 5 to 15 years of age, with a decline after adolescence and tapering off in adulthood (**Figure 2**) (Foy et al., 1979). This notion was corroborated in the recent CAP study in the U.S., where *M. pneumoniae* was detected significantly more frequent in children ≥5 years of age than in younger children (19% vs. 3%) (Jain et al., 2015).

In addition to the presentation at school-age, children with CAP due to *M. pneumoniae* have been found to present with a significantly longer duration of fever compared with other children with CAP (Fischer et al., 2002). Other symptoms that may be associated with *M. pneumoniae* CAP are the absence of wheeze and the presence of chest pain (Wang et al., 2012). However, there is still a paucity of high quality data regarding clinical signs and symptoms associated with *M. pneumoniae* infections. A recent Cochrane review therefore concluded that the absence or presence of individual clinical symptoms or signs cannot be used to help clinicians accurately diagnose *M. pneumoniae* in children and adolescents with CAP (Wang et al., 2012).

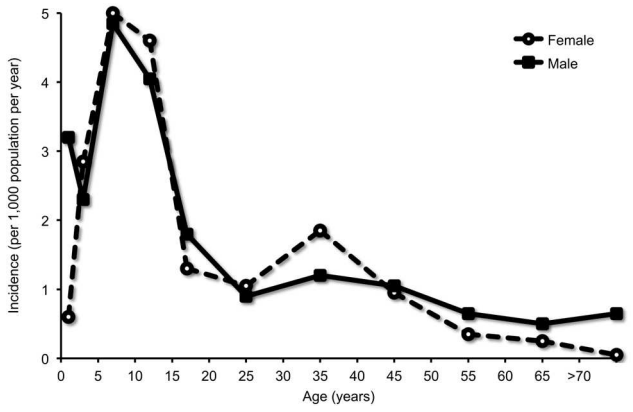

FIGURE 2 | Detection of *M. pneumoniae* in community-acquired pneumonia (CAP) according to age group. Infection was diagnosed by culture of respiratory specimens and/or a fourfold titer rise in complement fixation test (CFT). Adapted with permission from Foy et al. (1979).

Pathogenic effects in the respiratory tract may be caused by *M. pneumoniae* either directly (by active infection), indirectly (by infection-induced immune mechanisms), or both (Narita, 2010). *M. pneumoniae* causes direct injury through the generation of activated oxygen. A potential candidate protein of *M. pneumoniae* that may be involved in causing direct damage to the respiratory tract is a pertussis toxin-like protein termed Community-Acquired Respiratory Distress Syndrome (CARDS) toxin (Kannan and Baseman, 2006; Becker et al., 2015). A recombinant version of the CARDS toxin has been shown to bind with high affinity to surfactant protein A and to exhibit mono-ADP ribosyltransferase and vacuolating activities, which causes disruption of the respiratory epithelium in animal models (Kannan and Baseman, 2006).

In addition to the direct damage resulting from infection by *M. pneumoniae*, the immunological response following infection generates inflammatory reactions that may cause pulmonary and extrapulmonary symptoms. More severe symptoms of CAP have been observed in older children and adolescents (Waites and Talkington, 2004). This suggests that the age-dependent magnitude and nature of inflammatory responses in childhood may be a major factor contributing to the development of *M. pneumoniae*-associated disease, similar to what is observed, e.g., in infectious mononucleosis or rheumatic fever. In fact, the severity of *M. pneumoniae* CAP in children was closely associated with increased concentrations of interleukin (IL)-8 and IL-18 in acute phase serum and pleural fluid samples (Narita and Tanaka, 2007). In addition, it has been demonstrated that cell-mediated immunity contributes to the pathogenesis of *M. pneumoniae* CAP, as it was shown that the severity of CAP correlated positively with the size of a cutaneous induration following intradermal injection of *M. pneumoniae* antigens (Mizutani et al., 1971). This study described 20 patients with CAP, of which 19 were children 4–15 years of age, diagnosed by a significant rise in antibody titers against *M. pneumoniae* with CFT. The strongest skin reactions were seen in patients with severe CAP.

Asthma

Mycoplasma pneumoniae and other "atypical bacteria" have long been implicated in the pathogenesis of asthma (Atkinson, 2013). There are many studies that have addressed this issue in the recent past. In an observational study on children and adults with asthma, *M. pneumoniae* infection was diagnosed in 9% of children with asthma (24/256) and was found more frequent in patients with chronic asthma (14%) than in those with asthma exacerbations (7%; $p = 0.10$) (Bebear et al., 2014). The diagnosis of *M. pneumoniae* infection in this study was performed by PCR and/or serology. Another recent study diagnosed *M. pneumoniae* in children with acute asthma (64%, 34/53) and refractory asthma (65%, 17/26), as well as in healthy controls (56%, 36/64), but did not find significant differences between these three groups (Wood et al., 2013). The high detection rates reported in this study were obtained using novel diagnostic methods [CARDS

toxin enzyme immunoassay (EIA) and CARDS gene-specific PCR] (Wood et al., 2013). In a recent Taiwanese study (Yeh et al., 2015), 1591 children and adults with *M. pneumoniae* infection, diagnosed by positive immunoglobulin (Ig) M or fourfold IgG titer increase, but without prior asthma history were included from 2000 to 2008 and followed until the diagnosis of asthma or the end of 2011. Compared to matched 6364 patients without *M. pneumoniae* infection, the cumulative incidence of asthma was significantly higher in the *M. pneumoniae* cohort than in the control cohort ($p < 0.0001$). Patients with *M. pneumoniae* infection were at higher risk of having early-onset asthma (age at asthma diagnosis <12 years) and late-onset asthma (age at asthma diagnosis ≥12 years). These most recent findings suggested that *M. pneumoniae* can induce airway inflammation and contribute to incident asthma. Interestingly, exposure to recombinant CARDS toxin resulted in an allergic-type inflammatory response and airway hyperreactivity in mice and baboons (Hardy et al., 2009; Medina et al., 2012). It will be interesting to investigate whether CARDS toxins induce a similar allergic response during *M. pneumoniae* infections.

Extrapulmonary Manifestations

Apart from the respiratory tract infection, *M. pneumoniae* can cause extrapulmonary manifestations in almost every organ, including the skin and the hematologic, cardiovascular, musculoskeletal, and nervous systems (Narita, 2010). These manifestations may be caused either by direct local effects of *M. pneumoniae*, after dissemination of the bacteria throughout the body, or indirect effects, such as autoimmune reactions. The most frequent manifestations are diseases of the dermatologic and nervous system.

Skin manifestations occur in up to 25% of all *M. pneumoniae* infections, including mostly non-specific exanthems, erythema nodosum, urticaria, Stevens–Johnson syndrome, and a rare but distinct disorder with prominent mucous membrane involvement denominated as *M. pneumoniae*-associated mucositis (MPAM) (Schalock and Dinulos, 2009; Meyer Sauteur et al., 2012). This condition was first described by Fuchs (1876), and therefore also referred to as Fuchs syndrome (Meyer Sauteur et al., 2011). A recent review identified 32 patients with MPAM at a median age of 13.5 years at presentation (range 3–38 years, 23 children or young adolescents ≤18 years) (Meyer Sauteur et al., 2012). All patients presented with prodromal respiratory symptoms with a median duration of 7 days, and pneumonia was found in chest radiography in 79%. Oral lesions were present in all cases (**Figure 3**), ocular lesions in 97%, and urogenital lesions in 78%. There were no skin lesions in 69%. Although 12% of the patients were admitted to the intensive care unit, no one suffered from long-term sequelae.

Encephalitis and Guillain–Barré syndrome (GBS) constitute the most common and severe neurologic manifestations, where *M. pneumoniae* infection is established in up to 10 and 15% of patients, respectively (Bitnun et al., 2001; Sinha et al., 2007). In *M. pneumoniae*-associated encephalitis, both a direct infection of the central nervous system (CNS) and an immune-mediated process have been implied to be involved (Narita, 2009). Because the detection rate of *M. pneumoniae* by PCR in cerebrospinal fluid (CSF) of *M. pneumoniae* encephalitis patients is relatively low (0–14%) (Bitnun et al., 2001; Daxboeck et al., 2004; Christie et al., 2007a; Domenech et al., 2009), a significant proportion of the cases is believed to be immune-mediated. This is supported by the finding that various cases with *M. pneumoniae* encephalitis in which bacterial DNA could not be detected in CSF had a more prolonged duration of respiratory symptoms before the onset of encephalitis (>5–7 days) (Bitnun et al., 2001; Narita and Yamada, 2001; Daxboeck et al., 2004). These cases indicate that *M. pneumoniae* encephalitis represents a postinfectious disorder, which manifests after clearance of the bacteria from the CNS or respiratory tract by the immune system (Meyer Sauteur et al., 2014b). A recent study presented 365 children with *M. pneumoniae* detected in the respiratory tract or CSF by PCR, 22 (6%) of whom had encephalitis (1996–2013, Toronto, ON, Canada) (Al-Zaidy et al., 2015). Interestingly, patients in which *M. pneumoniae* was detectable in the respiratory tract but not in CSF showed pulmonary infiltrates on chest radiograph more frequently than patients with positive PCR in CSF (77% vs. 33%). This suggests that pneumonia may be an indicator for a remote inflammatory process in *M. pneumoniae* encephalitis patients, which was also shown in 83% (5/6) of children observed during a national surveillance, all with negative PCR in CSF (2010–2015, Switzerland) (Meyer Sauteur et al., 2016) (**Figure 4**).

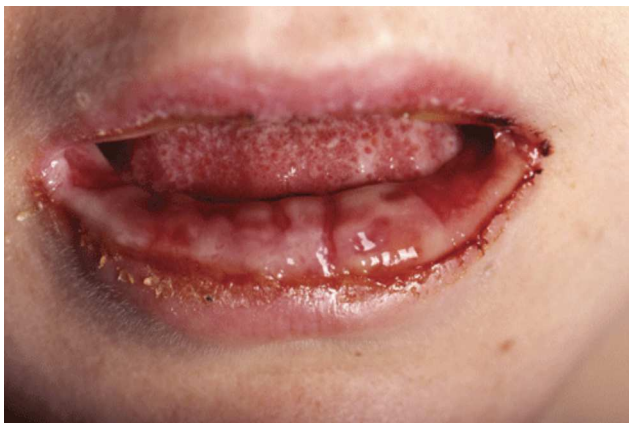

FIGURE 3 | *M. pneumoniae*-associated mucositis (MPAM). Erosive oral lesions limited to the mucosa in this form of MPAM in a 24-year-old woman. Reprinted with permission from Meyer Sauteur et al. (2012).

Mycoplasma pneumoniae expresses adhesion proteins and glycolipids that share structural homology with a variety of host cells (molecular mimicry) and may induce cross-reactive antibodies (Meyer Sauteur et al., 2014b). In children with *M. pneumoniae* encephalitis, intrathecal antibodies directed against galactocerebroside (GalC) were found (Christie et al., 2007b; Meyer Sauteur et al., 2015a). Of note, all these patients had a negative PCR in CSF. GalC is a major glycolipid antigen in the myelin sheath of both the peripheral and CNS neurons (Menge et al., 2005). In fact, antibodies against *M. pneumoniae* infection have been found to cross-react with GalC in GBS patients (Kusunoki et al., 2001; Ang et al., 2002). Moreover, anti-GalC antibodies caused demyelinating neuropathy in rabbits

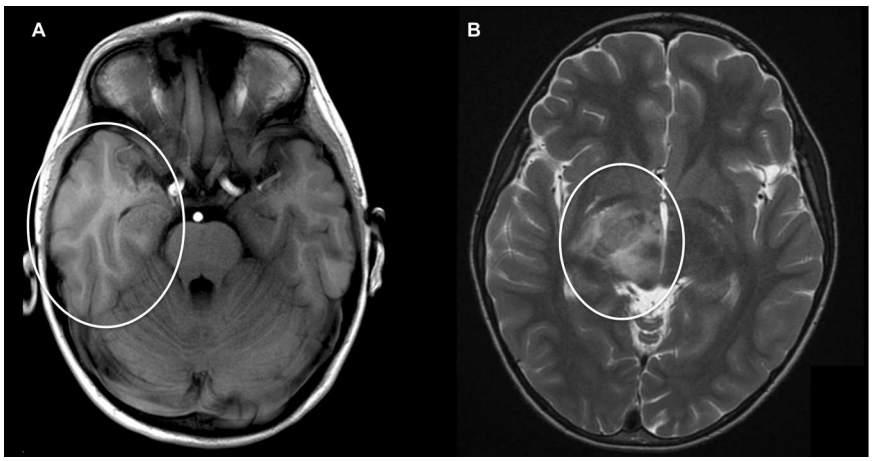

FIGURE 4 | *M. pneumoniae*-associated encephalitis. Axial cranial magnetic resonance imaging (MRI) in two children with encephalitis during *M. pneumoniae* infection: **(A)** 5-year-old boy with hyperintensity and generalized edema of the right temporal lobe [T1 weight MRI; patient 1 published in Meyer Sauteur et al. (2016)]. **(B)** 9-year-old boy with generalized edema of crus posterior of capsula interna [T2 weight MRI; reprinted with permission from Meyer Sauteur et al. (2014c)].

(Saida et al., 1979) and have been associated with demyelination in GBS (Ang et al., 2002), but also in encephalitis (Christie et al., 2007b) and encephalomyelitis (Samukawa et al., 2012). The detection of intrathecal antibodies against *M. pneumoniae* and GalC may also be regarded as a promising new diagnostic tool for *M. pneumoniae*-associated CNS disease (Meyer Sauteur et al., 2014b, 2015b).

CARRIAGE

Like many other respiratory pathogens, *M. pneumoniae* can be carried asymptomatically in the respiratory tract (Foy, 1993). Recent studies have demonstrated that asymptomatic carriage of *M. pneumoniae* is highly prevalent. Detection rates of *M. pneumoniae* DNA in the respiratory tract of healthy children without respiratory symptoms were 21% in a Dutch study (2008–2011, Rotterdam, The Netherlands) (Spuesens et al., 2013) and 56% in a U.S. study (2009–2011, San Antonio, TX, U.S.) (Wood et al., 2013). Longitudinal sampling of *M. pneumoniae*–positive asymptomatic children demonstrated that *M. pneumoniae* can be present in the upper respiratory tract without causing disease, for up to 4 months (Spuesens et al., 2013). The prevalence of *M. pneumoniae* in the upper respiratory tract of asymptomatic children varied considerably between years and seasons. For example, asymptomatic carriage rates of 3% and 58% were reported in the spring of 2009 and the summer of 2010, respectively (Spuesens et al., 2013). These data suggest that carriage follows an epidemic pattern. It is tempting to speculate that this fluctuation in prevalence is related to the cyclic epidemics of *M. pneumoniae* infections. Apart from *M. pneumoniae*, children were found to simultaneously carry many pathogens in their nose and throat (Spuesens et al., 2013). These pathogens include the bacteria *S. pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis,* and *Haemophilus influenzae*, and the viruses influenza A/B, human metapneumovirus, respiratory syncytial virus, parainfluenzavirus, rhinovirus, coronavirus, bocavirus, and adenovirus. The simultaneous presence of two or more of these pathogens was detected in 56% of asymptomatic children (Spuesens et al., 2013).

In children with *M. pneumoniae* CAP, co-existence of *M. pneumoniae* with other pathogens has also been described (Juven et al., 2000; Michelow et al., 2004), and was recently reported in 28% of the patients (Jain et al., 2015). The impact of co-infections in *M. pneumoniae* CAP on disease severity is not yet determined.

DIAGNOSIS

Diagnostic Tests

Because the mere presence of *M. pneumoniae* in the upper respiratory tract is neither indicative nor predictive for respiratory disease, the routine diagnostic procedures to detect acute respiratory infections with *M. pneumoniae* need to be reconsidered. An overview of diagnostic tests with their advantages and drawbacks is shown in **Table 1**.

Current guidelines (Bradley et al., 2011; Harris et al., 2011) recommend PCR and single-sample serological tests to diagnose *M. pneumoniae* infections. The sensitivity of serological tests depends on the time point of the first serum and on the availability of paired sera for seroconversion to IgG and/or rise in antibody titer. Specific serum IgM emerges within 1 week after initial infection and about 2 weeks before IgG (Meyer Sauteur et al., 2014b). Although specific serum IgA arises even earlier than IgM, it could be detected only in 2% of PCR-positive children with symptomatic respiratory tract infection (Spuesens et al., 2013). Cross-reactions with other pathogens and non-infectious disease has been described for CFT and particle agglutination assay, but also some EIAs lack the required sensitivity and specificity (Beersma et al., 2005). Further, it

TABLE 1 | Overview of diagnostic tests for *M. pneumoniae*.

Method	Test	Target/antigen	Antibodies	Specimen(s)	Performance	Value	Comments
Direct identification of *M. pneumoniae*	Polymerase chain reaction (PCR)	Different target genes (e.g., P1 gene, 16S rDNA, 16S rRNA, RepMP elements etc.)	–	Respiratory specimen Cerebrospinal fluid (CSF) Other bodily fluids or tissues	High sensitivity, high specificity	RD	- Validation and standardization required for routine diagnostic (Loens et al., 2010); - Epidemiological differentiation of clinical strains on the basis of differences in the P1 gene by PCR (Spuesens et al., 2009) or in the number of repetitive sequences at a given genomic locus by multiple-locus variable-number tandem repeat analysis (MLVA) (Chalker et al., 2015).
	Culture	–	–	Respiratory specimen	Low sensitivity, high specificity	AD	- Special enriched broth or agar media; - Isolation takes up to 21 days.
Non-specific serological tests for *M. pneumoniae*	Cold agglutinin test ("bedside test")	Erythrocytes (I antigen)	Cold agglutinins (IgM)	Serum	Low sensitivity, low specificity	–[1]	- Cold agglutinins target the I antigen of erythrocytes; - Positive in only about 50% and in the first week of symptoms; - Less well studied in children; - Cross-reactivity with other pathogens and non-infectious diseases.
Specific serological tests for *M. pneumoniae*	Complement fixation test (CFT)	Crude antigen extract with glycolipids and/or proteins	Igs (no discrimination between isotypes)	Serum	Sensitivity and specificity comparable to EIA	–[1]	- Positive criteria: fourfold titer increase between acute and convalescent sera or single titer $\geq$1:32; - Cross-reactivity with other pathogens and non-infectious diseases.
	Particle agglutination assay (PA)		IgM and IgG simultaneously			–[1]	
	Enzyme immunoassay (EIA)	Proteins (e.g., adhesion protein P1) and/or glycolipids	IgM, IgG, IgA	Serum CSF[2]	Moderate-high sensitivity, Moderate-high specificity	RD	- The sensitivity depends on the time point of the first serum and on the availability of paired sera (for seroconversion and/or rise in titer); - "Gold standard": fourfold titer increase as measured in paired sera.
	Immunoblotting				High sensitivity, high specificity	AD	- Confirmatory assay (Dumke et al., 2012).
	Immunofluorescent assay (IFA)				Less sensitive and less specific than EIA	AD	- Subjective interpretation.

Adapted from Meyer Sauteur et al. (2014b). AD, advanced diagnostic test; CFT, complement fixation test; CSF, cerebrospinal fluid; EIA, enzyme immunoassay; IFA, immunofluorescent assay; Ig, immunoglobulin; PA, particle agglutination assay; PCR, polymerase chain reaction; RD, routine diagnostic test; RepMP, repeated M. pneumoniae DNA. [1]*Largely replaced by EIA;* [2]*For the evaluation of an intrathecal antibody synthesis (Granerod et al., 2010), either by calculation of an antibody index (Reiber, 1994) or through parallel immunoblotting of simultaneously collected CSF and serum samples (Monteyne et al., 1997).*

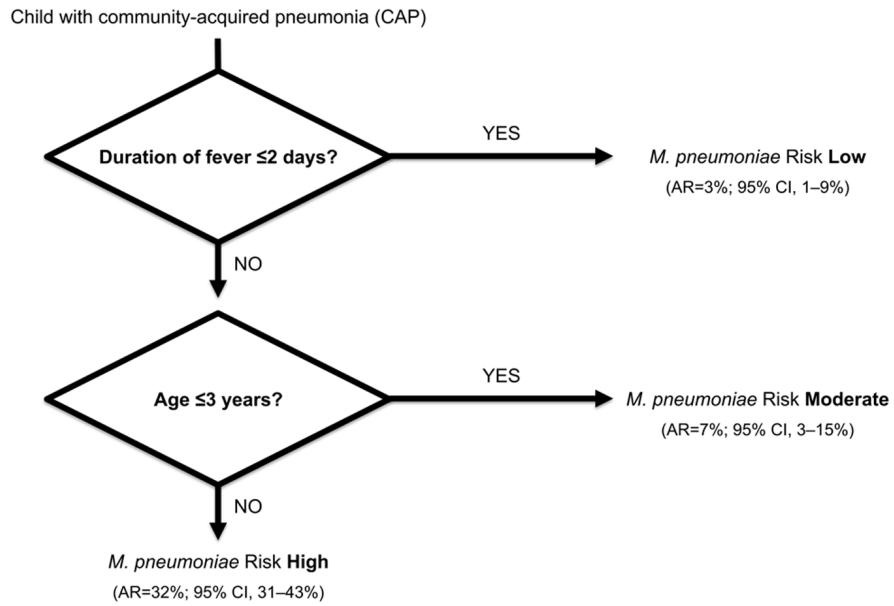

FIGURE 5 | A fast-and-frugal clinical decision tree for ruling out *M. pneumoniae* infection in children with community-acquired pneumonia (CAP). Clinical features are considered sequentially, with a possible stop decision after each question. Abbreviations: AR, absolute risk; CI, confidence interval. Adapted from Fischer et al. (2002).

is intriguing that the detection of IgM, as well as IgG and IgA by EIA could not discriminate between the asymptomatic and symptomatic groups of children (Spuesens et al., 2013). The demonstrated positive serological results in asymptomatic *M. pneumoniae* PCR-positive children ($n = 66$; IgM in 17%, IgG in 24%, and IgA in 6%) may simply reflect one or more previous encounters with *M. pneumoniae* and are not necessarily related to the presence of *M. pneumoniae* in the respiratory tract. Thus, it is questionable whether or not a positive result in these tests actually indicates the etiological role of *M. pneumoniae* in all cases. In that sense, the positive predictive value of these tests may be overestimated, whereas the negative predictive value may be acceptable (Bradley et al., 2011).

The "gold standard" for diagnosis of *M. pneumoniae* infections is still considered to be a fourfold increase in antibody titer as measured in paired sera (Gardiner et al., 2015). However, the use of convalescent sera is not useful in clinical practice because it is too time-consuming and does not allow clinicians to initiate treatment protocols in a timely fashion. Clinicians therefore need to be aware of the implications and clinical significance of a positive PCR or serology test result.

Clinical Assessment

While diagnostic tests may not be reliably predictive for a symptomatic *M. pneumoniae* infection, the clinical assessment of this entity is being revisited. The British Thoracic Society guidelines recommend that bacterial pneumonia should be considered in children when there is persistent or repetitive fever $>38.5°C$ together with chest recession and a raised respiratory rate (Harris et al., 2011). A chest radiograph should not be considered a routine investigation in children thought to have CAP. In fact, although bilateral, diffuse infiltrates are common, none of the radiographic findings associated with *M. pneumoniae* CAP is specific (John et al., 2001).

A fast-and-frugal clinical decision tree provided a rapid probability estimate of the cause of CAP in 253 children (1 months–16 years; 1997–1999, Zurich, Switzerland) (Fischer et al., 2002). *M. pneumoniae* infection was diagnosed in 13% ($n = 32$) of these children by PCR in respiratory specimens and serology (seroconversion and/or fourfold rise in antibody titer). Compared with other children with CAP, patients with *M. pneumoniae* were older and had a longer duration of fever ($p < 0.001$). Asking the simple question regarding the age of the child and the duration of fever allowed identification of the following group at high risk for CAP due to *M. pneumoniae*: children with CAP who have had fever >2 days and who were >3 years of age. The score model placed 75% of all patients with *M. pneumoniae* infection into the high-risk group (**Figure 5**). These simple rules may further aid physicians in prescribing appropriate first-line antibiotics.

TREATMENT AND VACCINES

Antibiotics

In consequence of the diagnostic uncertainty for *M. pneumoniae* infections, the British Thoracic Society guidelines suggest empiric macrolide treatment at any age if there is no response to first-line β-lactam antibiotics or in the case of very severe disease (Harris et al., 2011). The lack of a cell wall makes

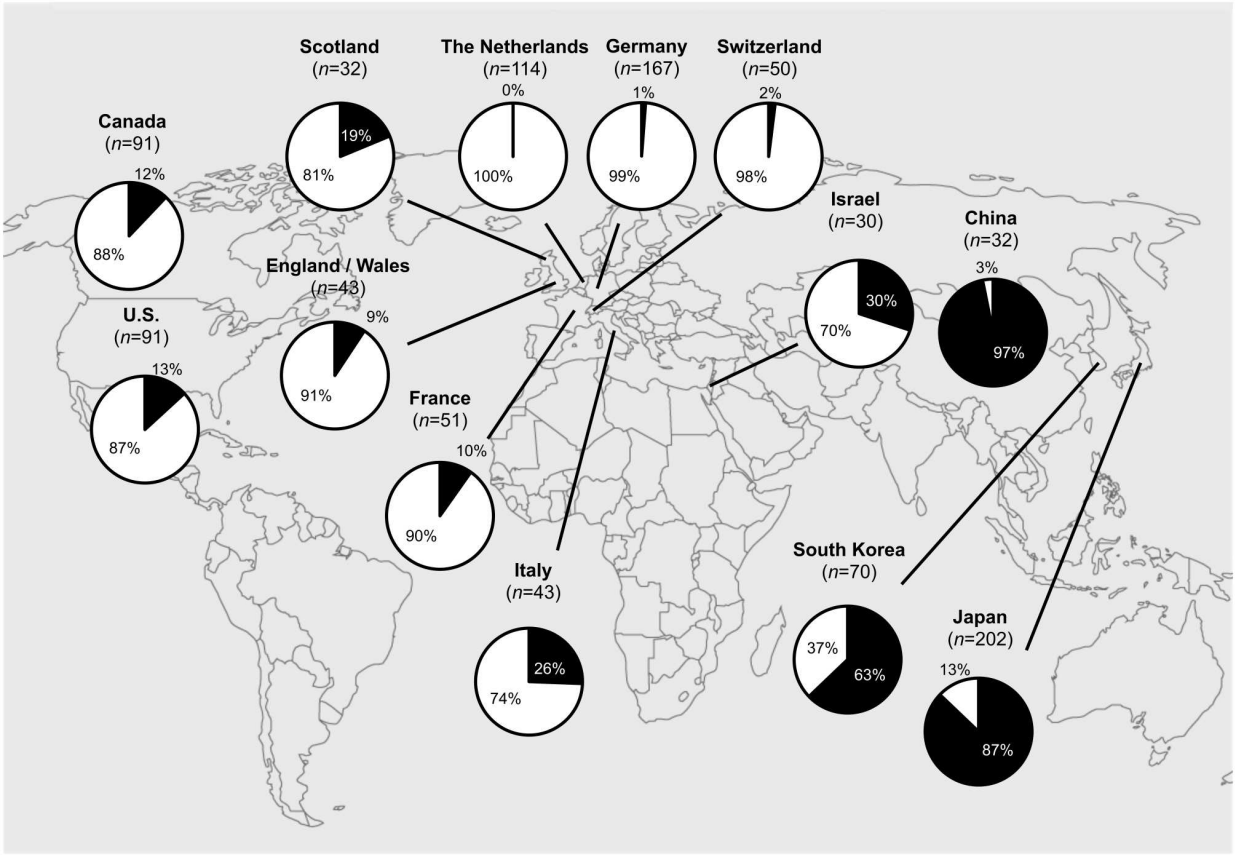

FIGURE 6 | Worldwide macrolide-resistant *M. pneumoniae* (MRMP) rates. Actual MRMP rates are punctually depicted in pie charts (in black) over the world map. **Asia:** Japan (2011): 87% (176/202) (Okada et al., 2012), South Korea (2011): 63% (44/70) (Hong et al., 2013), China (2012): 97% (31/32) (Zhao et al., 2013), Israel (2010): 30% (9/30) (Averbuch et al., 2011); **North America:** U.S. (2012–2014): 13% (12/91) (Zheng et al., 2015), Canada (2010–2012): 12% (11/91) (Eshaghi et al., 2013); **Europe:** The Netherlands (1997–2008): 0% (0/114) (Spuesens et al., 2012), Germany (2003–2008): 1% (2/167) (Dumke et al., 2010), France (2005–2007): 10% (5/51) (Peuchant et al., 2009), Italy (2010): 26% (11/43) (Chironna et al., 2011), Scotland (2010–2011): 19% (6/32) (Ferguson et al., 2013), Switzerland (2011–2013): 2% (1/50) (Meyer Sauteur et al., 2014a), England and Wales (2014–2015): 9% (4/43) (Brown et al., 2015).

M. pneumoniae resistant to cell wall synthesis inhibitors such as β-lactam antibiotics. The antibiotics with the best minimum inhibitory concentration values against *M. pneumoniae* include macrolides, tetracyclines, and fluoroquinolones (Waites and Talkington, 2004). Although the latter two have a good *in vitro* inhibitory effect against *M. pneumoniae*, tetracyclines may cause teeth discoloration (Waites and Talkington, 2004) and fluoroquinolones may affect the developing cartilage in young children (Adefurin et al., 2011). Thus, they are not recommended by current guidelines in young children; the age limit for tetracyclines is ≥8 years, while that of fluoroquinolones is adolescence with skeletal maturity (Bradley et al., 2011). The occurrence of arthropathy due to fluoroquinolones, however, is uncertain, and all musculoskeletal adverse effects reported in the literature had been reversible following withdrawal of treatment (Adefurin et al., 2011). The protein synthesis inhibitors of the macrolide class have a more favorable side effect profile and are therefore the first-line antibiotics for *M. pneumoniae* infections in children (Bradley et al., 2011).

Although antibiotics are effective against *M. pneumoniae* *in vitro* (Bebear et al., 2011), there is lack of evidence on their *in vivo* efficacy. Observational data indicated that children with CAP due to *M. pneumoniae* have a shorter duration of symptoms and fewer relapses when treated with an antimicrobial agent active against *M pneumoniae* (McCracken, 1986; Waites and Talkington, 2004). A recent Cochrane review evaluated seven studies on the effectiveness of antibiotic treatment for *M. pneumoniae* lower respiratory tract infections in children (Gardiner et al., 2015). However, the diagnostic criteria, the type and duration of treatment, inclusion criteria, and outcome measures differed significantly, making it difficult to draw any specific conclusions, although one trial suggested that macrolides may be efficacious in some cases (Esposito et al., 2005). It is clear that studies on the efficacy of antibiotics rely on a correct diagnosis of *M. pneumoniae* infections. Given the aforementioned shortcomings of current diagnostic tests, conclusions on the efficacy of antibiotic treatment will have to be re-examined.

Antibiotic Resistance

Since 2000, the extensive macrolide use led to an alarming worldwide increase in the prevalence of macrolide-resistant *M. pneumoniae* (MRMP) strains (Bebear et al., 2011). Resistance is based on specific point mutations in domain V of the *23S rRNA* (at positions 2063, 2064, and 2617), which reduce the affinity of macrolides to the large subunit (50S) of the bacterial ribosome (Bebear et al., 2011). MRMP has been observed during macrolide treatment as a result of antibiotic selective pressure (Cardinale et al., 2011; Chironna et al., 2011; Saegeman et al., 2012). To date, macrolide resistance has been detected on a worldwide scale. MRMP had developed in Asia (Hong et al., 2013), where MRMP rates have risen as high as 97% in China (Zhao et al., 2013). MRMP has now also been reported from North America and Europe (**Figure 6**).

The clinical relevance of macrolide resistance in hospitalized children with CAP may lie in prolonging the symptoms of the disease (Okada et al., 2012; Cardinale et al., 2013; Zhou et al., 2014). Zhou et al. (2014) found that an increase in MRMP may also have serious clinical consequences in children, leading to more severe radiological findings of CAP and even an increase in extrapulmonary manifestations. In this study, hospitalized children with CAP due to MRMP developed more often extrapulmonary disease than children with CAP caused by macrolide-sensitive strains (30% vs. 10%; $p = 0.03$) (Zhou et al., 2014). These manifestations included skin diseases and nervous system complications in 18% and 7%, respectively, of the MRMP-infected children. Serum inflammatory cytokine levels (INF-γ, IL-6, and IP-10) were higher in patients infected with MRMP than in patients infected with macrolide-sensitive strains (Matsuda et al., 2013). This suggests that the higher and more persistent inflammatory stimulation by MRMP may increase the possibility of severe lung lesions and extrapulmonary complications.

Vaccines

While previous attempts to produce vaccines on the basis of inactivated bacteria resulted in limited efficacy against CAP and various adverse effects (Linchevski et al., 2009), the recent use of recombinant proteins as potential vaccines was found to be promising: The immunization of mice with a immunogenic recombinant protein encompassing the C-terminal part of the P1 protein (RP14) induced strong mucosal and systemic antibody responses against *M. pneumoniae,* and reduced lung inflammation in infected mice (Zhu et al., 2012). Another study showed that immunization of guinea pigs with a chimeric protein consisting of RP14 and the P30 adhesion protein of *M. pneumoniae* resulted in a robust antibody response that led to a reduction in bacterial loads in the respiratory tract (Hausner et al., 2013).

CONCLUSION

The increasing prevalence of MRMP has become a significant issue, since MRMP can potentially cause more severe and even extrapulmonary disease. Because symptoms and radiologic features of *M. pneumoniae* CAP seem to be unspecific and current diagnostic procedures cannot discern between carriage and infection in a clinically relevant time frame, simple clinical rules may further aid physicians in prescribing appropriate first-line antibiotics. Thus, empiric macrolide treatment may be restricted to children at high risk for *M. pneumoniae* CAP, i.e., children with CAP who have fever >2 days and who are >3 years of age, or in the case of very severe disease. Future research should focus on novel aspects of *M. pneumoniae*-related pathogenesis resulting in more precise diagnostic tools and tailored treatment that prevents the emergence of antimicrobial resistance.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

PMMS received grants of the Swiss National Science Foundation (PBZHP3_147290), the Sophia Scientific Research Foundation (SSWO 2014–150/WO), the Promedica Foundation (Chur, Switzerland), and a Fellowship Award of the European Society for Paediatric Infectious Diseases (ESPID), outside the submitted work.

ACKNOWLEDGMENT

We thank Steve Gschmeissner (Bedford, UK) for performing scanning electron microscopy of the *M. pneumoniae* strain.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 13 June 2016
doi: 10.3389/fmed.2016.00028

Community-Acquired Pneumonia Caused by *Mycoplasma pneumoniae*: How Physical and Radiological Examination Contribute to Successful Diagnosis

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Mycoplasma pneumoniae is one of the most common causes of community-acquired pneumonia (CAP), particularly in young adults. Vital signs are usually normal except for temperature. On physical examination, general appearance is normal compared with that of typical pneumonia such as pneumococcal pneumonia patients. Mycoplasma sometimes causes ear infections such as otitis media. It is important to distinguish between typical pneumonia and atypical pneumonia such as mycoplasma pneumonia because having the right diagnosis allows for the use of the correct antibiotic to treat CAP while preventing development of drug-resistant bacteria and also decreasing medical cost. The symptoms and diagnosis of mycoplasma pneumonia is multi-fold. Auscultation of patients can demonstrate trace late inspiratory crackles or normal alveolar sounds; however, bilateral polyphonic wheezes can sometimes be heard because of bronchiolitis. With regard to radiological findings, a chest radiogragh often shows bilateral reticulonodular or patchy consolidation in both lower lobes. Pleural effusion is rarely observed in adult cases. Immunocompetent patients tend to reveal more extensive shadowing compared with immunocompromised patients. As serological diagnostic methods are not able to offer 100% reliable diagnosis, integration of physical and radiological examination is crucial to accurately diagnose mycoplasma pneumonia. Herein, I review the typical findings from physical examination and imaging patterns of patients with mycoplasma pneumonia.

Keywords: mycoplasma, physical examination, auscultation, radiological findings, atypical

OPEN ACCESS

Edited by:
Takeshi Saraya, Kyorin University School of Medicine, Japan

Reviewed by:
Shinichi Sasaki, Juntendo University Urayasu Hospital, Japan
Shusaku Haranaga, University of the Ryukyus, Japan

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Medicine

Received: *23 December 2015*
Accepted: *30 May 2016*
Published: *13 June 2016*

Citation:
Kishaba T (2016) Community-Acquired Pneumonia Caused by Mycoplasma pneumoniae: How Physical and Radiological Examination Contribute to Successful Diagnosis. Front. Med. 3:28. doi: 10.3389/fmed.2016.00028

INTRODUCTION

Mycoplasma pneumoniae is one of the most common causes of community-acquired pneumonia (CAP), particularly in young adults (1, 2). Atypical agents including *M. pneumoniae* pneumonia account for 7–20% of CAP (3–7). There is a unique diagnostic criterion for atypical pneumonia in Japan. The Japanese Respiratory Society (JRS) propose six parameters such as young age, absence of underlying disease, an intractable or non-productive cough, absence of crackles, and absence of leukocytosis as criteria for atypical pneumonia, particularly mycoplasma pneumonia (8). According to a Japanese multi-center study including 403 typical pneumonia cases, 62, 46, and 13 cases were caused by *M. pneumoniae*, *Chlamydophila pneumoniae*, and *Chlamydophila psittasi*,

respectively. The sensitivity and specificity of the JRS criteria were 88.6 and 69.8%, respectively, when only considering those aged <60 years (9).

A single high Mp-specific antibody titer is suggestive of recent infection; however, if the patient has been at hospital for less than a week, antibodies are usually negative. However, an increase in antibody titers by a factor of >4 in serum samples obtained during the acute and convalescent phases of the disease is indicative of a recent infection. In practice, this requires weeks of monitoring and is not practical. On the other hand, ImmunoCard (IC) Mycoplasma (Meridian) can detect IgM antibodies although this requires 3 or 4 days until a positive result is obtained. Furthermore, once it is positive, it will remain for some time. Ueda et al. reported that 31.3% IC Mycoplasma-positive cases showed a discrepancy when comparing the result of IC with that of the complement fixation (CF) test (10). Therefore, practical diagnosis requires a more rapid and reproducible test. Recently, more rapid detection of the Mycoplasma DNA, such as loop-mediated isothermal amplification (LAMP) system, has been reported that it is highly sensitive (11–14). Still, both physical findings and radiological patterns contribute to practical diagnosis of mycoplasma pneumonia. Herein, I review the typical physical findings and imaging patterns of mycoplasma pneumonia. In addition, I describe the crucially important differential diagnoses.

HISTORY TAKING: familial or school outbreaks of mycoplasma infection do occur, therefore, a detailed history regarding sick contacts are crucial for diagnosing mycoplasma infection.

VITAL SIGNS

Blood pressure and respiratory rate are usually within the normal range in mycoplasma infection, the patient will sometimes show pulse–temperature dissociation. However, relative bradycardia is observed less often compared with other atypical agents such as typhoid fever, Legionellosis, psittacosis, and rickettsia infection. Therefore, relative bradycardia has a low sensitivity in diagnosing mycoplasma pneumonia. The fever range is from a low to high grade such as 39°C (15–18).

PHYSICAL EXAMINATION

General

General appearance is the initial part of physical inspection. In patients with mycoplasma infection, the general appearance is normal compared with that in patients with typical pneumonia such as pneumococcal pneumonia and that caused by *Klebsiella pneumoniae* pneumonia.

Ear

We sometimes observe ear pain or transient deafness because of otitis media caused by mycoplasma (19, 20), although the deafness is usually unilateral and reversible. Clinical symptoms of otitis media caused by mycoplasma include ear pain without discharge, which is different from exudative otitis media. In addition, severity of pain is mild rather than bacterial infection. Because the nose, sinuses, ear, pharynx, and lower airway are connected, once an organ is affected by mycoplasma infection, we sometimes observe that sinusitis or otitis media coexist with the pneumonia (21). Frontal headache and tenderness is associated with sinusitis. And mycoplasma patients sometimes notice sore throat as presenting symptom of Mycoplasma-related pharyngitis.

Neck

Mycoplasma patients rarely show significant lymphadenopathy. Therefore, Epstein–Barr (EB) virus infection, acute human immunodeficiency virus (HIV), connective tissue disease (CTD), such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and adult-onset Still's disease (AOSD) need to be ruled out. If mycoplasma infection involves the posterior pharynx or tympanic membrane, non-prominent cervical adenopathy can be observed. Therefore, lymphadenopathy of mycoplasma infection shows without tenderness and hardness.

Lung

Majority of mycoplasma infection reveal only airway infection with intractable cough. However, only <10% showed pneumonia (22). Patients can exhibit either trace late inspiratory crackles or normal alveolar sound. Norisue et al. reported that among 74 mycoplasma patients, 58.1% showed no crackles and 33.8% showed late inspiratory crackles (23). Besides, the positive predictive value (PPV) of pneumococcal pneumonia ($n = 43$) and mycoplasma pneumonia ($n = 74$) based on crackles were 91 and 76%, respectively (**Table 1**). This means mycoplasma exclusively invades the airway or peribronchial interstitium without alveolar involvement. Therefore, most patients show an intractable, non-productive cough in the early phase. Sputum color change occurs late in the course. In addition, we sometimes detect bilateral polyphonic wheezes because of bronchiolitis (24). Presence of bronchiolitis is associated with hyperinflation or volume loss in radiological findings. Therefore, integration of auscultation and radiological findings provide useful information for pathogenesis of mycoplasma pneumonia. Mycoplasma patients experience dyspnea less often because the main target is the peribronchovascular interstitium and respiratory bronchiole, but not the alveolar septum. If patient report dyspnea, we consider pleural effusion or co-existence of asthma attack. Based on Cochrane Database Systematic Review, presence of chest pain is more than double the probability of Mp pneumonia. Wheeze was 12% more likely to be absent in children with Mp pneumonia [pooled positive likelihood ratio (LR+) 0.76, 95% CI 0.60–0.97; pooled negative likelihood ratio (LR−) 1.12, 95%

TABLE 1 | Sensitivity and specificity values based on crackles.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Pneumococcal pneumonia ($n = 43$)	83.1	85.7	90.7	75.0
Mycoplasma pneumonia ($n = 74$)	80.0	84.7	75.7	87.7

Ref. (23).

CI 1.02–1.23] (25). Chest pain and wheeze are both useful symptoms contributing to diagnosis of Mp pneumonia both in children and in adolescents.

Heart

Rhythm is regular and no extra sounds are heard, although patients can sometimes exhibit a rhythm disturbance with palpitation (18). We rarely observe mycoplasma-associated myocarditis, which is not fatal. And myocarditis has been reported rare autopsy reports. There are several case reports of mycoplasma that are associated with thrombosis of the heart. The pathogenesis is the formation of anticardiolipin antibodies (26). This suggests immunological-mediated reaction. This unusual presentation is associated with chest pain. The incidence and severity of most forms of cardiac involvement increase with age.

Abdomen

Gastrointestinal tract involvement is rarely observed, and symptoms are non-specific, although hepatitis and pancreatitis are possible because of cross-reacting antibodies to *M. pneumonia* (16). Compared to Legionellosis, mycoplasma infection rarely cause diarrhea.

Extremities

Common skin manifestation is macro-papular rash with itching as nodular erythema. These skin lesions are often detected on both thighs. The most severe form of skin involvement is Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (27), which are characterized by fever, rash, skin detachment, and mucositis (28). Medications such as sulfonamides, antiepileptics, non-steroidal anti-inflammatory drugs (NSAIDS), and allopurinol are associated with SJS. Therefore, a detailed drug history is required. Other manifestations such as arthralgia or muscle pain are rarely observed. And Raynaud phenomenon can be seen in *M. pneumoniae* infection secondary to cold agglutination formation (29) (**Table 2**).

TABLE 2 | Physical findings and clinical entity.

	Physical findings	Clinical entity
Ear, nose, throat	Ear pain without discharge Frontal headache Sore throat	Otitis media Sinusitis Pharyngitis
Neck	Mild lymphadenopathy without tenderness	Lymphadenitis
Lung	Late inspiratory crackles Polyphonic wheeze	Pneumonia Bronchiolitis
Heart	Palpitation Chest pain	Rhythm disturbance Myocarditis, thrombosis
Skin	Maculopapular or vesicular rash	Stevens–Johnson syndrome
Central nervous system	Headache, consciousness disturbance Tenderness of forehead Neck stiffness Progressive muscle weakness Back pain, paralysis Behavior change hemiparesis	Encephalitis Meningitis Guillain–Barrë Syndrome Acute transverse Myelitis Acute disseminated encephalomyelitis

Central Nervous System

We observed that 0.1% of all mycoplasma patients sometimes show central nervous system involvement (30). Fever, headache, and consciousness disturbance suggest encephalitis (31). Tenderness of forehead and neck stiffness is associated with meningitis. In addition, Guillain–Barrë syndrome may develop from mycoplasma infection if patients show progressive muscle weakness initiating in the lower extremities (32, 33), although this complication is quite rare. However, early recognition of neurological abnormality is important for prevention of neurological sequelae. In addition, acute transverse myelitis (ATM) and immunological infection, such as acute disseminated encephalomyelitis (ADEM), can result in some of the most severe complications associated with mycoplasma infection (34). Key symptoms of ATM are acute back pain and paralysis. ADEM usually show behavior change and hemiparesis with monophasic. The pathogenesis of CNS involvement of mycoplasma infection remains unknown. Direct infection or immune-mediated reactions are possible mechanisms (30).

When we examine a mycoplasma patient, we should exclude potential diagnoses based on key symptoms and characteristic physical findings and consider the clinical epidemiology.

RADIOLOGICAL FINDINGS

The chest radiograph shows bilateral reticulonodular or patchy consolidation across both lower lobes. In addition, mycoplasma tends to spread at the respiratory bronchiole resulting in alveolar collapse. Therefore, important findings from a chest radiograph are elevation of the diaphragm or downward shift of the minor fissure associated with volume loss. In children, volume loss is often associated with plastic bronchitis (35). A pleural effusion is rarely observed in adult cases. An increased risk of multilobar opacities was found among older or male patients with *M. pneumoniae* pneumonia (odds ratio, 1.065 and 3.279; 95% confidence interval, 1.041–1.089 and 1.812–5.934; $p < 0.001$ and $p < 0.001$, respectively). Patients with *M. pneumoniae* pneumonia showing multilobar opacities or consolidation had a significantly longer hospital length of stay ($r = 0.111$, $r = 0.275$; $p < 0.033$, $p < 0.001$, respectively) (36). Chest computed tomography (CT) without contrast material shows bronchovascular bundle (BVB) thickening and centrilobular nodules (37, 38) (**Table 3**). These two findings are consistent with the affinity of mycoplasma to airway cilia and the bronchioles. Other findings are consolidation, atelectasis,

TABLE 3 | Imaging findings from atypical pneumonia.

	BVB thickening	Centrilobular nodule	Volume loss	Consolidation	Pleural effusion
Legionella pneumonia	+	−	+	+++ (inhomogeneous)	+++
Mycoplasma pneumonia	++	+++	+	+	+ (child)
Chlamydophila pneumonia	−	+	+	++	−

Ref. (39).
BVB, bronchovascular bundle; GGO, ground glass opacity.

and ground glass opacity (GGO). In summary, imaging findings of mycoplasma are variable, although volume loss is often observed. For evaluation of volume loss, a serial chest radiograph is quite useful compared with chest CT, although if we use CT for detecting volume loss, displacement of the major fissure or minor fissure is key sign.

DIFFERENTIAL DIAGNOSIS

Legionella Pneumonia

With regard to Legionnaires' disease, the patient usually exhibits focal consolidation at the initial phase with these shadows bilaterally spread across the lung field later. One of the most common findings is peribronchovascular consolidation and bilateral pleural effusion (38). Approximately 70% of the patients show pleural effusion within 1 week (40).

Chlamydophila Pneumonia

Typical findings of chlamydophila pneumonia are pan-lobular or non-segmental consolidation (39). These radiological findings are similar to pneumococcal pneumonia. In addition, these patients sometimes show volume loss associated with organizing pneumonia.

Viral Pneumonia

Generally, primary viral pneumonia shows bilateral GGO (41). First, influenza A pneumonia often shows bilateral GGO and reticulation. However, both centrilobular nodule and bronchovascluar bundle thickening are usually absent. Second, human metapneumovirus (HMV) typically causes peribronchial thickening and linear shadowing. But, centrilobular nodule is rarely seen. In immunocompromised patients, we sometimes observe cytomegalo virus (CMV) pneumonia. In CMV pneumonia, bilateral GGO and a random distribution of small nodules is usually observed because the spread of infection of CMV is hematogenous. On the other hand, centrilobular nodule is often seen in Mp pneumonia. With these typical findings based on anatomical location, radiological distinction is possible from Mp pneumonia.

Pneumocystis Pneumonia

We sometimes observe pneumocystis pneumonia (PCP) in immunocompromised patients, particularly patients who receive prednisolone without PCP prophylaxis such as a sulfa-containing drug. Typical imaging patterns of PCP are bilateral perihilar GGO and reticulation, with these shadows usually showing peripheral sparing. However, HIV-associated PCP often causes multiple cavities in the upper lung field.

In conclusion, comprehensive examination of patients with mycoplasma pneumonia is quite important because these patients often show variable extra-pulmonary manifestations. From a radiological perspective, understanding the favorite site of infiltration in mycoplasma pneumonia is crucial, based on the understanding of mycoplasma pathogenesis and lung anatomy.

Using five senses for the comprehensive understanding of mycoplasma pneumonia is the key point.

AUTHOR CONTRIBUTIONS

The author wrote general findings from physical and radiological examination of patients with *Mycoplasma pneumoniae* pneumonia.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 28 January 2016
doi: 10.3389/fmicb.2016.00023

Classification of Extrapulmonary Manifestations Due to *Mycoplasma pneumoniae* Infection on the Basis of Possible Pathogenesis

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The list of extrapulmonary manifestations due to *Mycoplasma pneumoniae* infection can be classified according to the following three possible mechanisms derived from the established biological activity of *M. pneumoniae*; (1) a direct type in which the bacterium is present at the site of inflammation and local inflammatory cytokines induced by the bacterium play an important role (2) an indirect type in which the bacterium is not present at the site of inflammation and immune modulations, such as autoimmunity or formation of immune complexes, play an important role, and (3) a vascular occlusion type in which obstruction of blood flow induced either directly or indirectly by the bacterium plays an important role. Recent studies concerning extrapulmonary manifestations have prompted the author to upgrade the list, including cardiac and aortic thrombi as cardiovascular manifestations; erythema nodosum, cutaneous leukocytoclastic vasculitis, and subcorneal pustular dermatosis as dermatological manifestations; acute cerebellar ataxia, opsoclonus-myoclonus syndrome, and thalamic necrosis as neurological manifestations; pulmonary embolism as a respiratory system manifestation; and renal artery embolism as a urogenital tract manifestation. Continuing nosological confusion on *M. pneumoniae*–induced mucositis (without skin lesions), which may be called *M. pneumoniae*-associated mucositis or *M. pneumoniae*-induced rash and mucositis separately from Stevens-Johnson syndrome, is argued in the dermatological manifestations. Serological methods are recommended for diagnosis because pneumonia or respiratory symptoms are often minimal or even absent in extrapulmonary manifestations due to *M. pneumoniae* infection. Concomitant use of immunomodulators, such as corticosteroids or immunoglobulins with antibiotics effective against *M. pneumoniae*, can be considered as treatment modalities for most severe cases, such as encephalitis. Further studies would be necessary to construct a comprehensive therapeutic strategy, covering microbiology (antibiotics), immunology (immunomodulators), and hematology (anticoagulants). The possible influence of the emergence of macrolide-resistant *M. pneumoniae* on extrapulmonary manifestations, which can be considered of limited clinical threat in Japan where the resistant rate has currently decreased, is discussed on the basis of unique biological characteristics of *M. pneumoniae*, the smallest self-replicating organism.

Keywords: pneumonia, cytokine, interleukin-18, autoimmunity, immune complex, vasculitis, vasculopathy, macrolide resistance

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *19 November 2015*
Accepted: *11 January 2016*
Published: *28 January 2016*

Citation:
Narita M (2016) Classification of Extrapulmonary Manifestations Due to Mycoplasma pneumoniae Infection on the Basis of Possible Pathogenesis. Front. Microbiol. 7:23. doi: 10.3389/fmicb.2016.00023

INTRODUCTION

Mycoplasma pneumoniae has been known to cause a wide variety of extrapulmonary diseases, including several organs of the human body, but its pathomechanisms remain largely unknown. Following is a list of extrapulmonary manifestations due to *M. pneumoniae* infection classified according to the three possible pathomechanisms: (1) a direct type in which the bacterium is present at the site of inflammation and local inflammatory cytokines induced by the bacterium play an important role (2) an indirect type in which the bacterium is not present at the site of inflammation and immune modulations, such as autoimmunity or formation of immune complexes, play an important role (3) a vascular occlusion type in which obstruction of blood flow induced either directly or indirectly by the bacterium plays an important role (Narita, 2009, 2010, 2011a). Several years have passed since the initial list was presented and recent studies have prompted the author to upgrade the list (**Table 1**). While constructing the list, according to the primary policy (Narita, 2009, 2010, 2011a), diseases that can reasonably be considered true extrapulmonary manifestations due to *M. pneumoniae* infection on the basis of established biological ability of *M. pneumoniae* were preferentially selected, although it is hard to prove exactly the causal relation between *M. pneumoniae* infection and the development of diseases in indirect type manifestations. Because more recent studies are preferentially cited in this review, many fundamentally important matters are not mentioned here; frequent absence of pneumonia in the direct type manifestations, cold agglutinins in hematological manifestations, autoantibodies in neurological manifestations, and immunodeficiency in arthritis, among others. Also refer to the previous reviews (Narita, 2009, 2010, 2011a) for further discussions on those matters.

Cardiovascular System Manifestations

Cardiac thrombi in the left atrium (Bakshi et al., 2006), in the right ventricle (Nagashima et al., 2010), and an aortic thrombus (Flateau et al., 2013) have been reported as the vascular occlusion type manifestation of the cardiovascular system. Interestingly, all the cases revealed the existence of some type of antiphospholipid antibodies in the blood, such as anticardiolipin antibody and lupus anticoagulant, which can be raised during *M. pneumoniae* infection through molecular mimicry between *M. pneumoniae* cell components and human phospholipids (Narita, 2011a). A mechanism speculating about how these antibodies modulate the coagulation system leading to thrombosis is incompletely understood. These antibodies in most cases disappear during convalescence and the hypercoagulable state does not last for many months. Pneumonia may or may not be present. A short comprehensive review on this topic is presented in (Flateau et al., 2013).

While Kawasaki disease associated with *M. pneumoniae* infection is not unusual in Japan (Narita, 2010, 2011a) and may be found in Korea (Lee et al., 2011), the disease association is rarely reported outside Asia; however, few recent cases were observed in Italy (Vitale et al., 2010) and the United States (Ebrahim et al., 2011). Considering that pneumonia is not a hallmark of mycoplasmal infection, further surveys outside Asia would more precisely delineate the occurrence of this disease association among different ethnic groups. A short analytical review on this topic can be found in (Lee et al., 2011).

A recent report from China on myocardial damages during *M. pneumoniae* infection presented a little evidence for some type of immune modulation by *M. pneumoniae* (Fan et al., 2015).

Dermatological Manifestations

Erythema nodosum, which is considered to be an immune-mediated disease, mainly affects young women (<30 years old) and is characterized clinically by tender erythematous nodules (diameter > 1 cm) on lower legs and histologically by septal panniculitis (Cribier et al., 1998; Kakourou et al., 2001). While its frequency among mycoplasmal infections has been reported to be rather small, that is, in 3/27 (11%) patients with established etiology (Kakourou et al., 2001) or 1/32 (3.1%) patients undergoing mycoplasmal serology testing (Cribier et al., 1998), increasing awareness of the disease association (Kano et al., 2007; Schalock and Dinulos, 2009; Shimizu et al., 2012) allows it to become a subject of specific reviews (Greco et al., 2015; Terraneo et al., 2015). This disease must be included in the indirect type manifestations. Pneumonia is infrequent in this disease.

Cutaneous leukocytoclastic vasculitis is a pathological entity of skin disease characterized histologically by a neutrophilic perivascular infiltrate and clinically by erythematous macropapular rash mainly on lower extremities; it resembles erythema nodosum but is less tender and smaller in size (Kakourou et al., 2001). Several cases of this disease have been reported in association with *M. pneumoniae* infection (Van Bever et al., 1992; Perez et al., 1997; Perez and Montes, 2002; Greco et al., 2007; Trčko et al., 2012; Lee et al., 2015; Terraneo et al., 2015). Interestingly the reported cases were almost always accompanied by other organ involvement such as glomerulonephritis (Lee et al., 2015), arthritis (Perez et al., 1997; Lee et al., 2015), or arthralgia (Trčko et al., 2012), retinal vasculitis (Greco et al., 2007), encephalitis (Perez and Montes, 2002), and acute respiratory distress syndrome, erythema multiforme, and pancreatitis (Van Bever et al., 1992). Because circulating immune complexes are considered to play a critical role in the pathogenesis of cutaneous leukocytoclastic vasculitis (Van Bever et al., 1992; Perez and Montes, 2002; Trčko et al., 2012), this must be a partial manifestation of systemic vasculitic disease as a consequence of immune dysregulation elicited by an *M. pneumoniae* infection. Pneumonia may or may not be present.

Nosological confusion still exists concerning the spectrum of skin and mucous membrane diseases, including Stevens-Johnson syndrome (SJS), Fuchs syndrome, toxic epidermal necrolysis, and erythema multiforme major (Schalock and Dinulos, 2009; Wetter and Camilleri, 2010; Kunimi et al., 2011; Meyer Sauteur et al., 2012; Canavan et al., 2015a; Vujic et al., 2015). It has been acknowledged that *M. pneumoniae* is the most frequent infectious agent identified in "typical SJS" (Schalock and Dinulos, 2009; Wetter and Camilleri, 2010; Kunimi et al., 2011), presenting

TABLE 1 | Extrapulmonary manifestations due to *M. pneumoniae* infection classified according to the involved pathomechanisms.

Manifestations	Direct type[a]	Indirect type[b]	Vascular occlusion type[c]	Undetermined[d]
Cardiovascular system	Pericarditis, Endocarditis	Myocarditis, Kawasaki disease	Cardiac thrombus, Aortic thrombus	
Dermatological		Erythema multiforme, Urticaria, Anaphylactoid purpura, EN, CLV, SJS, MPAM, SPD		
Digestive organ	Early onset hepatitis	Late onset hepatitis	Pancreatitis	
Hematological/ Hematopoietic system		Autoimmune hemolytic anemia, Hemophagocytic syndrome, Thrombocytopenic purpura, Infectious mononucleosis	Disseminated intravascular coagulation, Splenic infarct	
Musculoskeletal system	Arthritis			Rhabdomyolysis
Neurological	Early onset encephalitis, Early onset myelitis, Aseptic meningitis	Late onset encephalitis, Late onset myelitis, Guillain-Barré syndrome, Cranial/peripheral neuropathies, Cerebellitis, Acute cerebellar ataxia, Opsoclonus-myoclonus syndrome	Stroke, Psychological disorders, Striatal necrosis, Thalamic necrosis	Acute disseminated encephalomyelitis
Respiratory system			Pulmonary embolism	
Sensory organ	Otitis media	Conjunctivitis, Iritis, Uveitis	Sudden hearing loss	
Urogenital tract		Glomerulonephritis, IgA nephropathy	Priapism, Renal artery embolism	

[a] *M. pneumoniae causes inflammation at the local site through the induction of cytokines.*
[b] *M. pneumoniae causes inflammation through immune modulation such as autoimmunity, or formation of immune complexes.*
[c] *M. pneumoniae causes vasculitic and/or thrombotic vascular occlusion with or without systemic hypercoagulable state.*
[d] *Either or all of the above three types of mechanisms may be involved.*
Underlines indicate diseases which are newly included in the panel or grouped into a different type of manifestation from the previous list (Narita, 2010).
EN, erythema nodosum; CLV, cutaneous leukocytoclastic vasculitis; SJS, Stevens-Johnson syndrome; MPAM, Mycoplasma pneumoniae-associated mucositis; SPD, Subcorneal pustular dermatosis.

with fever, conjunctivitis, stomatitis, generalized, often bullous cutaneous lesions (macules and flat atypical target lesions) involving <10% of body surface, and severe morbidity and substantial mortality (Meyer Sauteur et al., 2012). Moreover, an outbreak of *M. pneumoniae*-associated "typical SJS" was recently reported (Olson et al., 2015). In parallel with this, presence of another distinct form of diseases mimicking SJS and without skin lesions, is widely noticed and is often called as "atypical SJS" or "incomplete SJS." Although universal agreement has not yet been established, those can be a distinct entity and are presently called *M. pneumoniae*-associated mucositis (Schalock and Dinulos, 2009; Meyer Sauteur et al., 2012; Vujic et al., 2015) or *M. pneumoniae*-induced rash and mucositis (Canavan et al., 2015a,b; Norton, 2015). From a clinical point of view, *M. pneumoniae*-associated mucous membrane diseases, irrespective of whether they are "typical" or "atypical," have been considered less severe, often sensitive to corticosteroid therapy, and with fundamentally good prognosis when compared with drug-induced diseases (Schalock and Dinulos, 2009; Wetter and Camilleri, 2010; Kunimi et al., 2011; Meyer Sauteur et al., 2012; Canavan et al., 2015a; Vujic et al., 2015). These factors may be important especially for physicians in treating this spectrum of diseases, because to distinguish *M. pneumoniae*-associated diseases from drug-induced diseases early in the course allows them to predict the better prognosis for the *M. pneumoniae*-associated diseases. One factor which favors the *M. pneumoniae*-associated rather than the drug-induced diseases is younger age as it occurs more often in children and younger adults (Wetter and Camilleri, 2010; Kunimi et al., 2011; Canavan et al., 2015b; Norton, 2015). Meanwhile, three independent reports have similarly pointed out that severe ocular lesions were fairly frequent in the *M. pneumoniae*-associated diseases compared to the drug-induced diseases (Wetter and Camilleri, 2010; Kunimi et al., 2011; Olson et al., 2015), and might be an additional diagnostic indicator. Regarding pathogenesis, immunological mechanisms such as autoimmunity and immune complex-mediated vascular injury have been suspected irrespective of whether it is *M. pneumoniae*-associated

or not. Some authors have speculated that the synergistic effects of *M. pneumoniae* infection (and ensuing immune dysregulation) and drug exposure are important factors in developing mucous lesions (Schalock and Dinulos, 2009; Shimizu et al., 2012; Kurata et al., 2016). The reason why the *M. pneumoniae*-associated lesions are confined to the mucous membranes remains unclear. Lastly, the fact that *M. pneumoniae* was isolated from skin blister fluid on at least two independent occasions must not be ignored, which suggests the possibility of a direct type mechanism (Lyell et al., 1967; Meseguer et al., 1986). Because *M. pneumoniae* can never infect squamous cell epithelium, hematogenous transfer of *M. pneumoniae* from the respiratory tract to the skin might generate the inflammatory bullous lesions through the induction of cytokines.

Although very rare, subcorneal pustular dermatosis must be associated with *M. pneumoniae* infection (Lombart, 2014; Bohelay et al., 2015), which can be considered an indirect manifestation.

Digestive Organ Manifestations

A report on liver dysfunction in adults further substantiated the premise that hepatitis can be grouped into the two categories, consisting of the early- and late-onset types, the former being reported to occur at a median of 4 days from the respiratory onset and the latter at 13 days (Shin et al., 2012). Molecular mimicry between mycoplasmal cell components and sialo-oligosaccharides displayed on hepatic cell surfaces was speculated as a pathomechanism for the late-onset type. Another recent report on hepatic damages during *M. pneumoniae* infection also presented a little evidence for some type of immune modulation by *M. pneumoniae* (Fan et al., 2015).

A case report on necrotizing pancreatitis (Yang et al., 2015) favors vascular occlusion as the etiology of acute pancreatitis associated with *M. pneumoniae* infection as previously suggested (Van Bever et al., 1992; Narita, 2010).

Hematological/Hematopoietic System Manifestations

A case of splenic artery embolism was reported as a vascular occlusion type manifestation (Flateau et al., 2013).

Musculoskeletal System Manifestations

We have reported that production of tumor necrosis factor-α might play a role in the pathogenesis of rhabdomyolysis associated with *M. pneumoniae* infection (Oishi et al., 2012).

Neurological Manifestations

Opsoclonus-myoclonus syndrome is a rare neurological disorder characterized by involuntary, irregular, and multidirectional eye movements with myoclonus predominantly affecting the head and trunk and signs of cerebellar ataxia, especially the inability to stand and walk. While this syndrome has been known to occur in association with neuroblastomas in infants between 6 and 36 months and with various types of malignant tumors in adults, infectious etiologies are also known. Recent studies on this syndrome in association with *M. pneumoniae* infection in children (Huber et al., 2010; Shiihara and Takahashi, 2010) as well as in adults (Mesraoua et al., 2011; Nunes et al., 2011), which have suggested immune pathogenesis, have made it reasonable to assume that this syndrome can be included in the indirect type manifestations.

In addition to striatal necrosis (Narita, 2009, 2011a), brain diseases, in which characteristic bilateral lesions are observed on neuroimaging, have not infrequently been reported in association with *M. pneumoniae* infection. The affected areas include the pons (Perez and Montes, 2002), thalamus (Ashtekar et al., 2003), basal ganglia, and thalamus (Fusco et al., 2010), striatum and brain stem (Bae et al., 2011), and splenium of corpus callosum (Shibuya et al., 2012). Vasculitic necrosis is considered a presumptive etiology in some (Perez and Montes, 2002; Ashtekar et al., 2003), and immune pathogenesis is considered in others (Fusco et al., 2010; Bae et al., 2011). In this context, the clinical picture of a case presented in Perez and Montes (2002) fairly resembles that of acute necrotizing encephalopathy, which favors vasculitic etiology (Narita, 2002), whereas the intrathecal production of interleukins-6 and -8 was found in the patients with striatal necrosis (Yuan et al., 2015), as reported in the cases of encephalitis (Narita et al., 2005), which favors immune pathogenesis. A recent comprehensive study suggested that these manifestations are not peculiar but is a common form of encephalitis in children (Al-Zaidy et al., 2015). Representative of these diseases, both striatal and thalamic necrosis are included in the list of this review as vascular occlusion type manifestations. Meanwhile, some nosological alterations might be necessary concerning the use of the term "necrosis" for the fundamentally benign, reversible lesions, which are a characteristic of *M. pneumoniae* infection (Fusco et al., 2010).

Concerning disseminated encephalomyelitis, a recent paper reported that the genome of *M. pneumoniae* was detected in cerebrospinal fluid, suggesting the direct type mechanism (Matsumoto et al., 2009), whereas another paper reported a dramatic improvement after plasma exchange, suggesting the indirect type mechanism leading to vasculopathy (Gupta et al., 2009). The aforementioned study has suggested that a single etiology cannot explain the pathogenesis of acute disseminated encephalomyelitis (Al-Zaidy et al., 2015). In addition, the study also suggested that the classification for encephalitis can also be applied to transverse myelitis (the early onset, direct type or the late onset, indirect type).

Two cases of transient Parkinsonism in association with *M. pneumoniae* infections have recently been reported (Tay et al., 2014). With more attention given to *M. pneumoniae* infections even in the absence of respiratory symptoms, more cases of *M. pneumoniae* infection-associated psychological disorders would be diagnosed especially when it is transient and occurs in children or young adults.

Cerebellitis has been constantly reported as part of a disease involving multiple parts of the brain (Christo et al., 2010; Bae et al., 2011; Meyer Sauteur et al., 2014a) or as an isolated disease (Shkalim et al., 2009; Simpkins et al., 2012; Schmucker et al., 2014). Immune-mediated pathogenesis has been advocated for cerebellitis and is most likely an indirect type manifestation. The fact that lymphocytic infiltration was found in the cerebellar tissue in the late onset case (Simpkins et al.,

2012) is of some interest because the neutrophilic infiltration has typically been found in the cerebral tissues of early onset cases (Bruch et al., 2001; Stamm et al., 2008). Implication of these observations on pathogenesis remains unclear, but accumulation of histological investigations should provide us with clues for further understanding of the pathogenesis of neurological manifestations.

In acute cerebellar ataxia the evidence of neural inflammation is apparently absent and an immune-mediated pathogenesis has been suggested (Cimolai et al., 1994); this has been added to the list separately from cerebellitis.

Although it is still premature to conclude that it is a true manifestation of *M. pneumoniae* infection, a peculiar and rather pathologically identified disease entity called "mycoplasmal cerebral vasculopathy" has recently been suggested (Zu-Rhein et al., 2007; Ferreira, 2011; Rhodes et al., 2011). Because of its slowly progressive clinical course that occurs over several years with episodic encephalopathy and movement disorders, it is hard to determine whether the disease constitutes a distinct clinical entity. Nevertheless, the findings that *M. pneumoniae* antigens were found in the cytoplasm of brain microvascular endothelial cells as well as in microvascular lumina by histological investigations are highly interesting. This appears to further support the ability of hematogenous transfer of *M. pneumoniae* to the brain and to elicit vasculitis or vasculopathy at those local sites.

At the end of this section, a schematic presentation is shown in **Figure 1** for the neurological manifestations due to *M. pneumoniae* infection classified according to the possible pathomechanisms, which can be perplexing and sometimes overlapping. This figure suggests one possibility and is amenable to improvement by future studies. More information on neurological manifestations is presented in recent studies (Bitnun and Richardson, 2010; Meyer Sauteur et al., 2014b).

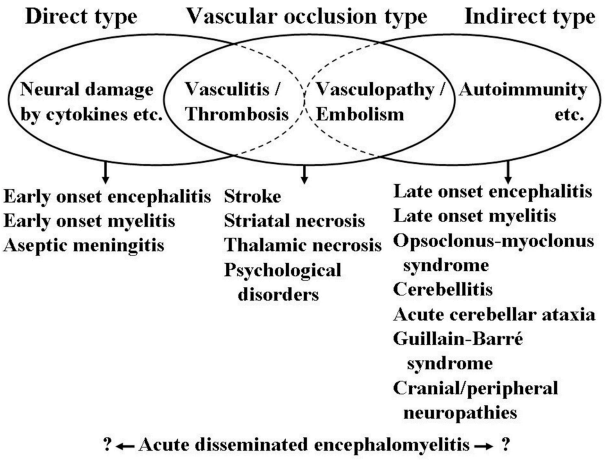

FIGURE 1 | Schematic presentation of neurological manifestations due to *M. pneumoniae* infection classified according to the possible pathomechanisms. Cytokines involving interleukin-6 and interleukin-8, among others. Vasculitis, thrombosis, vasculopathy, and embolism involving formation of immune complexes, production of antiphospholipid antibodies, among others. Autoimmunity involving molecular mimicry between mycoplasmal cell components and gangliosides, and galactocerebrosides, among others. These mechanisms are not mutually exclusive and can concomitantly work in a patient resulting in perplexed clinical features of neurological manifestations.

Respiratory System Manifestations

A few cases of pulmonary embolism have been reported in association with *M. pneumoniae* infection (Sterner and Biberfeld, 1969; Brown et al., 2008; Graw-Panzer et al., 2009). Production of antiphospholipid antibodies has been shown to be an underlying mechanism for thrombus formation (Brown et al., 2008; Graw-Panzer et al., 2009).

Urogenital Tract Manifestations

Each case of glomerulonephritis (Shimizu et al., 2012) or glomerulonephritis with interstitial nephritis (Lee et al., 2015) was recently reported along with multiple skin lesions mentioned in the previous dermatological section. The genome of *M. pneumoniae* was detected by polymerase chain reaction in a serum sample from a patient with glomerulonephritis (Chen et al., 2015), which further substantiated the assumption that circulating immune complexes containing mycoplasmal cell components are probably involved in the pathogenesis.

A case of renal artery embolism was reported in a patient with multiple embolisms (Flateau et al., 2013).

A new case of pediatric priapism was recently reported (Jacobs et al., 2015), which substantiated that this condition can be an extremely rare but reasonable vascular occlusion type manifestation.

DIAGNOSIS OF EXTRAPULMONARY MANIFESTATIONS DUE TO *M. pneumoniae* INFECTIONS

Since the primary site of infection and subsequent propagation of *M. pneumoniae* is restricted to the ciliated epithelium of the lower respiratory tract, any existing bacterial cells cannot be transferred to the upper respiratory tract in the absence of strong cough. In this context, as repeatedly mentioned in this and the previous reviews (Narita, 2009, 2010, 2011a), extrapulmonary manifestations due to *M. pneumoniae* infection often occur in the absence of pneumonia or even in the absence of respiratory symptoms. For this reason, molecular detection or culture methods using routine clinical samples obtained from the upper respiratory tract (such as pharyngeal swabs) are not always adequate for diagnosing extrapulmonary manifestations. Therefore, the diagnosis should be done by serological methods, which usually requires obtaining a second serum sample. On a few special occasions, molecular detection methods may be applied for non-respiratory samples, such as cerebrospinal fluid for encephalitis. For cases with abundant cough, point-of-care tests, which have recently been developed in Japan and include the loop-mediated isothermal amplification method (Kakuya et al., 2014; Petrone et al., 2015) or the antigen detection method (Miyashita et al., 2015b), should be used

because they may help in rapid diagnosis during the acute phase.

TREATMENT OF EXTRAPULMONARY MANIFESTATIONS DUE TO *M. pneumoniae* INFECTION

There is no doubt that aberrant host immune responses play a critical role in the development of extrapulmonary manifestations due to *M. pneumoniae* infections. Therefore, immunomodulators, such as corticosteroids or immunoglobulins, should be beneficial for the most severe cases, such as encephalitis or SJS. Moreover, anticoagulation therapy should be highly promising for the vascular occlusion type manifestations. In any of the cases, antibiotics effective against *M. pneumoniae* must be used concomitantly to reduce the amount of *M. pneumoniae* cells in the respiratory tract; this consequently results in the reduction of excessive antigenic stimuli. Larger studies would be necessary to construct the comprehensive therapeutic strategy covering microbiology (antibiotics), immunology (immunomodulators), and hematology (anticoagulants).

POSSIBLE INFLUENCE OF THE EMERGENCE OF MACROLIDE-RESISTANT *M. pneumoniae* ON EXTRAPULMONARY MANIFESTATIONS

Since 2000, when the first case of pneumonia due to macrolide-resistant *M. pneumoniae* was diagnosed in Japan (Okazaki et al., 2001), macrolide resistance has grown to be a significant problem in some countries, particularly in eastern Asia. A few cases of extrapulmonary manifestations due to macrolide-resistant *M. pneumoniae* have been reported (Atkinson et al., 2011; Koga et al., 2012; Oishi et al., 2012; Shen et al., 2013; Zhou et al., 2014). While the studies from China have reported that a substantial number of complications (most frequently liver and myocardial dysfunctions) occurred in patients infected by resistant strains (Shen et al., 2013; Zhou et al., 2014), no appreciable increase in the number of extrapulmonary manifestations has been observed in Japan in conjunction with a significant increase in the number of pneumonia patients infected by the resistant strains. This must be in part due to the impairment of the growth ability of resistant strains of *M. pneumoniae* when compared with the sensitive strains (Ohya et al., 2009, 2010; Pauchant et al., 2009) Some reasons for this are described in the following paragraphs.

M. pneumoniae is one of the smallest self-replicating organisms and has many peculiar biological characteristics. First of all, the most important characteristic associated with drug resistance is that extrinsic genes, such as plasmids or transposons, do not function within *M. pneumoniae* cells under natural conditions (Bébéar and Pereyre, 2005). Consequently, the resistant mechanism of *M. pneumoniae* is exclusively due to a point mutation in the domain V of 23S rRNA. Second, since *M. pneumoniae* has only one operon for constructing ribosomes (Himmelreich et al., 1996), the resistant strains that harbor a point mutation within their ribosome genes are exclusively mutants of ribosomes. Therefore, they suffer from less efficient protein synthesis and are deficient in growth ability (Narita, 2011b). In fact, no excessive morbidity has been observed particularly ascribed to the drug resistance in the reported cases (Atkinson et al., 2011; Koga et al., 2012; Oishi et al., 2012). In addition to that, serum levels of IL-18, which represent disease activity of *M. pneumoniae* infection (Narita et al., 2000; Tanaka et al., 2002; Oishi et al., 2011; Miyashita et al., 2015a), were rather lower in patients with pneumonia infected by the resistant strains than in patients infected by the sensitive strains (Matsuda et al., 2013). Taken together, the emergence of macrolide-resistant *M. pneumoniae* must not be a significant clinical threat concerning extrapulmonary manifestations of *M. pneumoniae* infection at least in Japan, where the resistant rate has fundamentally been decreasing according to the current data obtained from 2013 to 2015 (presented at domestic Japanese meetings).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 01 June 2016
doi: 10.3389/fmicb.2016.00800

Clinical Features of Severe or Fatal *Mycoplasma pneumoniae* Pneumonia

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Mycoplasma pneumoniae is one of the most common causes of community-acquired pneumonia in children and young adults. The incidence of fulminant *M. pneumoniae* pneumonia (MPP) is relatively rare despite the high prevalence of *M. pneumoniae* infection. This literature review highlights the clinical features of fulminant MPP by examining the most recent data in epidemiology, clinical presentation, pathogenesis, and treatment. Fulminant MPP accounts for 0.5–2% of all MPP cases and primarily affects young adults with no underlying disease. Key clinical findings include a cough, fever, and dyspnea along with diffuse abnormal findings in radiological examinations. Levels of inflammatory markers such as white blood cells and C-reactive protein are elevated, as well as levels of lactate dehydrogenase, IL-18, aspartate transaminase, and alanine transaminase. The exact pathogenesis of fulminant MPP remains unclear, but theories include a delayed hypersensitivity reaction to *M. pneumoniae* and the contribution of delayed antibiotic administration to disease progression. Treatment options involve pairing the appropriate anti-mycoplasma agent with a corticosteroid that will downregulate the hypersensitivity response, and mortality rates are quite low in this treatment group. Further research is necessary to determine the exact pathogenesis of severe and fulminant types of MPP.

Keywords: *Mycoplasma pneumoniae*, corticosteroids, fulminant pneumonia, hyperimmune response, LDH

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *01 December 2015*
Accepted: *11 May 2016*
Published: *01 June 2016*

Citation:
Izumikawa K (2016) Clinical Features of Severe or Fatal Mycoplasma pneumoniae Pneumonia. Front. Microbiol. 7:800. doi: 10.3389/fmicb.2016.00800

INTRODUCTION

Mycoplasma pneumoniae is a common cause of atypical pneumonia often seen in youths and accounts for 10–15% of cases in Japan (Ishida et al., 2004; Miyashita et al., 2005). *M. pneumoniae* pneumonia (MPP) is typically mild and characterized by a persistent dry cough, and sometimes self-limiting pneumonia cured with no medication, fulminant cases with severe complications such as respiratory failure, hypoxia, and others have been recognized. Key clinical findings of fulminant MPP involve respiratory failure with diffuse consolidation or an abnormal interstitial pattern on a chest radiograph. Material for this review is based on two review articles by Chan and Welsh (1995) and Izumikawa et al. (2014), along with one case series study by Miyashita et al. (2007). **Table 1** indicates the characters of these three articles. Although the definition of "fulminant MPP" has not been established, we defined "fulminant MPP"

TABLE 1 | Two review articles and one case series article.

Reference	Type of article	Number of cases	Unique points
Chan and Welsh, 1995	Review	46	Three category; Non-fatal respiratory failure ($n = 26$), fatal respiratory failure ($n = 13$), and fatal without respiratory failure ($n = 7$)
Miyashita et al., 2007	Case series	13	13 cases with acute respiratory failure (ARF) and 214 cases without ARF
Izumikawa et al., 2014	Review	52	All cases with respiratory failure

as confirmed MPP cases with respiratory failure or fatal cases without respiratory failure in this review.

EPIDEMIOLOGY

Approximately 0.5–2% of all MPP cases are the fulminant type, but the exact frequency of fulminant MPP is unclear (Chan and Welsh, 1995; Miyashita et al., 2007). Chan and Welsh (1995) reported that fulminant MPP occurs most frequently in adolescent males, especially in those with a history of smoking. Japanese fulminant MPP cases indicated no such trend (Miyashita et al., 2007; Izumikawa et al., 2014). Our data revealed that almost 50% of the 52 fulminant MPP cases occurred in patients aged 20–49 years, and 13.5% occurred in the elderly (age > 70 years). Only Four cases occurred in younger patients (age < 20 years). Although several cases of fulminant MPP were reported among children (Park et al., 2012), accurate frequency has not been described yet. The actual mortality rate in fulminant MPP cases is also unknown, with Chan and Welsh (1995) reporting 3–5% in 1980s and our review indicating a total of 2 deaths among 52 Japanese fulminant MPP cases, which is relatively lower (Izumikawa et al., 2014). Chan reported seven fatal cases of MPP without respiratory failure and causes of death included pulmonary thromboembolism, myocarditis, pneumonia, cerebritis, and psychosis. Extrapulmonary complications may occur, but there are no recent studies or reviews that address how these complications affected mortality rates.

CLINICAL AND LABORATORY FEATURES

The major clinical manifestations of fulminant MPP include a cough, high fever, and hypoxia along with diffuse abnormal findings on radiologic examination. In our review, all 52 patients presented with fever (>37.0°C) on their first visit to the hospital. A relatively high fever (>38.0°C) was observed in 88.5%. Respiratory symptoms such as a cough and dyspnea were observed upon admission in 97.3 and 83.3% of cases, respectively. The frequency of other upper respiratory symptoms and sputum production was lower in fulminant MPP (26.9% had sputum production; Izumikawa et al., 2014). The average duration from onset of infection to the development of respiratory failure was 11.2 days (range, 5–21 days; Izumikawa et al., 2014). Chan and Welsh (1995) and Miyashita et al. (2007) reported durations of 10–15 and 9.3 days, respectively, from onset to first administration of appropriate anti-mycoplasma agents. Both studies reveal a similar timeframe for developing respiratory failure.

Laboratory findings indicated elevated levels of white blood cells (WBC) and C-reactive protein (CRP; Miyashita et al., 2007; Izumikawa et al., 2014). The majority of severe MPP cases exhibited a moderate inflammation response. Liver dysfunction was common in fulminant MPP and indicated by elevations of alanine transaminase (ALT) and aspartate transaminase (AST). The observed liver dysfunction is not necessarily due to direct invasion of the liver tissue by *M. pneumoniae*, and may result from an indirect immunological response by liver tissue to the pathogen. Total protein (TP) and lactate dehydrogenase (LDH) levels are also elevated in fulminant MPP cases (Miyashita et al., 2007; Izumikawa et al., 2014). However, these abnormal findings were not specific to fulminant MPP cases and were present irrespective of disease severity. However, Miyashita et al. (2007) compared the laboratory findings of severe cases with and without acute respiratory failure (ARF), and found that WBC count and levels of CRP, LDH, AST, and ALT were all significantly higher in the ARF cases. Only TP levels were lower in fulminant MPP cases with ARF compared to cases without ARF.

RADIOLOGICAL FEATURES

Various radiological findings are observed in fulminant MPP cases. Miyashita et al. (2007) indicated that bilateral infiltrates and pleural effusion commonly present in MPP cases with ARF compared to those without ARF. Izumikawa et al. (2014) observed a diffuse interstitial pattern (e.g., reticular, nodular, linear) in 61.5% of cases. A diffuse alveolar pattern with or without air bronchogram was observed in 25.0% and a mixed interstitial and alveolar pattern was observed in 13.5%. Pleural effusion was noted in 13.5%, and these were categorized as either alveolar or mixed pattern cases. It is unclear what the differences between these radiological findings indicate, and observed findings may change throughout the course of the illness. Interestingly, the radiological findings and severity of inflammation reflected by inflammation markers indicated that mixed pattern cases had higher CRP levels compared to those of interstitial or alveolar pattern cases, though no statistical differences were observed. These findings indicate a possible correlation between inflammatory level and radiological findings. Furthermore, this indicated that radiological findings are closely related to the body's immunological response to *M. pneumoniae,* and not the local existence of *M. pneumoniae* itself in the affected region of the lung.

PATHOLOGICAL FEATURES

Few case studies describe the pathological findings found through transbronchial lung biopsy, open lung biopsy, and autopsy from fulminant MPP (Izumikawa et al., 2014). Acute bronchiolitis was identified in the early phase of infection, followed by organizing pneumonia and alveolitis with or without granuloma formation in the recovery phase. These findings, however, were not acquired from one specific case. Among fatal MPP cases, very few cases with diffuse alveolar damage have been reported (Chan and Welsh, 1995; Miyashita et al., 2007; Izumikawa et al., 2014).

PATHOGENESIS

The literature details how the host's cell-mediated immunity plays an important role in the development of typical MPP (Evengard et al., 1994; Chan and Welsh, 1995; Waites and Talkington, 2004). Factors associated with the virulence of *M. pneumoniae* include the following: (i) Toxic byproducts of inflammation can cause cell damage through direct interaction between human host cells (e.g., adherence to bronchoepithelial cells, toxin production, reactive oxygen species, and cytokines). (ii) indirect interaction of immunological or allergic reaction to *M. pneumoniae* infection, and this reaction can influence how the disease progresses (Fernald et al., 1981; Tanaka et al., 2002).

Although the mechanism and etiology of fulminant MPP are largely unknown, three possible hypotheses appeared in the literature: (i) a hyperimmune response that originates in the lung as a result of repeated childhood *M. pneumoniae* infections; (ii) loss of the ability to eradicate *M. pneumoniae* from the lung in primary infection resulting in longer-lasting *M. pneumoniae* infection in the lung, which may cause a hyperimmune response, and (iii) overactive innate immune response such as macrophage activation via heterodimerization of Toll-like receptors two and six of the bronchoepithelial cells to *M. pneumoniae* lipoproteins (Takeuchi et al., 2001). Tanaka et al. (2002) demonstrated that the levels of serum IL-18, but not of interferon, were higher in patients with fulminant MPP compared to those in mild cases, and this was correlated with the number of affected lung lobes. Recently, Miyashita et al. (2015) supported the notion of a positive correlation between IL-18 and LDH levels in severe MPP cases. Thus, an excessive host-cellular response with Th1 cytokines and IL-18 may play a critical role in the development of fulminant status.

TREATMENT

All previous reports under review indicated that the delayed use of anti-mycoplasma drugs, such as macrolides (erythromycin, clarithromycin, and azithromycin), tetracyclines, and quinolones contributed to the development of fulminant MPP (Chan and Welsh, 1995; Miyashita et al., 2007; Izumikawa et al., 2014). β-Lactams and aminoglycosides, both of which have no potent activity against *M. pneumoniae* infection, were used as the initial treatment in 61.5% of cases, and rates of both inappropriate and no-treatment cases reached 78.8% (Izumikawa et al., 2014). Corticosteroids are a reasonable treatment option, especially in fulminant MPP cases that present with a hyperactive immune response, given that this class of drugs acts by downregulating the cell-mediated immune response; numerous reports indicate a positive response (Evengard et al., 1994; Chan and Welsh, 1995; Takiguchi et al., 2001; Tsuruta et al., 2002). Currently, there is not enough data available to establish specific pharmacological guidelines to treat fulminant MPP. The literature describes a relatively high dose of methylprednisolone (>500 mg/day) combined with appropriate anti-mycoplasma agents that effectively improved symptoms in the majority of patients within 3–5 days of corticosteroid treatment (Izumikawa et al., 2014). Thirteen cases of severe or refractory MPP cases required mechanical ventilation, and nine of these patients received high dose corticosteroids and anti-mycoplasma agents (Miyashita et al., 2007). Corticosteroid dosage was gradually tapered within a week in almost all cases (Izumikawa et al., 2014). The prolonged or inappropriate usage of corticosteroids may cause excess downregulation of cell-medicated immunity and result in immunosuppression, making the individual more susceptible to a more severe

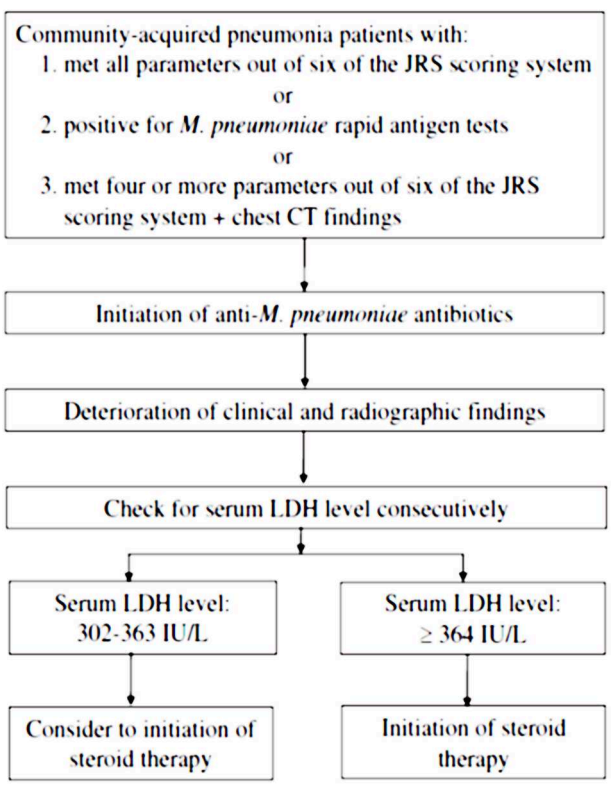

FIGURE 1 | A stepwise algorithm to correlate diagnosis, serum markers, and initiation of corticosteroid therapy in *Mycoplasma pneumoniae* pneumonia (MPP). The permission for usage of this figure was acquired from both Dr. Naoyuki Miyashita and the Journal of Infection and Chemotherapy. JRS, Japanese Respiratory Society. *M. pneumoniae* rapid antigen tests; Ribotest Mycoplasma (Asahi kasei Pharma Co., Tokyo, Japan) and PrimeCheck Mycoplasma Antigen (Alfresa Pharma Co., Osaka, Japan).

M. pneumoniae infection or opportunistic infections such as those by *Pneumocystis*, *Mycobacterium,* or *Cytomegalovirus* species. Careful consideration is required when determining corticosteroid usage.

A recent study recognized that serum LDH levels are significantly higher in cases of severe MPP compared to the control group at the initiation of corticosteroid therapy, and may represent disease severity among adolescents and adults. Lu et al. (2015) also revealed that serum LDH levels can be used as a biomarker to predict refractory MPP in children. A serum LDH level of 302–364 IU/L seems to be an appropriate criterion for the initiation of corticosteroid therapy in severe or refractory MPP among adolescents and adults (Miyashita et al., 2015). **Figure 1** indicates the algorithm of corticosteroid administration in severe and refractory MPP cases (Miyashita et al., 2015). The Japanese Respiratory Society (JRS) scoring system enables to differentiate atypical pneumonia from bacterial pneumonia by positivity of six factors as follows; (i) age < 60 years old, (ii) no or mild co-morbidity, (iii) paroxysmal cough, (iv) poor findings from chest physical examination, (v) no expectoration or no pathogens in rapid diagnostic tests, and (vi) white blood cell count $<10{,}000/mm^3$ (Miyashita et al., 2006). In addition, both of *M. pneumoniae* antigen, Ribotest Mycoplasma (Asahi kasei Pharma Co., Tokyo, Japan) and PrimeCheck Mycoplasma Antigen (Alfresa Pharma Co., Osaka, Japan) were used as rapid antigen tests for diagnosis of MPP (Miyashita et al., 2015).

CONCLUSION

Mycoplasma pneumoniae pneumonia usually causes a mild illness, and mortality is quite low. However, severe or fulminant cases do occur, and these cases require early administration of corticosteroids, along with administration of appropriate anti-mycoplasma agents. To date, there are no reports of an increase in cases of fulminant MPP during the recent outbreak of macrolide-resistant MPP in Japan, but future research is needed to examine the correlation between severity of *M. pneumoniae* infection and drug resistance, and also to establish clinical guidelines for management of fulminant MPP.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Conflict of Interest Statement: KI received honoraria from MSD K.K, Sumitomo Dainippon Pharma Co., Ltd, Pfizer Japan, Inc. and Taisyo Toyama Co., Ltd.

frontiers in Microbiology

REVIEW
published: 11 May 2016
doi: 10.3389/fmicb.2016.00695

Correlation between Radiological and Pathological Findings in Patients with *Mycoplasma pneumoniae* Pneumonia

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Studies focused on the pathological–radiological correlation of human *Mycoplasma* (*M*) *pneumoniae* pneumonia have rarely been reported. Therefore, we extensively reviewed the literature regarding pathological and radiological studies of *Mycoplasma* pneumonia, and compared findings between open lung biopsy specimen and computed tomography (CT). Major three correlations were summarized. (1) Peribronchial and perivascular cuffing characterized by mononuclear cells infiltration was correlated with bronchovascular bundles thickening on CT, which was the most common finding of this pneumonia. (2) Cellular bronchitis in the small airways accompanied with exudates or granulation tissue in the lumen revealed as centrilobular nodules on CT. (3) Neutrophils and exudates in the alveolar lumen radiologically demonstrated as air-space consolidation or ground-glass opacities. In *M. pulmonis*-infected mice model, pathologic patterns are strikingly different according to host cell-mediated immunity (CMI) levels; treatment with interleukin-2 lead to marked cellular bronchitis in the small airways and treatment with prednisolone or cyclosporin-A lead to neutrophils and exudates in the alveolar lumen. Patients with centrilobular nodules predominant radiologic pattern have a high level of CMI, measuring by tuberculin skin test. From these findings, up-regulation of host CMI could change radiological pattern to centrilobular nodules predominant, on the other hand down-regulation of host CMI would change radiological pattern to ground-glass opacity and consolidation. It was suggested the pathological features of *M. pneumoniae* pneumonia may be altered by the level of host CMI.

Keywords: radiological–pathological correlation, open lung biopsy, CT scan, centrilobular nodes, bronchovascular bundles thickening, host cell-mediated immunity

OPEN ACCESS

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *14 January 2016*
Accepted: *26 April 2016*
Published: *11 May 2016*

Citation:
Tanaka H (2016) Correlation between Radiological and Pathological Findings in Patients with Mycoplasma pneumoniae Pneumonia. Front. Microbiol. 7:695. doi: 10.3389/fmicb.2016.00695

INTRODUCTION

The majority of *Mycoplasma (M) pneumoniae* respiratory infection are self-limited. An estimated 3–13% of infected persons with infection experience pneumonia, and the remains are manifested as upper respiratory tract infection [Clyde, 1993; Waites and Talkington, 2004; Walter et al., 2008; Centers for Disease Control and Prevention (CDC), 2013]. Therefore pathological specimens of human *M. pneumoniae* pneumonia are rarely obtained. Pathological descriptions of this pneumonia include marked plasma cell-rich lymphocytic infiltration in peribronchial and perivascular areas, with accumulations of macrophages, neutrophils, and lymphocytes in the

alveolar spaces, foci of interstitial pneumonia, and hyperplasia of type II pneumocytes (Golden, 1944; Forsyth and Chanock, 1966; Meyers and Hirschman, 1972; Chan et al., 1999). Bronchiolitis and alveolitis with dense mononuclear cells infiltration, epithelioid cell granulation tissue filling alveolar ducts, organizing alveolar exudates, and hyaline membranes are characteristic findings in fulminant *M. pneumoniae* pneumonia (Koletsky and Weinstein, 1980; Rollins et al., 1986; Ito et al., 1995; Ebnother et al., 2001; Izumikawa et al., 2014). On the other hand, the patterns of *M. pneumoniae* pneumonia on chest radiography are non-specific segmental or lobar consolidation, bilateral diffuse reticular interstitial infiltrates (Putman et al., 1975). Computed tomography (CT) findings of this pneumonia are bronchovascular thickening, centrilobular nodules, ground-glass attenuation, or air-space consolidation (Tanaka et al., 1985; Tanaka N. et al., 1996; Reittner et al., 2000; Miyashita et al., 2009, 2014). However, there has been little report radiological–pathological correlation in human *M. pneumoniae* pneumonia (Heitzman, 1993). This review focuses on radiological–pathological correlation of *Mycoplasma* pneumonia in mice and humans, and the changes of pulmonary involvement patterns reflecting by host cell-mediated immunity (CMI) levels.

RADIOLOGICAL–PATHOLOGICAL CORRELATION IN ANIMAL MODELS

The pathogenesis of *Mycoplasma* infection has been studied in animal model. The pathological changes and patterns are similar to that seen in experimental infection in hamsters or mice. In **Figures 1A,B**, *M. pulmonis* inoculated mice model reveals that pathological changes consist with (1) peribronchial and perivascular mononuclear cells accumulation throughout large to small airways, (2) cellular bronchiolitis with lumen exudates and mononuclear cell in airway walls extending into adjacent alveoli (Tanaka et al., 1996a). Next, inflated lung specimens of *M. pulmonis* inoculated mice were prepared by Heitzman's (1993) method (Markarian and Dailey, 1993). Radiological findings of the infected lungs disclosed thickening of bronchovascular bundles, centrilobular nodules, and ground-glass opacities (**Figures 1C,D**). Pathological changes clearly reflect to radiological findings in *M. pulmonis* inoculated mice.

IMMUNOMODULATORS CHANGE THE PATHOLOGICAL PATTERN OF *Mycoplasma* PNEUMONIA

The role of T cells in the pathogenesis of *M. pneumoniae* infection can be defined by the apparent correlation of delayed-type hypersensitivity (DTH) skin reaction to *M. pneumoniae* in humans with the severity of disease (Mizutani et al., 1971). To elucidate immune-pathological mechanism of *Mycoplasma* pneumonia, the therapeutic effects of interleukin-2 (IL-2), cyclosporine A (CYA), and prednisolone (PSL) on mice model (Tanaka et al., 1996a). Mice were intra-nasally inoculated with *M. pulmonis* and were treated with IL-2,

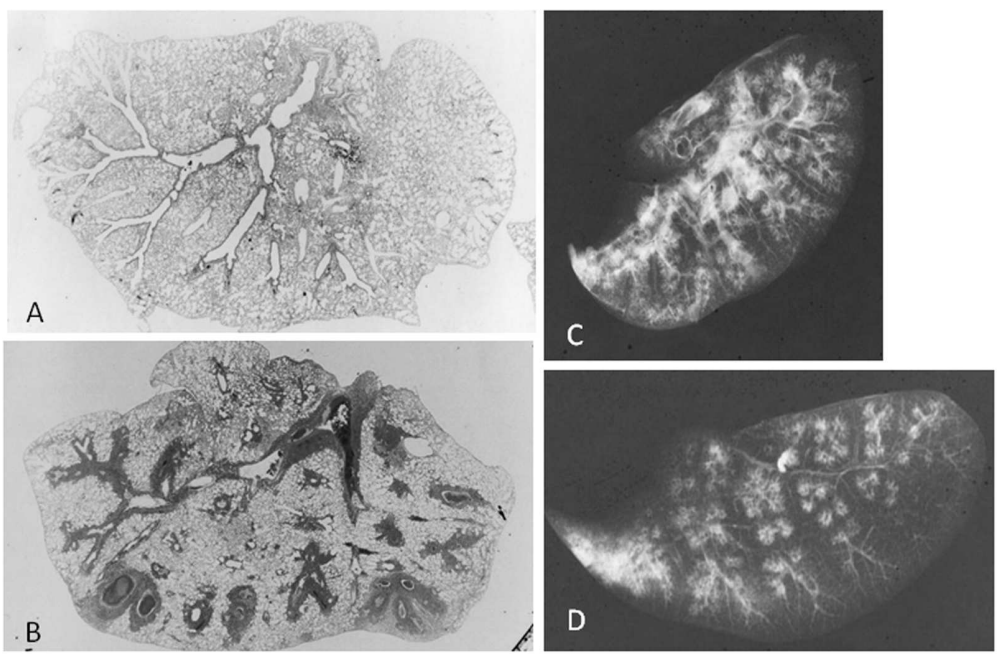


FIGURE 1 | *Mycoplasma pulmonis* infected mice, 2 weeks after inoculation. Low-magnification photomicrographs of non-infected lung **(A)** and infected lung **(B**; HE x17). **(C)** Radiograph of inflated lung of infected mice reveals bronchovascular bundles thickening, nodules, and ground-glass attenuation. **(D)** Radiograph of thin-sliced lung of infected mice shows nodules with centrilobular distribution and consolidation. Reproduced with permission from Tanaka (2016).

CYA, and PSL every day between Days 3 and 9, and were killed at Day 14. IL-2 is immunomodulator, especially up-regulate CMI, and CYA is immunosuppressant, especially down-regulate CMI of the host. PSL is a more powerful immunosuppressant. CMI level of the host was assessed by skin test by sheep red blood cell (SRBC). Peribronchial and perivascular mononuclear cell cuffing and accumulation of macrophages at the end of bronchiole were exacerbated in

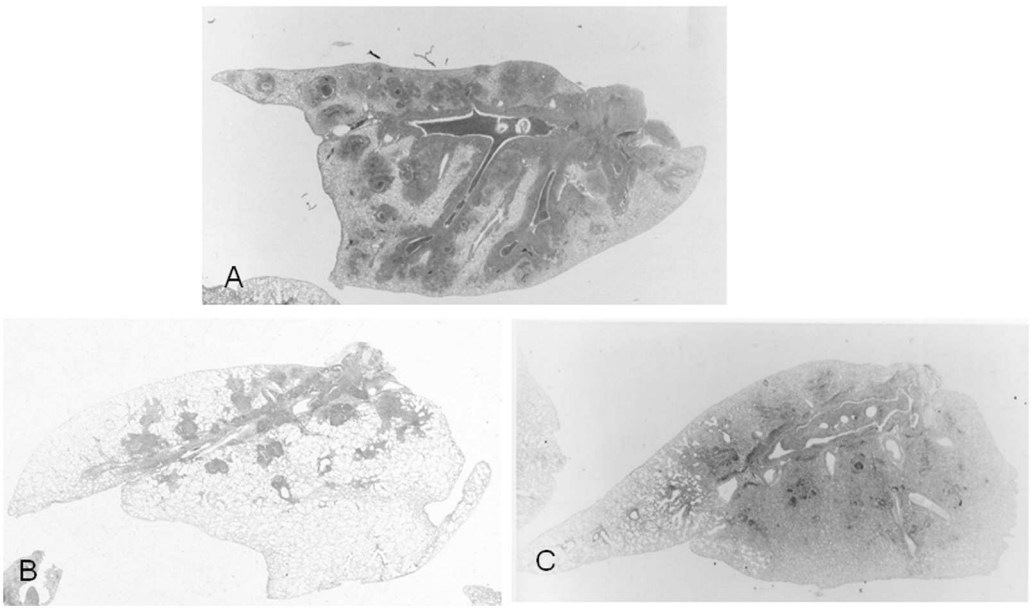


FIGURE 2 | Low-magnification photomicrographs of lung, 2 weeks after *M. pulmonis* inoculation (HE x17). (A) Mice without treatment. **(B)** Mice treated with interleukin-2, showing marked peribronchial and perivascular lymphocyte cuffing, and no intra-alveolar inflammation. **(C)** Mice treated with PSL, disclosing predominance of intra-alveolar inflammatory cell infiltration, and a little peribronchial and perivascular lymphocyte cuffing. Reproduced with permission from Tanaka and Tamura (1989).

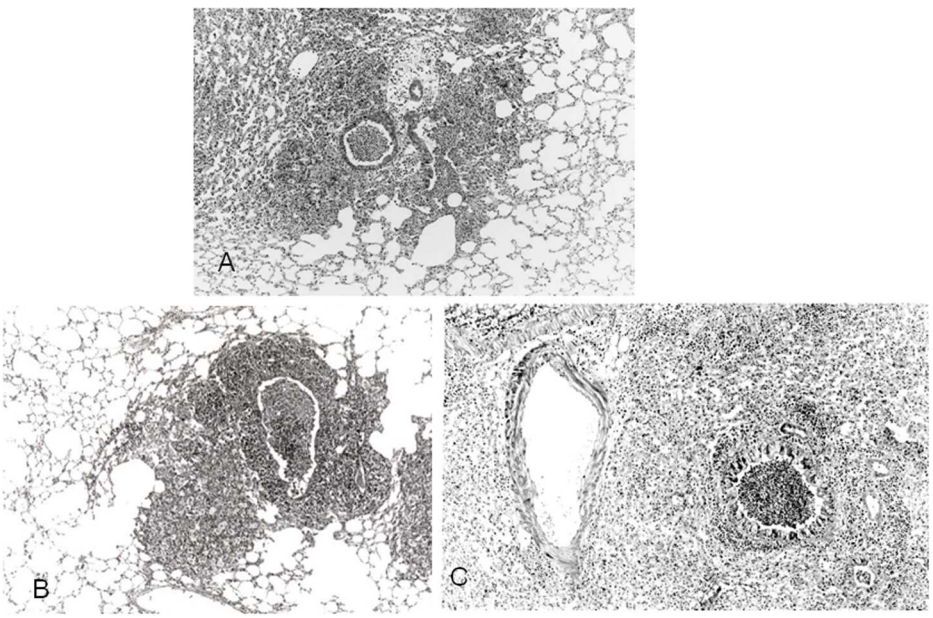


FIGURE 3 | Pathological observation of lung from *M. pulmonis* infected mice (HE x170). (A) Mice without treatment. **(B)** Mice treated with interleukin-2, showing marked peribronchial lymphocyte cuffing and macrophage accumulation at the end bronchiole. **(C)** Mice treated with PSL, disclosing predominant intra-alveolar inflammatory cell Infiltration and faint perivascular lymphocyte cuffing. Reproduced with permission from Tanaka and Tamura (1989).

IL-2 treated mice (**Figures 2** and **3**). On the other hand, prominent intra-alveolar inflammatory cell infiltration and faint peribronchial and perivascular mononuclear cell cuffing were observed on CYA or PSL treated mice (**Figures 2** and **3**). CMI to SRBC was increased in IL-2 treated mice, however, decreased in CYA or PSL treated mice. Another *M. pneumoniae* inoculated mice model exhibited host-dependent infection-related airway obstruction and airway hyperresponsiveness associated with chemokine and T-helper type 1 pulmonary host response and not T-helper type 2 response after *M. pneumoniae* infection (Fonseca-Aten et al., 2005). Recently, the severity of the *M. pneumoniae* pneumonia seemed to depend on the host innate immunity to the *M. pneumoniae*, which might be accelerated by antecedent *M. pneumoniae* exposure (re-exposure or latent respiratory infection) through up-regulation of Toll-like receptor 2 expression on bronchial epithelial cells and alveolar macrophages using mice model (Saraya et al., 2011, 2014).

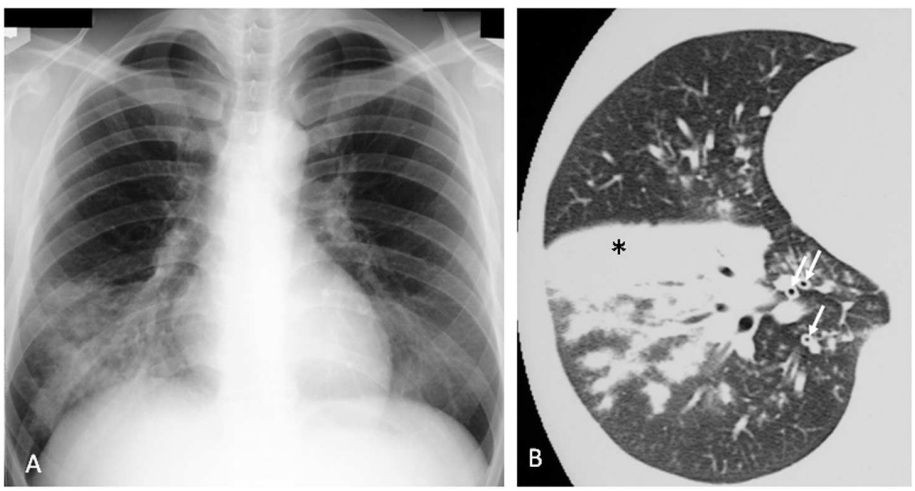


FIGURE 4 | *Mycoplasma pneumoniae* pneumonia in human. (A) Chest x-ray shows infiltrates in the right lower lobe. **(B)** Consolidation (*) and bronchovascular bundles thickening (↑) on CT scan. Reproduced with permission from Tanaka and Hayashi (2007).

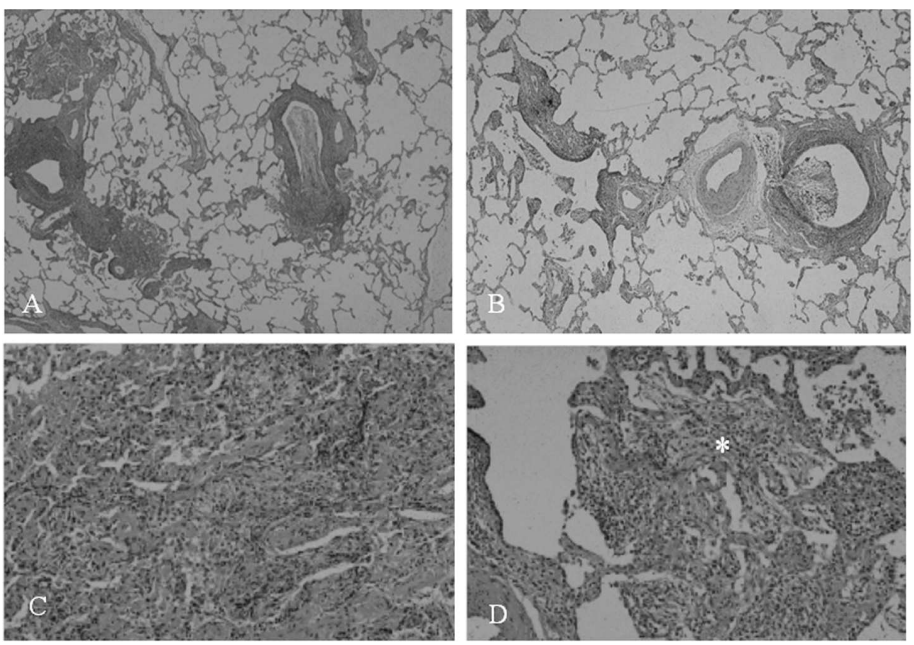
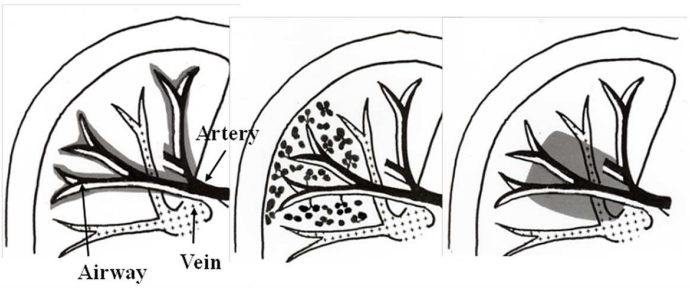


FIGURE 5 | Photomicrograph of open lung biopsy specimens in recovery phase of patients with *M. pneumoniae* pneumonia. Low-magnification views of small airways show cellular bronchiolitis and exudate in the lumen **(A,B)**. High-magnification of alveolar area disclose stuffed alveoli with exudate, fibrin, neutrophil, and granulation tissue in alveolar duct (*; **C,D**). Reproduced with permission from Tanaka (2016).



Radiological images: CT scan	Bronchovascular bundles thickening	Centrilobular nodules	Consolidation, Ground-glass attenuation
Pathological findings: biopsy specimen	Bronchitis characterized by inflammatory cells and plasma cells in the large airway walls	Cellular bronchitis in the small airways with exudate in the lumen	Neutrophils and exudate within bronchiolar and alveolar lumens
Frequency (n= 91)	75%	65%	66%

FIGURE 6 | Summary of radiological–pathological correlation in adult *M. pneumoniae* pneumonia. Reproduced with permission from Tanaka et al. (1997).

RADIOLOGICAL ASSESSMENT OF *M. pneumoniae* PNEUMONIA

The most common radiographic findings consist of unilateral or bilateral areas of air-space consolidation and ground-glass opacities. However, the findings are variable and can include reticular or nodular opacities. Associated features include bronchial wall thickening and occasionally small pleural effusion. CT shows more accurately the presence and extent of centrilobular nodules, the lobular distribution of ground-glass opacities and a small amount of pleural effusion not visible on chest radiograph. Typical findings of adult *M. pneumoniae* pneumonia on chest radiograph and CT are demonstrated in **Figure 4**. The findings of bronchiolitis and lobular consolidation seen in histopathological specimens were seldom apparent on radiography but were commonly evident on CT. The most distinct abnormality seen on CT consisted of poorly defined centrilobular nodules, suggesting bronchiolitis. A study using high-resolution CT shows the most frequent chest radiologic finding was air-space consolidation, seen in 86% of 28 patients, and most commonly involving the lower lobe and nodular opacities were detected in 14 patients (Reittner et al., 2000). The areas of patchy air-space consolidation or ground-glass attenuation frequently had a lobular distribution, a characteristic pathological feature of bronchopneumonia. Although the most common abnormalities were thickening of the axial interstitium appearing bronchovascular bundles thickening on radiograph and CT (Tanaka et al., 1985).

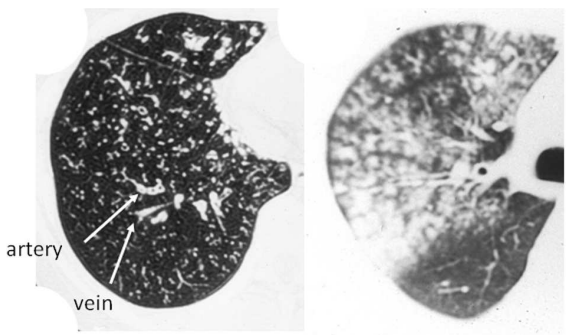


FIGURE 7 | Computed tomography of centrilobular nodules predominant pattern in two patients with *M. pneumoniae* pneumonia. Reproduced with permission from Tanaka et al. (2004).

PATHOLOGICAL FINDINGS IN OPEN LUNG BIOPSY

Mycoplasma pneumoniae organism selectively attaches airway ciliated epithelial cells (Tanaka et al., 2014) and therefore the pathological findings are usually limited to the airway walls as far down as small airways; the respiratory bronchioles. Histopathologically, *M. pneumoniae* pneumonia is characterized by acute cellular bronchiolitis with edematous and ulcerative lesions of bronchial walls and by peribronchial and perivascular interstitial opacities containing lymphocytes, plasma cells, and macrophages. The wall of bronchioles contains mononuclear cell and macrophage with a centrilobular distribution. In cases of severe pneumonia, diffuse alveolar damage with fibrinous exudates and hyaline membrane formation (Rollins et al., 1986; Izumikawa et al., 2014). Histopathological observation of open lung biopsy specimens from middle aged woman in recovery phase of *M. pneumoniae* pneumonia are showed in **Figure 5**. Low-magnification photomicrographs of small airways shows cellular bronchiolitis with thickening walls

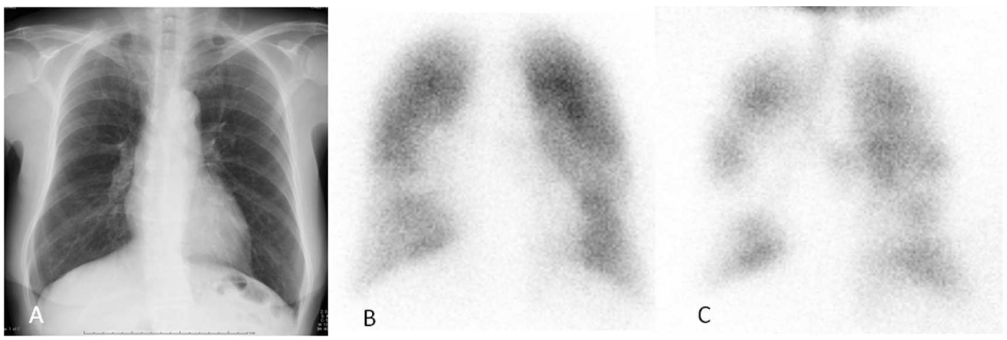


FIGURE 8 | Bronchiolitis obliterans following *M. pneumoniae* infection. (A) Chest X-ray showing normal. **(B)** ^{99m}Tc-MAA perfusion scan revealing slight defect. **(C)** ^{81m}Kr-aerosol ventilation demonstrating prominent multiple defects throughout the lung field. Reproduced with permission from Tanaka (2016).

and exudates in the lumen. High-magnification of alveolar area discloses intra-alveolar inflammatory-cell infiltration and organizing pneumonia with granulation tissue filling alveolar ducts.

Summary of pathological–radiological correlations and frequency of three major CT findings in 91 cases of adult *M. pneumoniae* pneumonia were shown in **Figure 6**. It was reported that *M. pneumoniae* pneumonia in the recovery phase showed predominantly centrilobular nodular patterns, which disclosed immunological inflammation remaining in the small airways (Tanaka et al., 1985). However, some patients demonstrated centrilobular nodules in the early phase of the pneumonia (**Figure 7**), which mimicking T-helper type 1 pulmonary host response in the mice model (Tanaka et al., 1996a).

HOST CMI AND RADIOLOGICAL PATTERN

The CMI of the host plays an important role in the development of *M. pneumoniae* pneumonia. *M. pneumoniae* pneumonia in patients with immunodeficiency syndrome had a rack of radiological chest findings (Foy et al., 1973). On the other hand, the radiographic appearance of *M. pneumoniae* pneumonia in patients with sarcoidosis showed a bilateral reticulonodular pattern (Putman et al., 1975). Ito et al. (1995) reported a married couple who developed *M. pneumoniae* pneumonia at the same time, and whose severity of pneumonia, radiological findings and serum soluble IL-2 receptor levels were marked different between wife and husband. The wife developed acute respiratory failure with high serum levels of soluble IL-2 receptor, on the other hand, the husband suffered from pneumonia with a moderate elevation of soluble IL-2 receptor. The difference may be reflected in the serum soluble IL-2 receptor levels, a marker of T cell activation *in vivo*. Serum IL-18 levels in patients with severe *M. pneumoniae* pneumonia were higher than those in mild cases (Tanaka et al., 2002), which suggested IL-18 and T-helper 1(Th1) cytokines may play a significant role in developing pneumonia. And IL-18 levels of pleural effusion in pediatric patients also elevated (Narita et al., 2000). In human *M. pneumoniae* pneumonia, positive rate of purified protein derivative (PPD) test in patients with nodular opacities predominant pattern on CT (group N) was higher than that in patients with air-space consolidation or ground-glass opacities predominant pattern on CT (group C) in *M. pneumoniae* pneumonia (Tanaka et al., 1996b). The PPD skin reaction; tuberculin skin test, is used not only to confirm past infection of *Mycobacterium tuberculosis* but also to determine the CMI of the host. In other words, patients with nodular opacities predominant on CT showed a more marked response to PPD than those with air-space consolidation predominant pattern on CT.

BRONCHIOLITIS OBLITERANS FOLLOWING *M. pneumoniae* INFECTION

The presence of centrilobular nodules in a patchy distribution is characteristic of infectious bronchiolitis, allowing distinction from non-infectious causes of bronchiolitis, which usually have a diffuse distribution throughout both lungs (Chan et al., 1999; Ebnother et al., 2001). We experienced a woman suffered *M. pneumoniae* infection. She complained dyspnea 2 months after the infection. Her chest radiograph and CT revealed overinflation with no centriacinar nodules (**Figure 8**). Her pulmonary function test revealed a vital capacity of 2469 ml, an forced effort in 1 s; FEV1 of 940 ml, and FEV1/FVC ratio of 41%, a V50/V25 ratio of 2.01, and a residual volume/total lung capacity ratio of 47%. ^{99m}Tc-MAA perfusion scan revealed slight defect and ^{81m}Kr-aerosol ventilation scan demonstrated prominent multiple defects, suggesting bronchiolitis obliterans. Centrilobular nodule of under 500 μm could not detected in high-resolution CT scan, therefore pulmonary functions are useful technique for detecting subtle small airway abnormality.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

Author gratefully acknowledges Hiroyuki Kobe, Teinekeijinnkai hospital, and Hiroshi Tamura, animal center of Teikyo University, for Pathological and radiological assists in *M. pulmonis* infected mouse model. The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 31 March 2016
doi: 10.3389/fmicb.2016.00448

Mycoplasma pneumoniae: Current Knowledge on Nucleic Acid Amplification Techniques and Serological Diagnostics

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Mycoplasma pneumoniae (*M. pneumoniae*) belongs to the class Mollicutes and has been recognized as a common cause of respiratory tract infections (RTIs), including community-acquired pneumonia (CAP), that occur worldwide and in all age groups. In addition, *M. pneumoniae* can simultaneously or sequentially lead to damage in the nervous system and has been associated with a wide variety of other acute and chronic diseases. During the past 10 years, the proportion of LRTI in children and adults, associated with *M. pneumoniae* infection has ranged from 0 to more than 50%. This variation is due to the age and the geographic location of the population examined but also due to the diagnostic methods used. The true role of *M. pneumoniae* in RTIs remains a challenge given the many limitations and lack of standardization of the applied diagnostic tool in most cases, with resultant wide variations in data from different studies. Correct and rapid diagnosis and/or management of *M. pneumoniae* infections is, however, critical to initiate appropriate antibiotic treatment and is nowadays usually done by PCR and/or serology. Several recent reviews, have summarized current methods for the detection and identification of *M. pneumoniae*. This review will therefore provide a look at the general principles, advantages, diagnostic value, and limitations of the most currently used detection techniques for the etiological diagnosis of a *M. pneumoniae* infection as they evolve from research to daily practice.

Keywords: ***Mycoplasma pneumoniae*, serology, nucleic acid amplification test, technological developments**

OPEN ACCESS

Edited by:
Cécile Bébéar, University of Bordeaux—Institut National de la Recherche Agronomique, France

Reviewed by:
Enno Jacobs, Technische Universität Dresden, Germany
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *29 January 2016*
Accepted: *18 March 2016*
Published: *31 March 2016*

Citation:
Loens K and Ieven M (2016) Mycoplasma pneumoniae: Current Knowledge on Nucleic Acid Amplification Techniques and Serological Diagnostics. Front. Microbiol. 7:448. doi: 10.3389/fmicb.2016.00448

About 50 years ago, an outbreak of *M. pneumoniae* in a pediatric chronic care facility was described (Baernstein et al., 1965). Twenty years earlier, the organism had been identified by Eaton and since the early 1960s, it was clearly identified as a bacterium which was associated both in children and adults with community-acquired infections of the respiratory tract (Lambert, 1964). Since then, numerous reports have been published on the association of *M. pneumoniae* with community-acquired infections (Waites and Talkington, 2004). Given the wide variations of data from studies with equally wide variation of and lack of standardized diagnostic methods, the true role of *M. pneumoniae* in RTIs still remains a challenge.

Since its discovery, scientists have explored several strategies for an optimal diagnosis of a *M. pneumoniae* infection in the laboratory to initiate an appropriate treatment. Because of its fastidious nature, *M. pneumoniae* is not routinely cultured from respiratory specimens. Culture methods have been the gold standard for diagnosis but are too insensitive producing a result after several

days or even several weeks and are therefore not relevant for the management of acute illness. Alternative diagnostic procedures were developed: Detection of IgM and/or IgG by ELISA, antigen detection by immunochromatography, and nucleic acid amplification techniques (NAATs), mainly PCR, although also isothermal amplification techniques such as LAMP (loop-mediated isothermal amplification method) have been developed. The utility of culture for *M. pneumoniae* was assessed by comparing it to PCR and IgM serology in a large study (She et al., 2010). Given the extremely low yield of culture and the wide availability of NAAT and serology, the authors concluded that culture for *M. pneumoniae* should be discontinued. Nowadays, most studies are serology and/or PCR-based. Different clinical specimens can be used as described in the review by Loens et al. (2009) for the latter.

APPLICATION OF NAATS

PCR is accepted as a rapid diagnostic test. Few of the currently available NAATs have been extensively validated against culture. The sensitivity of NAATs is almost always superior to that of traditional procedures and they are more and more considered as the "new gold standard."

An increasing body of literature describing the use of in-house NAATs for detection of *M. pneumoniae* DNA or RNA in various diseases is available with a great variation of methods used from study to study, including variability of target (P1 adhesin gene, 16S rRNA, ATPase gene, protease gene, CARDS toxin gene), NAAT (conventional, nested, real-time; monoplex vs. multiplex; PCR vs. isothermal amplification technologies), detection formats, and different platforms. An overview of the literature on the use of NAATs to detect *M. pneumoniae* since 1989 is given in two reviews (Loens et al., 2003, 2010a).

Lately, efforts have been mainly emphasized on the development of multiplex assays (Nummi et al., 2015; Shen et al., 2015) and on the evaluation of commercially available assays. Respiratory viruses and other so called "atypical bacteria" are all responsible for RTIs that may produce clinically similar manifestations. In order to reduce costs and hands-on-time, multiplex NAATs for the simultaneous detection of 2, 3, or up to more than 20 different respiratory pathogens in one tube with a mixture of primers have been developed by some groups. However, comparison between mono-and multiplex assays has been rarely performed. Findings and conclusions result frequently in contradictory and conflicting data concerning the sensitivity and specificity of the multiplex NAATs compared to the mono NAATs. This is not unexpected since the presence of several pairs of primers may increase the probability of mispairing resulting in non-specific amplification products and the formation of primer-dimers. Furthermore, enzymes, primers, and salt concentrations as well as temperature cyclings required for each target may be slightly different. The results of the proficiency panels (Loens et al., 2010b, 2012) described previously seem to confirm that multiplex assays are somewhat less sensitive than monoplex assays but until the number of organisms present in clinical specimens of diseased individuals is known, it is impossible to state whether the degree of sensitivity attained is clinically acceptable.

Since the previous review (Ieven and Loens, 2013) new NAATs became commercially available such as the Illumigene (Meridian Bioscience, USA) kit. It has been proposed that industry-produced assays in kit form result in better standardization. The analytical sensitivity of the Illumigene assay was evaluated by using 36 frozen stock cultures of *M. pneumoniae* reference strains, and a collection of other microorganisms and human DNA. (Ratliff et al., 2014). Serial dilutions of cultures with a known CFU/ml defined the analytical sensitivity at $\leq$88 CFU/ml. Based on the results obtained with 214 archived respiratory specimens, previously cultured for *M. pneumoniae,* the clinical sensitivity and specificity were found to be 100 and 99%, respectively, after resolving discrepancies by PCR and sequencing.

A second example of a test approved for the detection of a number of respiratory viruses by the US Food and Drug Administration is the Filmarray Respiratory panel (bioMérieux, France). The Filmarray is a small desktop closed single-piece flow real-time PCR system. It includes automation of nucleic acid extraction, an initial reverse transcription and multiplex PCR, followed by singleplex second stage PCR reactions for the detection of 15 viral agents including adenovirus, coronavirus HKU1, coronavirus NL63, human metapneumovirus, rhinovirus/enterovirus, influenza A/B, influenza A H1, AH1 2009, A H3, parainfluenza 1–4, and respiratory syncytial virus (Poritz et al., 2011). In May 2012, the US Food and Drug Administration expanded the use for the Filmarray respiratory panel with the addition of *B. pertussis*, *M. pneumonia,* and *C. pneumoniae*. The expanded panel detects now a total of 17 viruses and three bacteria. The test requires 5 min hands-on-time and 65 min instrumentation time. In 2013, a new version of the Filmarray (version 1.7) was released (Doern et al., 2013).

The Argene Respiratory MWSr-gene concept allows the detection of numerous pathogens (Influenza A/B, respiratory syncytial virus/human metapneumovirus, rhinovirus/enterovirus, adenovirus/bocavirus, *Chlamydia/ Mycoplasma pneumoniae*, human coronavirus/parainfluenza virus, *Bordetella, Bordetella parapertussis*) in the same run. In addition, the diagnostic strategy can be adapted to the season: searching for the most likely pathogens can be considered in 1st stage, the remaining pathogens being searched for systematically in a 2nd stage.

Pillet et al. (2013) compared six commercially available multiplex assays for the diagnosis of respiratory pathogens. Two out of six were also capable of detecting *M. pneumoniae*: the RespiFinder SMART 22 (PathoFinder, The Netherlands) and the Seeplex RV15 OneStep ACE detection and Pneumobacter ACE detection (Seegene Inc, South Korea). Sensitivities and specificities were calculated against the ArgeneChla/Myco pneumo assay (bioMérieux, France). Sensitivity and specificity were 70.0 and 100%, respectively, for the RespiFinder assay and 80.0 and 98.73% for the Seegene assay.

Dumke et al. compared four commercially available real-time PCR assays recommended for use with the Roche LightCycler

1.5 and 2.0 instruments [Diagenode *Mycoplasma/Chlamydophila pneumoniae* real-time PCR (Diagenode, Belgium), GeneProof *M. pneumoniae* (GeneProof, Czech Republic), BactoReal *M. pneumoniae* (Ingenetix, Austria), LightMix kit *M. pneumoniae* (TIB MOLBIOL, Germany)] for the detection of *M. pneumoniae* to results obtained with an in-house approach (Dumke and Jacobs, 2014) by using serial dilutions of a cultured *M. pneumoniae* strain tested in eight parallel runs and 37 clinical specimens, previously found to be *M. pneumoniae* positive by the in-house assay. All NAATs detected 20 colony forming units (CFU)/5µl sample. Only the in-house-test (repMP1-based approach) was able to detect 0.2 CFU/5µl sample. 37/37,35/37, 35/37, 34/37 *M. pneumoniae* positive clinical specimens were confirmed by the Diagenode test, the Ingenetix and Lightmix assay, and the GeneProof assay respectively.

An overview of commercially available NAATS for the detection of *M. pneumoniae* is presented in **Table 1**.

Since the calculation of the sensitivities of the commercial multiplex assays was mainly dependent on DNA copy number, further evaluation and standardization using an extended number of clinical specimens that may have a low bacterial load are needed. The use of an international standard developed by the WHO for harmonization of *Mycoplasma* NAAT (Nübling et al., 2015) or the yearly participation in the external quality assessment (EQA) panel for *M. pneumoniae* and *Chlamydophila pneumoniae* available from Quality Control for Molecular Diagnostics (QCMD, United Kingdom) should be considered.

So far, it is unclear whether asymptomatic carriage of *M. pneumoniae* in adults and children exists and if colonization could be differentiated from infection by the current diagnostic methods. There are only few data on the relation between the bacterial load and the severity of infection. 405 asymptomatic children and 321 children with a RTI were enrolled in a cross-sectional study (Spuesens et al., 2013). Nasopharyngeal washings and pharyngeal swabs were investigated by culture and quantitative real-time PCR (qPCR). Serum was collected for IgM and IgG ELISA. Neither qPCR, serology nor culture was capable of differentiating colonization from infection. In 21.2 and 16.2% of the asymptomatic and symptomatic children, *M. pneumoniae* DNA was detected. In addition, persistence of *M. pneumoniae* in the upper respiratory tract was shown for up to 4 months by longitudinal sampling. A retrospective study investigated the clinical significance of the *M. pneumoniae* bacterial load in children with a *M. pneumoniae* pneumonia (Jiang et al., 2014). The authors concluded that a high bacterial load was indicative for a *M. pneumoniae* infection, whereas for a low bacterial load the etiologic role of *M. pneumoniae* remains to be determined.

Edin et al. developed a qPCR with duplex reactions targeting eight bacteria, including *M. pneumoniae*, and six viruses (Edin et al., 2015). Clinical specimens from the upper and lower respiratory tract were used to compare the qPCR assay with standard microbiological methods. The use of the qPCR assay resulted in 113 positive identifications in 94 respiratory specimens compared with 38 by using standard diagnostics. The authors conclude that in parallel qPCR detection of the targeted respiratory bacteria and viruses is feasible since a good technical performance of the assay in clinical specimens was obtained.

In contrast to the above mentioned studies, Jain et al. (2015) examined specimens from 2222 hospitalized children with community-acquired pneumonia and 521 asymptomatic controls for the detection of a variety of respiratory pathogens. *M. pneumoniae* was detected in 8%, and in 3% or less of controls.

Another trend is the simultaneous detection of *M. pneumoniae* and mutations associated with macrolide resistance directly in clinical specimens (Ji et al., 2014; Liu et al., 2014; Nummi et al., 2015; Zhao et al., 2015).

APPLICATION OF SEROLOGY FOR THE DETECTION OF *M. PNEUMONIAE* INFECTIONS

Serological methods, in particular enzyme-linked immunosorbent assays (ELISA), are most widely used to diagnose a *M. pneumoniae* infection. The complement fixation test (CFT) has been replaced by assays which allow for quantification of IgM, IgA, or IgG. However, the most convincing evidence of an ongoing infection is a significant increase in IgG or an IgG seroconversion in paired sera, collected 3–4 weeks apart (Nir-Paz et al., 2006). Although IgM antibodies appear earlier than IgG antibodies, and are thus an attractive alternative for diagnosis of a *M. pneumoniae* infection, one should realize that IgM is not often produced in very young children, in a proportion of primary infections and during re-infections (Waites et al., 2008; Loens et al., 2010a).

Ten serological assays for the diagnosis of a *M. pneumoniae* infection were recently evaluated by using 145 sera from 120 patients (Busson et al., 2013): SeroMP IgM and IgG (Savyon Diagnostics), SeroMP Recombinant IgM, IgA and IgG (Savyon Diagnostics), LIAISON *M. pneumoniae* IgM and IgG (Biotrin International Ltd), *M. pneumoniae* IgM, IgA and IgG Medac (Medac GmbH). A low IgM specificity and cross-reactivity was noticed for the SeroMP recombinant and Liaison assay. For IgA, the Medac assay tended to be less specific than the SeroMP Recombinant assay. All four tests showed discrepancies in the IgG measurements confirming results of previous studies (Talkington et al., 2004; Beersma et al., 2005). In conclusion, serology remains a diagnostic tool of choice but improvement and standardization of the assays are still needed, especially for the determination of IgG.

The clinical significance of a serologic test, both for IgM and IgG, should be defined by studies of patients with a documented infection and for whom detailed information concerning the time lapses between onset of disease and the collection of the serum specimens are known.

A promising blotting technique improving the performance of the *M. pneumoniae* serological assays has been described (Dumke et al., 2012).

DETECTION OF *M. PNEUMONIAE* BY BOTH NAATS AND SEROLOGY

Data from recent studies using PCR based methods and serology published during the last decade in different patient populations

TABLE 1 | Summary of commercially available single and multiplex PCR assays for detection of *M. pneumonia*.

Kit	Manufacturer	Assay type	Detection procedure	Pathogens targeted
M. pneumoniae BDProbeTec ET	BD	SDA	Fluorescence	*M. pneumoniae*
ASR MPN	Cepheid	PCR	Real-time	*M. pneumoniae*
Simplex *M. pneumoniae*	Focus diagnostics	PCR	Real-time	*M. pneumoniae*
GeneProof *Mycoplasma pneumoniae*	GeneProof	PCR	Real-time	*M. pneumoniae*
Loopamp *Mycoplasma pneumoniae* DNA amplification kit	Eiken chemical	LAMP	Turbidity	*M. pneumoniae*
BactoReal *Mycoplasma pneumoniae*	Ingenetix	PCR	Real-time	*M. pneumoniae*
Illumigene *Mycoplasma*	Meridian BioScience	LAMP	Turbidity	*M. pneumoniae*
Venor MP	Minerva BioLabs	PCR	Agarosegel electrophoresis and real-time	*M. pneumoniae*
M. pneumoniae LightMix kit	TIB MolBIOL	PCR	Real-time	*M. pneumoniae*
Cp/Mp tracer	Affigene	MX-PCR	Real-time	*M. pneumoniae, C. pneumoniae*
AID CAP bacterial assay	AID GmbH	MX-PCR	ICT	*S. pneumoniae, H. influenzae, M. catarrhalis, C. pneumoniae, M. pneumoniae, L. pneumophila*
Chlamylege	Argene, bioMérieux	MX-PCR	Hybridization	*C. pneumoniae, M. pneumoniae, Legionella spp*
EasyPlex respiratory pathogens B and C	Ausdiagnostics	MX-PCR	Real-time	Influenza A, influenza A H1, influenza A H3, influenza A H5, influenza B, RSV, rhinovirus, enterovirus, PIV 1-3, HAdV, hMPV, HCoV 229E and OC43, *B. pertussis, M. pneumoniae, C. pneumoniae, L. pneumophila, L. longbeachae, Pneumocystis, H. influenzae, S. pneumoniae*
Respiratory Multi Well System Chla/Myco pneumo r-gene	BioMérieux	5 duplex PCRs	Real-time	Influenza A, influenza B, RSV, hBoV, HAdV, hMPV, rhino/enterovirus, *C. pneumoniae*, *M. pneumoniae*
Diagenode *Mycoplasma/Chlamydophila pneumoniae* real-time PCR kit	Diagenode	Duplex PCR	Real-time	*M. pneumoniae, C. pneumophila*
Fast Track Respiratory Pathogen assay	Fast-track diagnostics	MX-PCR	Real-time	*S. pneumoniae, S. aureus, H. influenzae, M. catarrhalis, Legionella spp, M. pneumoniae, C. pneumoniae*
ProPneumo-1	Hologic	MX-PCR	Real-time	*M. pneumoniae, C. pneumoniae*
ProPneumo1+	Hologic	MX-PCR	Real-time	*M. pneumoniae, C. pneumoniae*
Filmarray	BioMérieux.	MX-PCR	Microarray	Influenza A H1N1, influenza A H1, influenza A H3, influenza B, RSV, hMPV, HCoV NL63, OC43, 229E, HKU1, HAdV PIV 1-4, HBoV, rhino/enterovirus, *B. pertussis, M. pneumoniae, C. pneumoniae*
RespiFinder plus	Patho Finder	MX-PCR	Capillary electrophoresis	*M. pneumoniae, C. pneumoniae, L. pneumophila, B. pertussis,* Influenza A (H5, non-specific) and B, RSV A/B, PIV 1-4, rhinovirus, 3 HCoV, hMPV, HAdV
RespiFinder focus	Patho Finder	MX-PCR	Capillary electrophoresis or microfluidics	*M. pneumoniae, C. pneumoniae, L. pneumophila, B. pertussis,* influenza A and B, RSV A/B, hMPV, HAdV
SmartFinder	Patho Finder	MX-PCR	Real-time	*M. pneumoniae, C. pneumoniae, L. pneumophila, B. pertussis,* influenza A and B, RSV A/B, PIV 1-4, HAdV, rhinovirus, 3 HCoV, hMPV, HBoV
Seeplex PneumoBacter ACE	Seegene Inc.	MX-PCR	Capillary electrophoresis	*S. pneumoniae, H. influenzae, M. pneumoniae, C. pneumoniae, L. pneumophila, B. pertussis*
Seeplex RV/PB18 ASE	Seegene Inc.	MX-PCR	Capillary electrophoresis	*S. pneumoniae, H. influenzae, M. pneumoniae, C. pneumoniae, L. pneumophila,* Influenza A and B, RSV A/B, PIV 1-3, rhinovirus, 3 HCoV, HAdV, HBoV, enterovirus

Bold, FDA-approved test.

HAdV, human adenovirus; HBoV, human bocavirus; HCoV, human coronavirus, hMPV, human metapneumovirus; ICT, immunochromatographic test; LAMP, Loop-Mediated isothermal amplification method; MX-PCR, multiplex PCR; PIV, parainfluenzavirus; qPCR, quantitative PCR.

from around the world are summarized in the recent reviews published by Ieven and Loens (Loens et al., 2010a; Ieven and Loens, 2013) and updated in **Table 2**.

The availability of the very sensitive NAATs has in recent years also put the often used serological tests in their right perspective and allow a better interpretation of the serological test results and their limitations such as the low sensitivity of IgM antibodies in acute phase specimens and importance of the delay between two serum samples. Studies in which also NAAT's are used on respiratory specimens should allow a better interpretation of the serological test results.

A rapid response report from the Canadian Agency for Drugs and Technologies in Health (Canadian Agency for Drugs and Technologies in Health, 2015) presents the results of a literature search in order to identify the diagnostic test accuracy, clinical effectiveness, and cost-effectiveness of serum IgM and molecular tests for the detection of *M. pneumoniae* in patients with a respiratory infection[1]. Six relevant studies were identified, but no evidence regarding the clinical effectiveness or cost-effectiveness of a serum IgM test compared with molecular tests was identified. Zhang et al. conducted a systematic review and meta-analysis on the diagnosis of *M. pneumoniae* by PCR and serology (Zhang et al., 2011) and reported a significant heterogeneity between the studies and inconsistent results as well.

Two studies compared the application of real-time PCR and serology in children with pneumonia. In 2011, 54/290 children were found to be positive by PCR (Chang et al., 2014), 44/182 were *M. pneumoniae* IgM positive. 12.6% of patients were found to be *M. pneumoniae* positive by both tests at the same time. Using PCR as gold standard, a sensitivity and specificity of resp. 62.2 and 85.5% were obtained. The specificity could be increased to 90.3% by increasing the cut-off without changing the sensitivity of the IgM assay. A study conducted by Medjo et al. (2014) applied PCR, culture, IgM and IgG in paired sera for the detection of a *M. pneumoniae* infection in 166 children. Using IgG serology as gold standard, the sensitivity of IgM, PCR, and culture was found to be equal (81.8%), specificity was found to be 100, 98.6, and 100% respectively. It was concluded that during the acute phase of disease, detection of IgM antibodies in combination with PCR allowed for a precise and reliable *M. pneumoniae* diagnosis. A prospective study in children with community-acquired CAP (Kakuya et al., 2014) compared loop-mediated isothermal amplification, (LAMP), culture and serology at first visit. Patients were defined positive if positive by culture and/or sero-conversion or a four-fold increase in IgG in paired sera. 31/191 patients met the criteria. Thirteen were positive by culture and serology, 17 on culture only, and one by serology only. A positive LAMP result was obtained for all patients that were culture positive. The sensitivity and specificity for LAMP, EIA, and the particle agglutination test, were 96.8, 38.7, 19.4, and 100%, 76.9 and 93.1%, respectively.

When establishing the etiology in 267 adult CAP-patients in Norway, 10 were found to be *M. pneumoniae* positive: two by serology, seven and one by PCR applied to a nasopharyngeal flocked swab and an oropharyngeal flocked swab, respectively (Holter et al., 2015).

AMPLIFICATION-FREE AND OTHER TECHNOLOGICAL DEVELOPMENTS

Newer technologies such as microfluidics and the application of nanotechnology offer the potential to an even more rapid detection of important pathogens allowing even near-patient testing. Since these technologies, as NAATs, do not require viable organisms, and thus avoid any adverse effect of longer specimen transport, they can be successfully applied to both the in- and outpatient settings. Several companies currently possess the technical expertise and research infrastructure to bring a useful diagnostic testing approach to the clinical trial stage shortly.

Li et al. (2015) developed a colloidal gold-based immune-chromatographic assay by using a pair of monoclonal antibodies targeting a region of the P1 gene. When applied to 303 clinical specimens from children suspected with a *M. pneumoniae* infection, the sensitivity and specificity against real-time PCR were 100 and 97.4%. This is in contrast to the results obtained with a commercially available rapid antigen test targeting the ribosomal protein L7/L12 (Ribotest Mycoplasma). Compared to real-time PCR, a sensitivity and specificity of respectively 62.5 and 90.9% were obtained when applied to clinical specimens (Miyashita et al., 2015). Based on these results, the authors concluded that treatment decisions should not be taken based on the Ribotest results alone.

Other amplification-free detection methodologies are currently being developed as biosensing detection strategies: A proto-type of an enzyme-free electrochemical genosensor on nanostructured screen-printed gold electrodes (Garcia-Gonzalez et al., 2015); A silver nanorod array-surface enhanced Raman Spectroscopy biosensing platform was successfully applied for the detection of *M. pneumoniae* in simulated and clinical throat swabs (Henderson et al., 2014, 2015).

CONCLUSIONS

With the use of tools such as NAATs a greater understanding of the etiology and epidemiology of *M. pneumoniae* is possible. Taken into account the results obtained in recent studies, there is more evidence that real-time NAATs are superior to other *M. pneumoniae* detection strategies during the early phase of infection. NAATs, however, cannot completely replace serology. In epidemiological studies, serology is certainly more useful than for the management of individual patients with LRTI or even CAP since results are often delayed by the need for paired sera to detect a seroconversion or a significant rise in titer; early in the course of an infection, false-negative results often occur.

In case a specific IgM test is used, serology should not completely be abolished despite the fact that IgM serology shows a moderate sensitivity. Nowadays, a combination of the detection of IgM antibodies and PCR may be the most optimal approach

[1](2015). Serum IgM and Molecular Tests for Mycoplasma pneumoniae Detection: A Review of Diagnostic Test Accuracy, Clinical Effectiveness, Cost-Effectiveness, and Guidelines, Ottawa, ON.

TABLE 2 | Summary of recent single and multiplex NAATs for detection of *Mycoplasma pneumoniae* published since the previous review, and previously validated assays used as comparators.

Monoplex assays

Assay year (references)	Assay type	Detection format	Gene target (product size)	PCR assay used as comparator for new assay	Non-PCR comparator test	Specimens tested for validation of sensitivity and or specificity
2012 (Zhao et al., 2012)	PCR	Real-time	P1-gene (72)	repMp1 and Mp181		Various bacterial species, bacterial dilution series, well-defined clinical specimens
2012 (Gotoh et al., 2012)	LAMP	Turbidity	P1 operon (NS) (Eiken Chemical)		IgG seroconversion/ significant rise	Samples from 368 pneumonia patients
2013 (Chaudhry et al., 2013)	PCR	Real-time	P1-gene (534)	Conventional PCR (NS)	IgM, IgG and IgA serology	Dilution series, respiratory samples from CAP-patients,
2013 (Schmitt et al., 2013)	PCR	Real-time	ptsI (160)	LightMix kit *M. pneumoniae* (TIB MOLBIO), *M. pneumoniae* analyte specific reagent (Focus diagnostics), (Dumke et al., 2007)		Bacterial dilution series, spiked clinical specimens, well-defined clinical specimens
2014 (Liu et al., 2014)	PCR	Cycleave	23S rDNA (Takara BioInc)	(Ieven et al., 1996)		Various bacterial species, bacterial dilution series, clinical specimens
2014 (Ratliff et al., 2014)	LAMP	Turbidity	Illumigene assay (208)	2nd real-time PCR and sequencing	Culture	Various bacterial species, bacterial dilution series, 214 culture positive/negative specimens
2014 (Medjo et al., 2014)	PCR	Real-time	P1 (125)		IgM and IgG serology, culture	Specimens from CAP-patients

Multiplex assays

Assay year (references)	Assay type	Detection format	Specimens tested for validation of sensitivity and or specificity
2013 (Puppe et al., 2013)	MX-PCR	ELISA	Culture supernatans of the organisms, clinical specimens from frozen stocks, prospectively included nasopharyngeal aspirates
			Pathogens targeted: enterovirus, influenza A, influenza B, RSV, PIV 1-4, HAdV, rhinovirus, hMPV, HCoV, reovirus, *M. pneumoniae, C. pneumoniae, B. pertussis, B. parapertussis, L. pneumophila*
2013 (Simões et al., 2013)	MX-PCR	Affimetrix Chip-image file	Clinical specimens simultaneously investigated by culture and two commercially available assays: the Eragen assay and the Luminex RVP
			Pathogens targeted: 72 pathogens
2013 (Weinberg et al., 2013)	MX-PCR	TAC-array	Well-defined clinical specimens analyzed by individual real-time PCRs
			Pathogens targeted: HAdV, hMPV, PIV1-4, influenza A, influenza B, influenza C, RSV, rhinovirus, HCoV OC43, 229E, NL63, HKU1, enterovirus, *B. pertussis, C. pneumoniae, H. influenza, L. pneumophila, M. pneumoniae, S. pneumoniae, S. pyogenes*
2014 (Hirama et al., 2014)	MX-PCR	Real-time	DNA dilution series, welldefined clinical specimens from CAP-patients
			Pathogens targeted: *S. pneumoniae, H. influenza, M. catarrhalis, P. aeruginosa, K. pneumoniae, E. coli, S. aureus, M. pneumoniae, C. pneumoniae, C. psittaci, C. burnetii, Legionella spp, L. pneumophila, B. pertussis, M. tuberculosis, M. intracellulare, M. avium, M. kansasii, P. jeroveci, Nocardia spp*, metallo-beta-lactamase, MRSA
2014 (Ji et al., 2014)	MX-PCR	Agarose gel electrophoresis	Various bacterial pathogens, bacterial dilution series, confirmation by sequencing, well-defined clinical specimens
			Pathogens targeted: *M. pneumoniae* and associated macrolide resistance
2015 (Zhao et al., 2015)	Duplex PCR	Real-time	Pathogens targeted: *M. pneumoniae* and genotyping
2015 (Shen et al., 2015)	MX-PCR	Resequencing microarray	Pathogens targeted: *S. pneumoniae, M. pneumoniae, H. influenza, K. pneumoniae, M. catarrhalis, S. aureus, P. aeruginosa, M. tuberculosis, N. meningitidis*, Group A Streptococci
2015 (Nummi et al., 2015)	MX-PCR	Real-time	Pathogens targeted: *M. pneumoniae, C. pneumoniae* and mutations associated with macrolide resistance

HAdV, human adenovirus; HBoV, human bocavirus; HCoV, human coronavirus; hMPV, human metapneumovirus; PIV, parainfluenzavirus; MX-PCR, multiplex PCR; LAMP, Loop-mediated isothermal amplification; TAC, Taqman Array Card.

for early diagnosis of a *M. pneumoniae* infection, especially in children.

The implementation of quantitative tests could shed further light on the relation between bacterial load and the seriousness of the disease, produce useful prognostic information and help in the differentiation between colonization and infection. More information could be gathered on the length of the post infection carrier state as well as on the importance of subclinical infections and how prone these are for spreading infection.

It remains important to recognize the urgent need for the adoption of a more unified and consistent diagnostic approach for current and future investigations. Therefore, a common set of recommendations should be developed.

AUTHOR CONTRIBUTIONS

KL drafted the manuscript. GI revised and approved the final manuscript.

ACKNOWLEDGMENTS

KL is supported through The Belgian National Reference Centre for Respiratory Pathogens which is partially supported by the Belgian Ministry of Social affairs through a fund within the Health Insurance System. KL is also supported by the EU FP7 project PREPARE (602525).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 08 March 2016
doi: 10.3389/fmicb.2016.00232

The Evolution of Advanced Molecular Diagnostics for the Detection and Characterization of *Mycoplasma pneumoniae*

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Over the past decade there have been significant advancements in the methods used for detecting and characterizing *Mycoplasma pneumoniae,* a common cause of respiratory illness and community-acquired pneumonia worldwide. The repertoire of available molecular diagnostics has greatly expanded from nucleic acid amplification techniques (NAATs) that encompass a variety of chemistries used for detection, to more sophisticated characterizing methods such as multi-locus variable-number tandem-repeat analysis (MLVA), Multi-locus sequence typing (MLST), matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS), single nucleotide polymorphism typing, and numerous macrolide susceptibility profiling methods, among others. These many molecular-based approaches have been developed and employed to continually increase the level of discrimination and characterization in order to better understand the epidemiology and biology of *M. pneumoniae*. This review will summarize recent molecular techniques and procedures and lend perspective to how each has enhanced the current understanding of this organism and will emphasize how Next Generation Sequencing may serve as a resource for researchers to gain a more comprehensive understanding of the genomic complexities of this insidious pathogen.

Keywords: *Mycoplasma pneumoniae*, molecular diagnostics, molecular epidemiology, molecular characteristics, whole genome sequencing

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *15 January 2016*
Accepted: *15 February 2016*
Published: *08 March 2016*

Citation:
Diaz MH and Winchell JM (2016) The Evolution of Advanced Molecular Diagnostics for the Detection and Characterization of Mycoplasma pneumoniae. Front. Microbiol. 7:232. doi: 10.3389/fmicb.2016.00232

INTRODUCTION

Mycoplasma pneumoniae is a common cause of respiratory infections in all age groups worldwide (Waites and Talkington, 2004; Atkinson et al., 2008; Waites and Atkinson, 2009; Winchell, 2013). *M. pneumoniae* infections vary dramatically in clinical presentation, ranging from mild, self-limiting upper respiratory symptoms to radiographically confirmed pneumonia requiring hospitalization (Waites and Talkington, 2004; Atkinson et al., 2008). In some cases, infection may result in severe clinical syndromes involving other organ systems (Waites and Talkington, 2004; Atkinson et al., 2008; Narita, 2010; Olson et al., 2015; Magun et al., 2016). Localized outbreaks of *M. pneumoniae* have been reported frequently, especially in closed settings, and transmission between household contacts is known to occur (Foy et al., 1966; File et al., 1998; Walter et al., 2008; Waites and Atkinson, 2009; Winchell, 2013). The long incubation period of up to 3 weeks

and prolonged shedding after infection allows outbreaks to often go unnoticed and extend for long periods of time (Foy et al., 1966; File et al., 1998; Hammerschlag, 2001; Atkinson et al., 2008; Nilsson et al., 2008). Larger community-wide outbreaks are frequently not identified or are recognized late. This underrecognition is due, in part, to a lack of pathogen-specific testing for mild respiratory illness in the primary care setting. However, increased incidence of *M. pneumoniae* infections in Europe, Asia, and the United States has been reported since 2010 (Lenglet et al., 2012; Diaz et al., 2015b; Kim et al., 2015). Such epidemic seasons of *M. pneumoniae* have been reported to occur every 4–7 years (Foy et al., 1979; Waites and Talkington, 2004; Atkinson et al., 2008; Winchell, 2013).

Despite the diversity and complexity of the clinical and epidemiological characteristics of *M. pneumoniae* infections, the bacterium itself is one of the smallest and simplest known organisms capable of living outside of a host cell. The genome of *M. pneumoniae* is approximately 800 kb in size, maintaining a set of approximately 700 annotated protein-coding genes (Guell et al., 2009; Lluch-Senar et al., 2015; Xiao et al., 2015) plus additional non-coding RNA genes (Dandekar et al., 2000; Xiao et al., 2015). The reduced genome is deceivingly complex as it contains tracts of tandemly repeated sequences at numerous loci (Degrange et al., 2009) and has been proposed to utilize sophisticated transcriptional regulatory mechanisms and antigenic variation to control gene expression (Guell et al., 2009; Spuesens et al., 2009, 2011; Citti et al., 2010). Variation of surface-exposed immunogenic epitopes, including the P1 adhesion molecule, have been reported (Spuesens et al., 2009, 2011) and may be a driving force for the cyclic occurrence of epidemic seasons of *M. pneumoniae* infections (Dumke et al., 2004).

Methods for identification of *M. pneumoniae* infection include culture, serological analysis, or molecular detection of pathogen-specific antigen(s) or nucleic acid. Culture is a definitive method for diagnosis of *M. pneumoniae*, and ongoing maintenance of a collection of clinical isolates is critical for monitoring trends in the epidemiology of this organism. However, culture is slow and requires specialized media and trained personnel, and, most importantly, recovery of isolates is highly variable and may be low, even in specialized laboratories (Ieven et al., 1996; Dorigo-Zetsma et al., 1999; Morozumi et al., 2004; Thurman et al., 2008). Serological analysis has proven problematic for the specific identification of *M. pneumoniae* infection due to poor diagnostic sensitivity and specificity and the requirement for paired acute and convalescent sera, and it does not allow for characterization of the *M. pneumoniae* strain causing the infection (Beersma et al., 2005; Nir-Paz et al., 2006; Thurman et al., 2008). Neither culture nor serology is practical for rapid detection of acute infection, limiting the utility of these methods to retrospective investigations.

Molecular methods for nucleic acid or antigen detection have emerged as the primary techniques for identification of *M. pneumoniae* in surveillance programs. However, adoption of these methods has lagged in the clinical setting in the United States with many physicians continuing to rely on serological tests or opting for no pathogen-specific testing in concordance with the guidelines from the Infectious Diseases Society of America (IDSA) for the treatment of community-acquired pneumonia (CAP; Mandell et al., 2007; Bradley et al., 2011). Beyond the methods for detection of *M. pneumoniae* in clinical specimens, numerous molecular methods have been developed in recent years that exploit the limited genomic diversity of *M. pneumoniae* isolates in order to characterize isolates for epidemiological purposes, although no clear correlation of strain type with clinical presentation, disease severity, or patient outcome has been identified to date. The emergence of macrolide resistance in this species has also spurred the development of molecular methods for determining susceptibility to this frontline antibiotic to improve appropriate prescribing. More recently, whole genome sequencing (WGS) has emerged as a more accessible and thorough approach for investigating the biological and epidemiological characteristics of *M. pneumoniae*. This review summarizes the various molecular methods for both detection and characterization of *M. pneumoniae* with a focus on WGS and the potential of this approach to transform the field in coming years.

DETECTION OF *M. pneumoniae* USING NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAATs)

Respiratory infections, including CAP, may be caused by a wide variety of pathogenic microorganisms that are indistinguishable by clinical evaluation alone. Nucleic acid amplification techniques (NAATs) have been increasingly recognized and implemented as the preferred method for identification of respiratory bacteria and viruses, including *M. pneumoniae*, in clinical specimens as a result of the high level of sensitivity and specificity and rapid turnaround time afforded by these methods. NAATs for *M. pneumoniae* were reviewed by Loens et al. (2003b), at which time only two of the 34 assays described for detection of *M. pneumoniae* were real-time PCR methods. Since that time, numerous real-time PCR assays encompassing a variety of chemistries have been developed and have largely replaced conventional PCR for research and diagnostic purposes. The predominant real-time PCR chemistries utilized for *M. pneumoniae* detection are intercalating dyes and 5′ hydrolysis (TaqMan®) assays. The most common genetic target regions within the *M. pneumoniae* genome are 16S rRNA, P1 gene, or the ATPase operon (Loens et al., 2003b). More recently, the gene encoding the community-acquired respiratory distress syndrome (CARDS) toxin, first described in Kannan et al. (2005), has also proven to be a useful target sequence for *M. pneumoniae* detection by real-time PCR (Winchell et al., 2008; Thurman et al., 2011).

A subsequent review of the status of *M. pneumoniae* diagnostics in 2010 detailed the rapid rise in laboratory-developed NAATs, specifically real-time PCR, multiplex or multi-pathogen PCR, and isothermal amplification methods, and emphasized the need to properly evaluate new assays prior to implementation (Loens et al., 2010). A lack of qualified standards used to evaluate new assays makes an accurate comparison

of performance characteristics impossible. The external quality assessment (EQA) panel for *M. pneumoniae* available from Quality Control for Molecular Diagnostics (Glasgow, Scotland, United Kingdom) provides a useful metric for assessment of new laboratory-developed NAATs. Implementation of controlled standards for assessment of new methods will be beneficial for advancing *Mycoplasma* diagnostics. In addition to the numerous in-house laboratory-developed tests, several real-time PCR assays are now commercially available. A few recent studies have been conducted to evaluate the performance of these products, which overall were found to have comparable sensitivity and specificity albeit at a higher per specimen cost compared to LDTs (Dumke and Jacobs, 2009, 2014; Touati et al., 2009). Still, testing practices for detecting *M. pneumoniae* in the primary care setting are unlikely to change without modifications to guidelines for pathogen-specific testing established by relevant medical professional organizations, such as the IDSA. Periods of high incidence of *M. pneumoniae* infections, such as the recent worldwide epidemic period described in 2010–2012 (Lenglet et al., 2012; Diaz et al., 2015b; Kim et al., 2015), serve to bring *M. pneumoniae* to the attention of primary care providers and key decision-makers in the creation of guidelines for the treatment of CAP (Jacobs, 2012).

Multiplex and Multi-Pathogen NAATs

Clinical presentation of *M. pneumoniae* infection can vary significantly and may be indistinguishable from respiratory infections caused by other bacterial and viral agents. Like *M. pneumoniae*, the *Chlamydophila* species are fastidious and extremely slow and difficult to culture. For these reasons, assays for detection of other atypical causes of bacterial pneumonia, including *Chlamydophila pneumoniae* or *C. psittaci*, as well as *Legionella* species, are often combined with *M. pneumoniae* into multiplex PCR formats (Miyashita et al., 2004; McDonough et al., 2005; Thurman et al., 2011). Diaz and Winchell (2012) described a rapid real-time PCR assay for detection of *M. pneumoniae* and *C. pneumoniae* that can be performed directly from clinical specimens without a nucleic acid extraction step about six times faster compared to standard real-time PCR methods. Although the sensitivity of the direct PCR was lower compared to extracted nucleic acid, it is possible that improved sensitivity could be achieved through further optimization of the method along with immediate testing of an upper respiratory swab at the time of collection, thus allowing for performance of this assay at the point-of-care. The requirement for a thermocycler instrument and procedural separation to avoid laboratory contamination and potential false positive results remain the most significant barriers to implementation of even the simplest and fastest real-time PCR methods in the clinical setting.

Mycoplasma pneumoniae has also been included in multi-pathogen panels for detection of a diverse array of bacterial, viral, and fungal agents capable of causing pneumonia, such as the BioFire FilmArray respiratory panel (BioFire Diagnostics, Salt Lake City, UT, USA), which is cleared for diagnostic use by the U.S. Food and Drug Administration (FDA; Poritz et al., 2011), and the Fast-track Diagnostics Respiratory Pathogens multiplex real-time RT-PCR assay kits (Fast-track Diagnostics, Luxembourg, Belgium) and Seegene Allplex Respiratory Full Panel Assay (Seegene, Inc., Seoul, Korea), which are CE certified in Europe. The U.S. Centers for Disease Control and Prevention (CDC) includes *M. pneumoniae* in a multi-pathogen testing panel on the TaqMan Array Card (TAC; ThermoFisher Scientific, CA, USA) used for investigating unexplained respiratory disease outbreaks in the United States (Kodani et al., 2011; Cieslak et al., 2012). Since 2011, TAC has been used by our laboratory for surveillance testing at the U.S. CDC to identify at least seven outbreaks of *M. pneumoniae* and was used to implicate *M. pneumoniae* as the cause of a cluster of severe CAP cases (Rhea et al., 2014; Waller et al., 2014; Diaz et al., 2015b; Hastings et al., 2015). Implementation of multi-pathogen detection methods could profoundly improve determination of incidence of infections caused by *M. pneumoniae* and impact appropriate antibiotic prescribing during CAP.

It is important to note that multiplex detection approaches for respiratory infections, including CAP, introduce additional complexity into the determination of etiology since the mere presence of an organism does not indicate a contribution to disease. Many bacterial and viral agents with pathogenic potential may also be present in the upper respiratory tract in a carriage state or for a prolonged period of shedding after resolution of infection in apparently healthy individuals (Hammitt et al., 2006; Roberts et al., 2012; Tenenbaum et al., 2012; Self et al., 2015; Skevaki et al., 2015). Frequent detection of *M. pneumoniae* in asymptomatic controls has been reported among children in the Netherlands (Spuesens et al., 2013), although this same phenomenon was not observed in a recent study of CAP etiology among children in the United States (Jain et al., 2015). Co-detections of other bacterial and viral pathogens along with *M. pneumoniae* have been reported in children (Michelow et al., 2004; Peng et al., 2009; Chiu et al., 2015). However, the significance of the presence of additional pathogens in the upper respiratory tract and their potential interplay with *M. pneumoniae* is not known. Expanded testing of respiratory specimens to include a wide collection of potential pathogens will present a challenge to clinicians for interpreting the true etiology of disease. Further investigation is needed to fully understand these interactions, and the movement of next generation sequencing techniques to clinical microbiology laboratories may help resolve some of these questions.

Real-time PCR has become a mainstream diagnostic procedure in reference laboratories and in some clinical laboratories. However, substantial barriers to implementation of this testing method in all clinical laboratories remain. Equipment for real-time PCR is expensive and requires routine preventative maintenance to ensure proper function. Laboratories need to have proper procedural separation of space and training of laboratory personnel, and all clinical laboratories offering patient testing in the United States must comply with the Clinical Laboratory Improvement Amendments (CLIA). Even when real-time PCR is available as a diagnostic test order, it is often not requested by physicians since current treatment guidelines recommend empiric antibiotic therapy without testing

for suspected *M. pneumoniae* infection (Mandell et al., 2007; Bradley et al., 2011). As a result, significant effort has recently been invested in developing simpler NAATs or other detection approaches that may be utilized at the point-of-care, which will be summarized in the following sections.

Isothermal Amplification Assays

Isothermal NAATs that do not require thermal cycling and are amenable to simple visual readout have also been developed for detection of *M. pneumoniae*, although these are less common than real-time PCR methods. Visualization of target amplification can be achieved using a fluorescent intercalating dye, chemiluminescent reporter, or even simple optical density (turbidity) measurement. These assays can be performed using minimal equipment, requiring only a standard heat block instead of a thermocycler with optical capability. Isothermal amplification assays have potential utility as point-of-care testing methods since they require only basic inexpensive equipment and minimal operator training, and they are relatively rapid. The two primary isothermal amplification methods that have been described for detection of *M. pneumoniae* are nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP).

Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA is a unique method in that it is used for detection of RNA only through a process in which double stranded DNA (dsDNA) is generated from RNA through the use of avian myeloblastosis virus (AMV) reverse transcriptase (AMV-RT), T7 RNA polymerase, and RNase H while maintaining a constant reaction temperature of approximately 41°C. The dsDNA product is detected through an electrochemiluminescent readout for conventional assays or, more recently, through detection of fluorescent signal from a molecular beacon probe. Similar to PCR, real-time NASBA methods have largely replaced conventional NASBA assays. Loens et al. (2003a) described a real-time NASBA assay for detection of *M. pneumoniae* that performed comparably to a previously described conventional NASBA method in terms of analytical and clinical sensitivity (Loens et al., 2002). This assay was subsequently expanded to a multiplex format for simultaneous detection of *M. pneumoniae*, *C. pneumoniae*, and *Legionella* species (Loens et al., 2008).

Despite favorable assay performance characteristics and advantages of minimal equipment and operator expertise, NASBA has not been widely implemented in clinical laboratories to date. It may be that clinical laboratories have not yet updated test offerings from traditional methods such as serological analysis; alternatively, it is possible that laboratories prefer to offer real-time PCR for *M. pneumoniae*, for which there are numerous well-validated assays available and the technology is now widely accepted in diagnostic microbiology compared to NASBA. Procurement, inventory management, and quality control of additional specific reagents for NASBA testing for a single diagnostic test represents a significant investment of financial and personnel resources for a laboratory. Rather, it is more likely a NASBA assay could be implemented at the point-of-care than in clinical or reference laboratories, although substantial procedural optimization is required to make this feasible.

Loop-Mediated Isothermal Amplification (LAMP)

LAMP utilizes four to six primers and the strand-displacing DNA polymerase *Bst* to generate concatenated amplicons through a process in which stem-loop structures are introduced flanking the amplified target sequence. Several LAMP assays have been reported for the detection of *M. pneumoniae* (Saito et al., 2005; Yoshino et al., 2008; Gotoh et al., 2012; Petrone et al., 2015), and these vary in sensitivity and specificity due to both major and minor differences between the assays. Variables such as genetic target, reaction composition, and readout mechanism can significantly impact assay performance. Due to the complexity of the amplification events and high number of oligonucleotides, extensive optimization and validation is required to ensure reliability of results (Petrone et al., 2015).

The *illumigene* Mycoplasma DNA amplification assay (Meridien Bioscience, Inc., Cincinnati, OH, USA) is the only standalone assay to be cleared by the U.S. FDA for detection of *M. pneumoniae* in clinical specimens. This assay, which targets the intracellular protease gene, displayed 100% sensitivity and 99% specificity compared to culture (Ratliff et al., 2014). Still, the requirement for extraction of nucleic acid from the primary specimen hinders the implementation of this assay for point-of-care diagnostic use. Petrone et al. (2015) demonstrated successful detection of *M. pneumoniae* in clinical specimens directly without a nucleic acid extraction step using a novel LAMP assay targeting the CARDS toxin gene. The sensitivity of this LAMP assay for detection of *M. pneumoniae* using primary specimen in place of extracted nucleic acid was 82% compared to real-time PCR. This assay was optimized to utilize calcein as a fluorescent readout and can be performed in approximately 1 h from time of specimen collection to results. Improved sensitivity would be expected with appropriate modification of specimen collection procedures and immediate testing of specimens after collection. Further optimization is needed to formulate a simple user-friendly reaction setup in order for this method to be feasible for point-of-care testing.

DETECTION OF *M. pneumoniae* USING ANTIGEN DETECTION METHODS

Some detection methods rely on capture and detection of *M. pneumoniae*-specific antigen, rather than nucleic acid, present in clinical specimens. However, antigen detection methods such as enzyme-linked immunosorbent assay (ELISA) and hybridization assays have been largely replaced by NAATs due to the improved sensitivity, specificity, and rapid turnaround time. A complete review of these methods is outside of the scope of this review. Nonetheless, there remains some interest in antigen detection methods for diagnosis of *M. pneumoniae*, particularly those that may be amenable to point-of-care use. In addition to being sensitive and specific, point-of-care diagnostic tests must be extremely rapid (on the order of minutes), simple

to perform, require little to no equipment, and be relatively inexpensive.

Immunochromatographic (Lateral Flow) Assays

Immunochromatographic assays, also known as lateral flow assays, represent one of the simplest test formats available and have been widely implemented for various purposes, including pathogen detection, at the point-of-care (Posthuma-Trumpie et al., 2009). Several immunochromatographic assays for detection of *M. pneumoniae* antigen are currently available in Japan. These tests provide results within 15 min and are easily interpreted by visual observation of the presence or absence of a colored line on the test strip. Diagnosis and appropriate treatment of *M. pneumoniae* is particularly important in Asia where the majority of strains are resistant to macrolides (Cao et al., 2010; Okada et al., 2012), the recommended first-line antimicrobial therapy, as described in further detail in section "Macrolide Susceptibility Genotyping of *M. pneumoniae*" below.

Ribotest Mycoplasma^{TM} (Asahi Kasei Corporation, Tokyo, Japan), is one commercially available rapid diagnostic lateral flow assay for qualitative detection of the ribosomal protein L7/L12 of *M. pneumoniae* in pharyngeal swab specimens. However, Miyashita et al. (2015) recently reported a diagnostic sensitivity of only 60% for Ribotest^{TM} compared to real-time PCR, indicating that this assay does not meet the level of sensitivity required for detection of *M. pneumoniae* in clinical specimens. Recently, Li et al. (2015) reported a novel colloidal gold immunochromatographic antigen assay for detection of the P1 gene of *M. pneumoniae* that was 100% sensitive and 97.4% specific compared to real-time PCR. A highly sensitive and specific assay with broad market distribution will be required to achieve widespread implementation in clinical laboratories.

Nanorod Array Surface-Enhanced Raman Spectroscopy (NA-SERS)

Another alternative approach that has been proposed for detection of *M. pneumoniae* is nanorod array surface-enhanced Raman spectroscopy (NA-SERS). This method involves generation of a metallic nanorod array substrate and application of Raman spectroscopy to detect the unique vibrational spectral profile of biomolecules in an applied sample. Hennigan et al. (2010) demonstrated the ability of NA-SERS to detect *M. pneumoniae* in mock or true clinical specimens with sensitivity comparable to real-time PCR methods. Further evaluation revealed the ability of this method to differentiate *M. pneumoniae* from other commensal and pathogenic *Mycoplasma* species and to further differentiate strain types (Henderson et al., 2015), as described further in section "Nanorod Array Surface Enhanced Raman Spectroscopy" below. While this method provides a unique and promising alternative strategy for *M. pneumoniae* detection, significant challenges remain for implementation in clinical laboratories, including required equipment, specimen processing, and application of statistical analysis to evaluate the spectral profile identified in clinical specimens. Furthermore, substantial testing will be necessary to evaluate the feasibility of this approach for *M. pneumoniae* detection in the presence of co-detected pathogens and normal microbial flora of the upper respiratory tract.

Mass Spectrometry

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is a useful technique for the rapid identification of pathogenic microorganisms, including both Gram-positive and Gram-negative bacteria, based on the unique spectral profile of proteins in bacterial lysate. Pereyre et al. (2013) generated peptide mass fingerprint product ion spectra for 10 human and 13 ruminant *Mycoplasma* species or subspecies in order to develop a main spectra (MSP) database for identification of clinically relevant *Mycoplasma* species, including *M. pneumoniae*. The dendrogram based on 29 MSPs from 23 mycoplasmas was consistent with 16S rRNA phylogeny (Pereyre et al., 2013). This method was sufficiently sensitive to discriminate closely related *Mycoplasma* species, but is limited by the requirement for a culture isolate, which may take weeks to obtain due to the slow growth of *M. pneumoniae*. Furthermore, a large volume of culture (30–100 mL) was required for extraction of proteins for successful generation of MSPs; the time required and means to generate such a high volume culture are not practical in most clinical laboratories. Nonetheless, MALDI-TOF MS has been shown to be useful for the detection of anaerobic, fastidious, and slow-growing bacterial isolates from clinical specimens (Biswas and Rolain, 2013). In order for MALDI-TOF MS to meet the rapid turnaround time possible with NAATs, technical optimization is needed to achieve detection of *M. pneumoniae* directly from respiratory specimens.

CHARACTERIZATION OF *M. pneumoniae*

Mycoplasma pneumoniae is a highly genetically conserved species; genomic comparisons have revealed >99% sequence similarity between isolates (Lluch-Senar et al., 2015; Xiao et al., 2015). Still, several methods have been developed to characterize *M. pneumoniae* strains based on various genetic elements and allowing for classification of *M. pneumoniae* for epidemiological purposes. In the absence of substantial sequence diversity, strain differentiation efforts have focused largely on variability in the relatively high number of repetitive elements within the genome. Approximately 8% of the *M. pneumoniae* genome is comprised of repetitive sequences, some of which are present in multiple copies throughout the genome (Himmelreich, 1996). Variation in the nucleotide sequence or the number of tandem repeats at these genetic loci underlie some of the most commonly used methods for *M. pneumoniae* typing.

Recent advances in WGS and access to an increasing collection of publicly available complete *M. pneumoniae* genomes

(discussed in depth in section "Whole Genome Sequencing" below) have aided in identifying areas within the genome that can be targeted to achieve greater discriminatory power. With the expansion of WGS to the clinical sector, there is little doubt that genomic characterization of *M. pneumoniae* will become more reliable and robust, and WGS analysis may be routinely used during outbreak investigations or as part of surveillance programs. In the following sections, we review the current approaches for typing of *M. pneumoniae* and describe the methods for each characterization scheme, culminating in a review of recent WGS advancements and a discussion of the future application of WGS to *M. pneumoniae* diagnostics.

P1 Typing

Typing based on sequence variation within repetitive elements located in the gene encoding the P1 adhesion molecule was first described in 1990 and has the longest history of use for distinguishing the two main subtypes of *M. pneumoniae*, types 1 and 2 (Dallo et al., 1990; Su et al., 1990b). Two of the repetitive elements found in the *M. pneumoniae* genome, RepMP2/3 and RepMP4, are located within the gene encoding the 170 kDa adhesin protein P1, and sequence variation between types 1 and 2 strains occurs largely within these repetitive regions (Su et al., 1990a). In addition, 7 copies of RepMP4 and nine copies of RepMP2/3 have been identified at various loci in the genome outside of the transcriptionally active operon that includes the P1 gene (Ruland et al., 1990; Himmelreich, 1996). Evidence of recombination of these sequence copies into the transcribed P1 gene has been reported, yet the exact mechanisms underlying these recombination events and the frequency of such events are not known (Spuesens et al., 2009; Musatovova et al., 2012). Notably, Spuesens et al. (2009) found that isolates contain either types 1 or 2-specific RepMP sequences within their genome, but not both, suggesting an early divergence in the phylogeny of *M. pneumoniae*. Variants of each type have also been described (Schwartz et al., 2009a; Spuesens et al., 2009; Zhao et al., 2011; Kenri et al., 2012).

The P1 adhesin is a major virulence determinant of *M. pneumoniae*, facilitating adherence of the bacteria to respiratory epithelial cells during infection (Baseman et al., 1996; Baseman and Tully, 1997; Razin et al., 1998; Waites and Talkington, 2004; Atkinson et al., 2008). P1 is a primary immunogenic component of *M. pneumoniae*, and thus sequence variation within the P1 gene can be expected to result in alteration in the surface-exposed protein thereby potentially affecting the infectious process. In fact, the alternating predominance of types 1 or 2 strains circulating in a population during epidemic seasons has been documented previously (Lind et al., 1997; Kenri et al., 2008; Kogoj et al., 2015), and the cyclic pattern was potentially attributed to the development of temporary immunity to one type, thus allowing reemergence of the other type (Dumke et al., 2004). However, recently co-circulation of both P1 types and multiple variants have been reported during the same epidemic period and even during discrete outbreaks (Waller et al., 2014; Diaz et al., 2015b; Jacobs et al., 2015). These findings suggest that P1 typing alone is likely not adequate to classify *M. pneumoniae*.

Furthermore, the lack of any association of strain type with disease characteristics, particularly severity of illness or patient outcomes, calls into question the utility of P1 typing and other typing schemes included in this review. However, there remains continued interest and benefit in monitoring strain types using existing methods in order to understand the epidemiological shifts in circulating *M. pneumoniae* strains over time and across geographic locations. Newer methods have been developed with superior discriminatory power compared to P1 typing alone, yet, even using WGS analysis, the most comprehensive characterization method available, strains are still classified into 2 main clades corresponding to P1 types, although further separation within these clades has been observed using WGS (Lluch-Senar et al., 2015). Although P1 typing is likely to be augmented or replaced by newer methods to better characterize *M. pneumoniae*, it will be used for some time still for epidemiological investigations and surveillance programs. Here we review the primary methods used for typing *M. pneumoniae* based on the P1 adhesin (**Table 1**).

Restriction Fragment Length Polymorphism (RFLP) and Sequencing Analysis

One widely used approach to typing *M. pneumoniae* based on P1 is PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis (Sasaki et al., 1996; Cousin-Allery et al., 2000; Kenri et al., 2008; Musatovova et al., 2008). Results are compared to prototypical type strains of each type, M129 (type 1) and FH (type 2). Using this method, *M. pneumoniae* isolates can be identified as type 1 or 2 or a number of unique variants. Dumke et al. (2006) utilized amplification and sequencing to distinguish P1 types directly from clinical specimens. Spuesens et al. (2010) reported the use of pyrosequencing for molecular typing of *M. pneumoniae* into the two main subtypes based on sequence variation in the MPN141 (P1) and MPN528a genes. Both of these methods require post-PCR processing, which increase time to results and potential for contamination of laboratory space with PCR amplicon, which may lead to false positive results. This risk can be mitigated by meticulous separation of space and equipment for post-PCR processing steps.

High Resolution Melt (HRM) Analysis

Schwartz et al. (2009a,b) reported a novel PCR assay with High Resolution Melt (HRM) analysis to differentiate types 1 and 2 and to identify variants. This approach uses amplification of a 1900 bp sequence followed by melting curve analysis in a one-step reaction to clearly distinguish types 1 and 2 isolates based on alteration in the melting temperature created by multiple single nucleotide polymorphisms (SNPs) located within the amplicon. Variants of type 1 or 2 are also identifiable by virtue of further sequence variation in the amplified target region (Schwartz et al., 2009a). The major advantages of this method are a more rapid turnaround time and no requirement for post-PCR reaction manipulation,

TABLE 1 | Molecular methods for characterization of *Mycoplasma pneumoniae*.

Characterization scheme	Method(s)	Reference(s)
P1 gene typing	PCR-RFLP	Cousin-Allery et al., 2000
	PCR-high-resolution melt (HRM)	Schwartz et al., 2009b
	NASBA	Ovyn et al., 1996
	Sequencing	Dumke et al., 2006
	Pyrosequencing	Spuesens et al., 2010
	MALDI-TOF MS	Xiao et al., 2014
	NA-SERS	Henderson et al., 2015
Multilocus Variable-Number Tandem-Repeat Analysis (MLVA)	MLVA	Degrange et al., 2009
	MLVA (nested PCR)	Dumke and Jacobs, 2011
Multilocus sequence typing (MLST)	MLST	Brown et al., 2015b
Single nucleotide polymorphism (SNP) genotyping	SNaPshot minisequencing assay	Touati et al., 2015
Macrolide susceptibility genotyping	Sequencing	Lucier et al., 1995
	Pyrosequencing	Spuesens et al., 2010; Chan et al., 2013
	PCR-melting curve analysis	Wolff et al., 2008; Chan et al., 2013
Whole genome sequencing (isolates)	Shotgun sequencing	Himmelreich et al., 1996
	High-throughput sequencing	Lluch-Senar et al., 2015; Xiao et al., 2015
	Single-molecule long-read sequencing	Lluch-Senar et al., 2013

dramatically reducing the risk of amplicon contamination in the laboratory.

Nucleic Acid Sequence-Based Amplification (NASBA)

In addition to typing based on analysis of the P1 gene, it has been reported that there is one SNP in the 16S rRNA gene that can be used to differentiate types 1 and 2 strains. Ovyn et al. (1996) developed a conventional NASBA assay which allows differentiation of the two main P1 types based upon binding of the electrochemiluminescent-labeled hybridization probe in the region containing this SNP. This SNP could be easily detected using targeted resequencing methods or identified in whole genome sequences in order to simply and reliably identify the main P1 types.

Multi-Locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA)

MLVA is a technique applied to many bacterial species for strain differentiation based upon the number of tandemly repeated sequences located at designated loci throughout the genome. Approximately 8% of the *M. pneumoniae* genome is comprised of repetitive elements (Himmelreich, 1996), making this species well-suited for characterization using MLVA. In 2009, Degrange et al. (2009) developed a five-loci MLVA scheme for differentiation of *M. pneumoniae* strains through identification and selection of VNTR regions in *M. pneumoniae* that were polymorphic between isolates, yet stable upon passage in broth culture. Using this five-loci typing scheme, 26 MLVA types were identified, and these were assigned alphabetical identifiers, A through Z. Shortly after the development of this method, a slightly modified protocol was applied which enabled testing of nucleic acid extracted from primary clinical specimens, thus eliminating the need for a culture isolate (Dumke and Jacobs, 2011; Benitez et al., 2012). Since the introduction of this method for *M. pneumoniae* characterization, it has been widely implemented for investigating outbreaks as well as characterizing historical strain collections. However, in the course of implementing this method, the first locus, Mpn1, was shown to be unstable, rendering it impractical for classification of *M. pneumoniae* (Benitez et al., 2012; Sun et al., 2013). Multiple research groups proposed the exclusion of this marker and modification to a four-loci MLVA scheme (Sun et al., 2013; Waller et al., 2014). The removal of the Mpn1 locus from the typing scheme reduced the discriminatory power of this method, allowing classification of *M. pneumoniae* strains into fewer unique types. Nonetheless, the modified approach is generally considered to be more robust and has been accepted as the new international standard for *M. pneumoniae* MLVA typing (Chalker et al., 2015).

Adaptation to the four-loci MLVA typing scheme revealed a few predominant MLVA types circulating concurrently in the past 5–7 years, a period during which increased *M. pneumoniae* cases were documented on multiple continents, including Europe, Asia, and North America (Chalker et al., 2011, 2012; Blystad et al., 2012; Eibach et al., 2012; Lenglet et al., 2012; Nir-Paz et al., 2012; Polkowska et al., 2012; Eshaghi et al., 2013; Sun et al., 2013; Diaz et al., 2015b). The three most common types identified during this period were 4572, 3562, and 3662 (Sun et al., 2013; Diaz et al., 2015b). Adaptation to the four-loci MLVA typing scheme in our laboratory revealed a correlation between MLVA type and P1 type; isolates identified as MLVA type 4572 were always P1 type 1 while MLVA type 3562 or 3662 were always P1 type 2 (Waller et al., 2014; Diaz et al., 2015b). While this correlation has held up for all isolates and specimens tested to date in our laboratory, other investigators have reported a small number of P1 type 1 strains that are not MLVA type 4572 and P1 type 2 strains that are not MLVA type 3X62, suggesting that there are few exceptions to this correlation (Degrange et al., 2009; Dumke and Jacobs, 2011; Sun et al., 2013). Nonetheless, the biological reasons underlying the observed correlation are not well understood,

particularly since these four VNTR regions are located either within an intergenic region (Mpn13) or in an open reading frame (ORF) encoding a hypothetical protein [Mpn14 (MPN 501), Mpn15 (MP 524), and Mpn16 (MPN 613)] (Degrange et al., 2009). However, recent whole genome SNP and indel analysis of numerous *M. pneumoniae* isolates also substantiates the separation of these two main groups (Lluch-Senar et al., 2015; Xiao et al., 2015), supporting the early phylogenetic divergence of two main lineages of *M. pneumoniae* (Musatovova et al., 2012).

While MLVA has higher discriminatory power compared to P1 typing (Pereyre et al., 2012), researchers have continued to pursue the development of typing methods with even higher discriminatory power or that are clinically or epidemiologically informative. Some recent reports have suggested a correlation of MLVA type 4572 with macrolide resistance and disease severity (Qu et al., 2013; Ho et al., 2015). Other studies have reported an association between macrolide resistance and disease severity or clinical course (Cardinale et al., 2013; Zhou et al., 2014); thus, additional studies are needed to determine strain attributes that may impact the course of *M. pneumoniae* infection. Further investigation is necessary to verify any potential associations since these findings may influence testing practices for *M. pneumoniae* in clinical laboratories and could ultimately improve patient management.

Multi-Locus Sequence Typing (MLST)

MLST is a widely used tool for strain differentiation in many genera of bacteria. Initial attempts to categorize *M. pneumoniae* isolates into MLST types using housekeeping and structural genes were generally unsuccessful due to limited sequence variation within these regions (Dumke et al., 2003). Using a growing set of whole genome sequence data, a new MLST method was recently reported by Brown et al. (2015b) that exploited sequence polymorphisms of eight housekeeping genes (*ppa*, *pgm*, *gyrB*, *gmk*, *glyA*, *atpA*, *arcC*, and *adk*). SNPs were identified in the type strains of *M. pneumoniae* (M129 and FH) and 35 clinical isolates. Further sequencing and PCR experimentation with an additional 20 isolates allowed for 12 distinct sequence types (STs) to be established. This is substantially more discriminating than the previous MLST scheme that only found slight sequence variation in the three housekeeping genes selected for discrimination (Dumke et al., 2003). The authors also confirmed the relative stability of these MLST loci after performing 10 sequential subculture passages of isolates, finding no change in the SNP patterns. Like other typing methods, no link between the reported STs and isolation year, patient age, or geographic origin of the clinical specimen, was found. However, two distinct genetic clusters were observed that correlate with MLVA type 4572 and 3X62. The two clonal complexes resulting from this more comprehensive MLST study underscore the significant differences between these two genetically distinct lineages.

Although this typing scheme is more discriminating than the commonly used MLVA and P1 typing methods, it still requires PCR and sequencing of the isolate to generate the ST. Greater utility of this procedure will surely be realized when this methodology is applicable for testing directly on clinical specimens. Nonetheless, this method is of value for typing isolates for epidemiological investigations and can greatly enhance the understanding of strain circulation, transmission dynamics, and relative persistence in a population or geographic location. The creators of this scheme also established a web-based database for *M. pneumoniae* MLST data that can also be linked to an isolate database that contains epidemiological information (Jolley and Maiden, 2010). New data can be submitted to the database in order to track the number of unique profiles identified to date.

Single Nucleotide Polymorphism (SNP) Genotyping

Another molecular typing approach used to more clearly define and genotype *M. pneumoniae* isolates and positive clinical specimens is based upon SNPs that were identified after performing WGS on eight strains. Touati et al. (2015) showed that nine SNP types can be determined from eight reliable SNPs identified within housekeeping, predicted lipoprotein, and P1 adhesin genes using a "SNaPshot" mini-sequencing assay. This approach uses a single-base extension (SBE) method which allows an unlabeled mini sequencing primer to anneal one base upstream of the specified SNP using a fluorescently labeled ddNTP that can be easily detected after the separation and extension of the product has occurred. This technology was used to characterize 140 *M. pneumoniae* strains previously typed using five-loci MLVA and P1 methods. These previously used typing schemes had mixed correlation when compared to the SNaPshot mini-sequencing procedure; SNP genotyping correlated poorly with 5-loci MLVA types but strongly with P1 types (Touati et al., 2015). The poor correlation with the 5-loci MLVA type may be a result of the instability of the first locus, Mpn1, which introduces artefactual differences between strains. The SNP typing method had a higher Hunter and Gaston diversity index compared to other typing methods, including 4-loci MLVA. Other major advantages of this technology are that it can be highly multiplexed, has increased sensitivity, and can be used directly on clinical specimens (Sobrino et al., 2005; Touati et al., 2015). Identification of SNPs in nucleic acid from clinical specimens without performing any sequencing procedure affords a significant savings in time and cost while also mitigating any potential contamination within laboratories since minimal manipulation of the specimen is required. Furthermore, this method allows for discrimination of strains without the need to generate and handle WGS data, which requires substantial computing power and bioinformatics expertise. This SNP typing method may prove to be useful for epidemiological analysis, but is likely to only be performed at highly specialized academic and reference laboratories.

MALDI-TOF MS + ClinProTools

Initial evaluation of MALDI-TOF MS method for identification of *M. pneumoniae* (described in section "Identification of *M. pneumoniae* Using Mass Spectrometry" above) also revealed

that strains clustered by P1 type based upon the MSP (Pereyre et al., 2013). Xiao et al. (2014) utilized an analysis software tool, ClinProTools (Bruker Daltonics, Bremen, Germany) to differentiate P1 types using a genetic algorithm based upon seven biomarker peaks identified using MALDI-TOF MS. These investigators demonstrated that the genetic algorithm model was able to correctly identify the P1 type of 43 *M. pneumoniae* isolates based upon the peptide mass fingerprints. Thus, P1 typing can be successfully performed either by analysis of the nucleic acid or protein composition of an isolate. To date, nucleic acid-based approaches have been preferred methods by most laboratories, but MALDI-TOF MS is rapidly gaining acceptance in the clinical microbiology field and thus represents a potential future avenue for *M. pneumoniae* diagnostics.

Nanorod Array Surface Enhanced Raman Spectroscopy (NA-SERS)

The NA-SERS method described in section "Nanorod Array Surface-Enhanced Raman Spectroscopy" was also capable of differentiating *M. pneumoniae* strains into three classes corresponding to P1 types 1, 2 and 2 variant (2V) (Henderson et al., 2015). Partial least squares-discriminatory analysis (PLS-DA) modeling was applied to differentiate *M. pneumoniae*-specific spectra from background and the spectra of other pathogenic or commensal mycoplasmas. Subsequently, three unique PLS-DA models were built to distinguish strains based on P1 type. Although this method does not provide a higher discriminatory power compared to other typing methods, it does allow for simultaneous detection and typing of *M. pneumoniae* in a rapid and reliable manner. However, unless a clinically relevant association of strain type with disease is identified, performance of typing assays in a clinical laboratory is of little value for patient care, and typing methods are likely to remain an offering only by specialized reference laboratories. In addition, further validation is needed to assess performance of this method for detection and typing of *M. pneumoniae* strains in complex clinical specimens. Nonetheless, NA-SERS represents a unique alternative approach to the identification of *M. pneumoniae* and other respiratory pathogens, particularly if this method can be modified for multi-pathogen detection in clinical specimens.

MACROLIDE SUSCEPTIBILITY GENOTYPING OF *M. pneumoniae*

Macrolides, primarily azithromycin, are the recommended first-line antibiotic for treatment of *M. pneumoniae* (Waites and Talkington, 2004; Mandell et al., 2007; Atkinson et al., 2008; Bradley et al., 2011; Waites, 2011). Since the first report in 2001 of macrolide-resistant *M. pneumoniae* (Okazaki et al., 2001), the prevalence of this trait has emerged worldwide, reaching dangerously high levels upward of 90% in Asia (Liu et al., 2009; Xin et al., 2009; Cao et al., 2010; Okada et al., 2012). In the United States and Europe, macrolide resistance has persisted over the past decade, albeit at relatively low levels (∼10%; Steffens et al., 2012; Diaz et al., 2015a,b; Zheng et al., 2015). However, diagnostic testing for detection of *M. pneumoniae* is not routinely performed in the United States, so specimens are not often available for susceptibility testing. Estimates of the prevalence of macrolide resistance in the United States are generally obtained from outbreak investigations and limited surveillance studies. Three recent studies have reported macrolide resistance rates ranging from 3.5 to 13.2% among *M. pneumoniae*-positive clinical specimens in the United States (Diaz et al., 2015a,b; Zheng et al., 2015). The highest rate reported in the United States, 27%, occurred during a discrete outbreak in Rhode Island in 2009, although this was based on only 11 specimens (Wolff et al., 2008). Resistance rates from large-scale multi-site surveillance studies likely represent a more accurate estimate, but it remains possible that macrolide resistance is more likely to develop during prolonged outbreaks.

Some studies suggest that resistance develops in an individual patient in response to macrolide treatment (Dumke et al., 2014), although the frequency with which this occurs is not well defined and may be low (Nilsson et al., 2014). Development and expansion of a resistant subpopulation within an individual patient in response to macrolide therapy rather than transmission of a resistant isolate within a population is supported by investigations of outbreaks and transmission among household contacts in which only a few sporadic clinical isolates were found to be resistant (Diaz et al., 2015b). Still, the rapid emergence of macrolide resistant *M. pneumoniae* in Asia compared with the relatively low and stable presence of these strains in Europe and North America underscore that this trait must be studied at the population level rather than only within an individual patient. Further investigation including longitudinal studies are needed in order to understand how the resistance trait emerges and expands within a population.

The mechanism of resistance to macrolides in *M. pneumoniae* is well understood; a single SNP at one of several key residues within or adjacent to the binding site in the peptidyl transferase loop of the 23S rRNA large subunit prevents the macrolide from binding and inhibiting protein synthesis (Bebear et al., 2011). Mutations at positions 2063 and 2064 in *M. pneumoniae* result in high level resistance to macrolide antibiotics, whereas a mutation at position 2067 or 2617 is associated with a lower level of resistance (Morozumi et al., 2010). Mutations that occur in the 23S rRNA gene are dominant as there is only a single rRNA operon in the *M. pneumoniae* genome (Gobel et al., 1984). Since only a single base change confers resistance, it is biologically plausible that this event may happen frequently, especially since *M. pneumoniae* is known to have limited DNA repair mechanisms in the reduced genome (Carvalho et al., 2005). Furthermore, the relatively long biological half-life of macrolide antibiotics, particularly azithromycin, may also contribute to the development of resistance *in vivo* (Stevens et al., 1997; Kastner and Guggenbichler, 2001).

Molecular methods have been developed using a variety of techniques to rapidly determine susceptibility of a *M. pneumoniae* isolate or primary clinical specimen extract. While these methods vary in complexity, all require a substantial investment of equipment, laboratory space, and highly trained staff for performance and, therefore, are generally restricted

to reference and research laboratories. However, some studies indicate that infection with macrolide-resistant *M. pneumoniae* may be of longer duration or severity (Cardinale et al., 2013; Zhou et al., 2014), supporting the value of macrolide susceptibility testing for informing patient management, particularly in severe cases.

Sanger Sequencing

Perhaps the most straightforward approach to identifying sequence polymorphisms in the 23S rRNA gene is to amplify the target region by conventional PCR and perform nucleotide sequencing analysis. Lucier et al. (1995) performed broth dilution tests, ribosomal binding studies, and DNA sequencing analysis to identify SNPs within 23S rRNA gene of *M. pneumoniae* that confer resistance to macrolide antibiotics. Subsequent studies have used Sanger sequencing as a comparative method to validate novel molecular assays for detection of known polymorphisms (Matsuoka et al., 2004; Wolff et al., 2008; Chan et al., 2013).

Recently, Dumke et al. (2014) reported the emergence of a macrolide-resistant subpopulation of *M. pneumoniae* within an individual patient by collection and testing of multiple specimens during the course of the infection. In this report, the investigators cloned PCR amplicons of 23S rRNA into a plasmid, selected colonies, and performed sequencing to identify the genotype present in the specimen. Using this method, only sensitive *M. pneumoniae* were detectable in the specimen collected on day 1, but a mixture of both sensitive and resistant sequences were detected in a specimen collected 18 days later (Dumke et al., 2014). Interestingly, resistant quasispecies containing either the A2063G or A2064G mutations (46 and 28%, respectively) were identified in the same specimen, along with the wildtype genotype (26%). While this method may be used to identify mixed populations of macrolide-sensitive and –resistant *M. pneumoniae* in a patient specimen, it is prohibitively cumbersome and time-consuming to be feasible for clinical testing or even as a routine procedure in specialty reference laboratories.

Pyrosequencing

Pyrosequencing has been used in several studies to evaluate macrolide susceptibility of *M. pneumoniae* isolates or primary specimen extracts (Cao et al., 2010; Spuesens et al., 2010, 2012). For pyrosequencing, PCR is performed using a set of oligonucleotide primers, one of which has a biotin label. The resulting biotinylated PCR product is purified using streptavidin-coated beads, denatured, and subjected to sequencing. Pyrosequencing is the only method developed to date that is capable of quantifying the proportions of macrolide-sensitive and -resistant quasispecies within a clinical specimen (Chan et al., 2013) and is a more feasible approach for determination of mixed genotypes in clinical specimens compared to the cloning and sequencing method described in section "Sanger Sequencing." Using pyrosequencing, Chan et al. (2013) determined that nearly 80% of *M. pneumoniae*-positive clinical specimens contained some proportion of macrolide-resistant quasispecies. In specimens that were previously identified as having the wildtype (macrolide-susceptible) genotype using another method, up to 44% of the *M. pneumoniae* population was found to be macrolide-resistant. Among specimens identified as macrolide-resistant by other methods, pyrosequencing revealed that the resistant quasispecies comprised 52–100% of the total population. These results underscore the potential for development of macrolide resistance during the course of infection in an individual patient. Longitudinal studies in which multiple specimens are collected from the same patient throughout the duration of illness will be necessary to demonstrate the emergence of macrolide-resistant *M. pneumoniae* resulting from macrolide therapy at an individual level. Adoption of methods capable of identifying quasispecies within a patient specimen, including pyrosequencing and potentially digital droplet PCR, could help monitor the emergence of resistance in this organism or to identify infections that are less likely to be resolved by macrolide therapy.

Restriction Fragment Length Polymorphism (RFLP) Analysis

Matsuoka et al. (2004) established RFLP methods for analysis of point mutations in 23S rRNA in *M. pneumoniae*. RFLP has traditionally been used for typing of *M. pneumoniae* based on the P1 adhesion molecule as described in section "Restriction Fragment Length Polymorphism (RFLP) and Sequencing Analysis" above. Digestion of a 210 bp PCR product amplified from 23S rRNA with either BceAI or BsaI results in multiple fragments when either the A2063G or A2064G mutation is present compared to a single uncut fragment for amplified product containing the wildtype genotype (Matsuoka et al., 2004). While this method is reliable, it is not well-suited for use in clinical microbiology laboratories.

Melting Curve Analysis

Wolff et al. (2008) described a PCR assay using high-resolution melt (HRM) analysis to rapidly differentiate macrolide-resistant and –susceptible isolates. Two versions of the assay were developed, using a specific primer set with an intercalating dye or a self-quenched fluorogenic LUX primer. The substitution of G for A at position 2063 or 2064 causes the amplicon to melt at a slightly higher temperature, thus the melting profile can reliably distinguish macrolide-resistant isolates by comparison to sensitive and resistant controls included in the run. However, this method does not identify the exact mutation present within the amplicon. Subsequently, the HRM assay was modified to include a nested PCR step, allowing for testing of nucleic acid from primary clinical specimens (Diaz et al., 2015b). Eliminating the need to obtain an isolate allowed the assay to be performed in sufficient time to inform patient treatment decisions.

Similarly, Chan et al. (2013) developed a SimpleProbe real-time PCR assay with melting curve analysis for detection of SNPs in 23S rRNA of *M. pneumoniae*. The SimpleProbe format consists of a single-labeled hybridization probe that emits higher fluorescence when bound to the specific target sequence containing the SNP of interest compared to emission in the unhybridized state. Binding of the probe to the PCR product that contains the SNP is less stable, causing it to melt at a lower

temperature. This reaction can be performed with relatively rapid cycling conditions resulting in a turnaround time under 1 h (Chan et al., 2013), which represents a substantially faster time to results compared to other methods for macrolide susceptibility determination.

Recently, Nummi et al. (2015) reported the development of a multiplex real-time PCR assay for simultaneous detection of *M. pneumoniae*, *C. pneumoniae*, and the two most common mutations that confer macrolide resistance in *M. pneumoniae*. This method utilizes post-PCR dissociation curve analysis to identify macrolide-resistant 23S rRNA sequences amplified from clinical specimens. This type of assay, which provides simultaneous identification of *M. pneumoniae* and determination of macrolide susceptibility in patient specimens, would improve appropriate antibiotic prescribing for respiratory infections caused by this pathogen. Implementation of this type of method at the point-of-care would provide the best opportunity to impact prescribing and patient management. On a population level, widespread implementation of methods like this in surveillance programs would improve monitoring of macrolide resistance patterns, particularly as these may change rapidly and vary substantially based on geography.

WHOLE GENOME SEQUENCING (WGS)

The reduced genome makes *M. pneumoniae* amenable to high throughput WGS and other "omics" analyses. Vast improvements in WGS over the past decade have made this a more accessible approach for identification and characterization of bacteria. Sequencing platforms have evolved from whole genome shotgun sequencing (Sanger) to high-throughput sequencing (Roche 454 and Illumina) and finally to single-molecule long-read sequencing (PacBio SMRT sequencing and Oxford Nanopore sequencing; Loman and Pallen, 2015). The availability of benchtop sequencers has expanded WGS capacity in academic, clinical, and public health laboratories. This expansion in sequencing capability has resulted in a rapid increase in the number of bacterial genomes, including *M. pneumoniae*, made publicly available in the last several years.

Figure 1 shows a timeline highlighting the major milestones in *M. pneumoniae* WGS. The genome of *M. genitalium* was one of the first bacterial whole genome sequences obtained in 1995 (Fraser et al., 1995). The *M. pneumoniae* type 1 reference strain M129 followed soon after in 1996, making *Mycoplasma* the first bacterial genus to have whole genome sequences from two different species (Himmelreich et al., 1996). The genome of M129 was subsequently re-annotated in 2000 and found to have 816,394 bp and 730 genes (Dandekar et al., 2000). This served as the only available reference genome for *M. pneumoniae* until the first sequence of a type 2 *M. pneumoniae* strain, the reference strain FH, was reported 10 years later (Krishnakumar et al., 2010). This was followed by the report of the whole genome of a type 2a strain (309) in 2012 (Kenri et al., 2012). Demonstrating the rapid advancement in technical improvements and accessibility to WGS technology, two studies were published in 2015 reporting comparative genomic analysis of 15 and 23 *M. pneumoniae* strains, respectively (Lluch-Senar et al., 2015; Xiao et al., 2015). While a discussion of all "-omics" analysis of *M. pneumoniae* is outside the scope of this review, in the following sections we

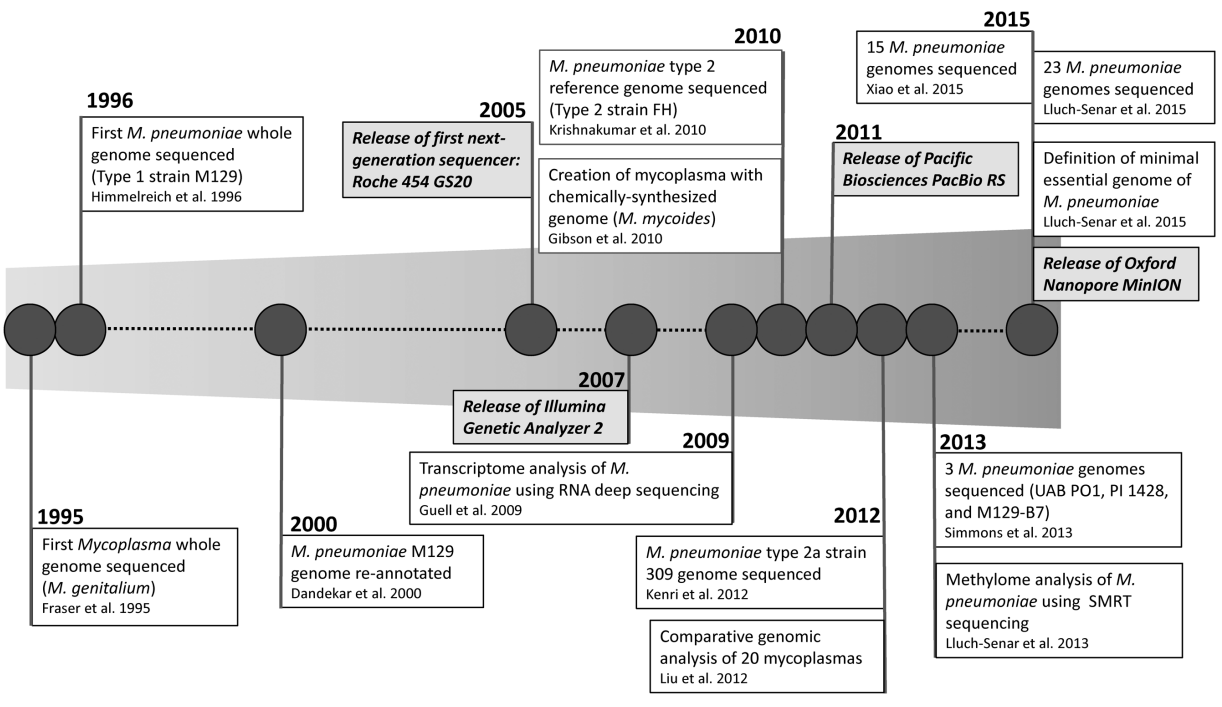

FIGURE 1 | Timeline of milestones in whole genome sequencing of *Mycoplasma pneumoniae*.

will discuss recent findings of genomic analyses and how this technology may impact *M. pneumoniae* diagnostics in the future.

Comparative Genomics

Xiao et al. (2015) analyzed 15 *M. pneumoniae* genomes obtained by Illumina sequencing, including 11 clinical isolates and 4 reference strains. They observed over 99% sequence similarity between all genomes, with the most variation occurring in specific regions within each of the P1 and ORF6 genes, two genes encoded in the same operon which encode components of the adhesin complex. Phylogenetic trees built on whole genome SNP analysis revealed two major clusters based on P1 type. This analysis also suggested that the genome of *M. pneumoniae* is extremely stable over time and geographic distribution, and no evidence of horizontal gene transfer was found in the sequenced isolates (Xiao et al., 2015).

Lluch-Senar et al. (2015) reported a multi-"omics" analysis of *M. pneumoniae*, including comparative genomic analysis of 23 *M. pneumoniae* isolates. Classification of diverse *M. pneumoniae* isolates based on SNPs and indels revealed new subclasses within the broader P1 types 1 and 2 classifications, including four subtypes within type 1 (1a–1d) and five within type 2 (2a–e). The authors concluded that some of these subtypes were associated with country of isolation, but a more comprehensive study including a higher number of isolates representing additional geographic origins is necessary to confirm this observation. Interestingly, this analysis revealed that the frequency of genomic rearrangements was higher than that of SNPs or indels in *M. pneumoniae*. In addition, it was observed that SNPs, indels, and non-synonymous mutations were enriched within genes encoding for proteins involved in virulence, including adhesion molecules. These findings support the purported rearrangement of adhesion genes present in multiple copies within the *M. pneumoniae* genome during infection as a mechanism to circumvent host immune responses (Citti et al., 2010).

Beyond the vast clinical, epidemiological, and microbiological interest in *M. pneumoniae*, it is also commonly used as a model organism in systems biology. The genome of *M. pneumoniae* has been compared to other Mycoplasmas, and the core genome defined for this genus represents the minimal genetic requirements for a prokaryotic organism (Liu et al., 2012). Researchers have even been able to create a viable mycoplasma cell (*M. mycoides*) containing a completely synthetic genome (Gibson et al., 2010). Others have characterized the transcriptome (Guell et al., 2009; Lluch-Senar et al., 2015), proteome (Ueberle et al., 2002; Kuhner et al., 2009; Catrien and Herrmann, 2011; Lluch-Senar et al., 2015), phosphoproteome (Su et al., 2007; Schmidl et al., 2010), methylome (Lluch-Senar et al., 2013), and metabolome (Maier et al., 2013) of *M. pneumoniae*, all of which add to the vastly increasing field of systems biology. The incredibly rapid accumulation of "-omics" data prompted the creation of MyMpn, an open access database for *M. pneumoniae* datasets, including complete genome sequences (Wodke et al., 2015). It is expected that large-scale datasets, including WGS data from many isolates, will continue to grow and be mined for data to investigate *M. pneumoniae* as a pathogen as well as a model organism.

Impact of Whole Genome Sequencing on *M. pneumoniae* Diagnostics

Whole genome sequencing has the potential to permanently change the field of *M. pneumoniae* biology and epidemiology by allowing improved characterization of strains and better discriminatory power compared to any previous typing method. These data can be used to inform development of newer methods to improve strain discrimination that are accessible to all laboratories. In 2015 alone, two new methods, MLST and SNaPshot mini-sequencing assays, were reported in which whole genome sequence data was used to inform the assay design (Brown et al., 2015b; Touati et al., 2015). Eventually, WGS directly from clinical specimens may become the standard method for determination of etiology of respiratory infections. While the cost of sequencing a bacterial genome has dropped dramatically in recent years, sequencing is still primarily performed on bacterial isolates. Recently, WGS directly from clinical specimens has been demonstrated for detection of respiratory viruses (Zoll et al., 2015) and for *Mycobacterium tuberculosis* (Brown et al., 2015a). Continued technical improvements could allow for direct metagenomics analysis of the entire composition of microbial flora within a patient specimen, which will be critical for implementation of deep sequencing as a primary diagnostic method. This will allow for detailed epidemiological tracking of temporal and geographical trends in strain circulation and will fundamentally change how outbreaks of respiratory disease are investigated.

CONCLUSION

Over the past decade, advanced molecular methods for the detection and characterization of *M. pneumoniae* have grown exponentially in regards to both the number and variety of available methods. More widespread implementation of these methods globally has revealed new trends, such as the rapid emergence of macrolide resistance in some parts of the world and the co-circulation of multiple strain types during a discrete period, which challenges a long-standing belief about *M. pneumoniae* epidemiology. Numerous studies in which novel methods were utilized have also highlighted the inadequacy of existing typing strategies, particularly with regards to the inability to definitively link any particular type with clinical characteristics or patient outcomes. The improved accessibility of WGS at the clinical laboratory level and rapidly growing wealth of bioinformatics tools for sequence analysis from clinical specimens is likely to result in a paradigm shift toward WGS analysis for *M. pneumoniae* diagnostics and in clinical microbiology overall.

AUTHOR CONTRIBUTIONS

MD and JW contributed to the literature review and interpretation, drafted the work and revised for intellectual content, provided final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Microbiology

MINI REVIEW
published: 22 June 2016
doi: 10.3389/fmicb.2016.00974

Mycoplasma pneumoniae: Current Knowledge on Macrolide Resistance and Treatment

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *13 February 2016*
Accepted: *06 June 2016*
Published: *22 June 2016*

Citation:
Pereyre S, Goret J and Bébéar C (2016) Mycoplasma pneumoniae: Current Knowledge on Macrolide Resistance and Treatment. Front. Microbiol. 7:974. doi: 10.3389/fmicb.2016.00974

Mycoplasma pneumoniae causes community-acquired respiratory tract infections, particularly in school-aged children and young adults. These infections occur both endemically and epidemically worldwide. *M. pneumoniae* lacks cell wall and is subsequently resistant to beta-lactams and to all antimicrobials targeting the cell wall. This mycoplasma is intrinsically susceptible to macrolides and related antibiotics, to tetracyclines and to fluoroquinolones. Macrolides and related antibiotics are the first-line treatment of *M. pneumoniae* respiratory tract infections mainly because of their low MIC against the bacteria, their low toxicity and the absence of contraindication in young children. The newer macrolides are now the preferred agents with a 7-to-14 day course of oral clarithromycin or a 5-day course of oral azithromycin for treatment of community-acquired pneumonia due to *M. pneumoniae*, according to the different guidelines worldwide. However, macrolide resistance has been spreading for 15 years worldwide, with prevalence now ranging between 0 and 15% in Europe and the USA, approximately 30% in Israel and up to 90–100% in Asia. This resistance is associated with point mutations in the peptidyl-transferase loop of the 23S rRNA and leads to high-level resistance to macrolides. Macrolide resistance-associated mutations can be detected using several molecular methods applicable directly from respiratory specimens. Because this resistance has clinical outcomes such as longer duration of fever, cough and hospital stay, alternative antibiotic treatment can be required, including tetracyclines such as doxycycline and minocycline or fluoroquinolones, primarily levofloxacin, during 7–14 days, even though fluoroquinolones and tetracyclines are contraindicated in all children and in children <8 year-old, respectively. Acquired resistance to tetracyclines and fluoroquinolones has never been reported in *M. pneumoniae* clinical isolates but reduced susceptibility was reported in *in vitro* selected mutants. This article focuses on *M. pneumoniae* antibiotic susceptibility and on the development and the evolution of acquired resistance. Molecular detection of resistant mutants and therapeutic options in case of macrolide resistance will also be assessed.

Keywords: *Mycoplasma pneumoniae*, macrolides, resistance, molecular detection, treatment

INTRODUCTION

Mycoplasma pneumoniae is responsible for community-acquired respiratory tract infections, such as tracheobronchitis and pneumonia, particularly in school-aged children and young adults. These infections occur both endemically and epidemically at 3-to-7-year intervals worldwide (Atkinson et al., 2008). Numerous extra-respiratory manifestations of variable severity have also been associated with *M. pneumoniae* infections including dermatological manifestations and neurological complications. Before 2000, *M. pneumoniae* infections were easily treated using macrolides because only rare cases of resistance to macrolides had been reported in clinical isolates. Since 2000, macrolide resistance rates have been rising up to 90–100% in Asia, hindering the efficacy of common antibiotic regimens.

This mini-review focuses on *M. pneumoniae* intrinsic resistance, antibiotic susceptibility and on the development and the evolution of acquired macrolide resistance worldwide since the last published review (Bébéar et al., 2011). Methods for molecular detection of macrolide resistance-associated mutations and therapeutic options in case of infections with macrolide-resistant *M. pneumoniae* strains are also assessed.

ACTIVE ANTIBIOTICS AND INTRINSIC RESISTANCE

Like all microorganisms that lack cell wall, *M. pneumoniae* is intrinsically resistant to beta-lactams and to all antimicrobials targeting the cell wall, such as glycopeptides and fosfomycin. *M. pneumoniae* is also resistant to polymixins, sulfonamides, trimethoprim, rifampicin and linezolid (Bébéar and Kempf, 2005; Bébéar et al., 2011). Antibiotics with potential activity against *M. pneumoniae* that are used in clinical practice include macrolides, lincosamides, streptogramin combinations and ketolides (MLSK), tetracyclines and fluoroquinolones. These drugs achieve high intracellular concentration in mammalian cells and are thereby able to reach intracellular mycoplasmas. The MICs of the main antibiotics belonging to the MLSK group are the lowest against *M. pneumoniae* compared with those of the two other classes, except MIC of lincomycin that is high (see MIC of the sensitive reference strain M129 (ATCC 29342) in **Table 1**; Bébéar et al., 2011). MICs of tetracyclines and fluoroquinolones are about 10 times higher than those of MLSK, but newer fluoroquinolones such as levofloxacin and moxifloxacin show an enhanced activity against *M. pneumoniae.* Only fluoroquinolones and ketolides have a potential bactericidal action. Other antibiotics such as aminoglycosides and chloramphenicol show some activity against *M. pneumoniae* (MICs 2–10 μg/ml for chloramphenicol and MIC 4 μg/ml for gentamicin, Bébéar et al., 2011) but are not recommended for *M. pneumoniae* infections.

The *in vitro* activity of a few new agents was recently reported. AZD0914, a spiropyrimidinetrione DNA gyrase inhibitor, showed a MIC_{90} of 1 μg/ml, comparable to that of levofloxacin (Waites et al., 2015). ACH-702, a novel isothiazoloquinolone, and BC-3781, a semi-synthetic pleuromutilin antibiotic, showed

TABLE 1 | MICs of MLSK, tetracycline and fluoroquinolone antibiotics for *M. pneumoniae* clinical isolates resistant to macrolides and genetically characterized.

	14-membered macrolides		15-membered macrolide		16-membered macrolides		Lincosamides		S.C.	Ketolide	Tetracycline	Fluoroquinolones		
Isolates	**ERY[a]**	**CLA**	**AZM**	**JOS**	**MDM**	**RKI**	**LIN**	**CLI**	**Q–D**	**TEL**	**MIN**	**CIP**	**LEV**	**MXF**
Sensitive reference strains M129 (ATCC 29342)	0.004–0.03	0.002–0.015	0.002–0.06	0.03–0.12	0.008–0.06	0.01–0.06	8	4	0.25	0.002	0.25	1	0.12–1	0.03–0.25
CLINICAL STRAINS WITH MUTATION IN DOMAIN V OF 23S rRNA:														
A2058G[b]	32–>256	32–>256	2–>64	0.06–64	2–>64	0.01–16	>256	16–256	0.06–1	16–>64	0.016–1	0.125–2	0.125–2	<0.008–0.03
A2058C	>256	>256	16	64	64	4	64	32	1	ND[c]	ND	ND	ND	ND
A2058T	32–64	16–64	0.064–0.25	16	ND	4	ND	256	ND	ND	0.25–1	0.5–1	0.25–1	0.032
A2059G	>64–>256	16–>256	4–64	>64–256	>64–>256	8–32	64	32	0.06–0.25	1–16	0.03–1	0.5–1	0.25–1	0.06–0.12
C2611G	8	1	0.03	0.25	0.25	0.06	16	4	0.25	ND	ND	ND	ND	ND
C2611A	1	0.5	0.03	0.06	ND	0.03	ND	ND	ND	0.06	1	ND	1	0.125

Adapted from Bébéar et al. (2011), Cao et al. (2010), Zhao et al. (2013b), Matsuda et al. (2013), Yamazaki et al. (2007), Pereyre et al (2004b), Waites et al. (2011), Morozumi et al. (2010).

[a]ERY, erythromycin; CLA, clarithromycin; AZM, azithromycin; JOS, josamycin; MDM, midecamycin; RKI, roktamycin; LIN, lincomycin; CLI, clindamycin; S.C., Streptogramin combination, Q-D, quinupristin-dalfopristin; TEL, telithromycin; MIN, minocycline; CIP, ciprofloxacin; LEV, levofloxacin; MXF, moxifloxacin.

[b]E. coli numbering.

[c]ND, not determined.

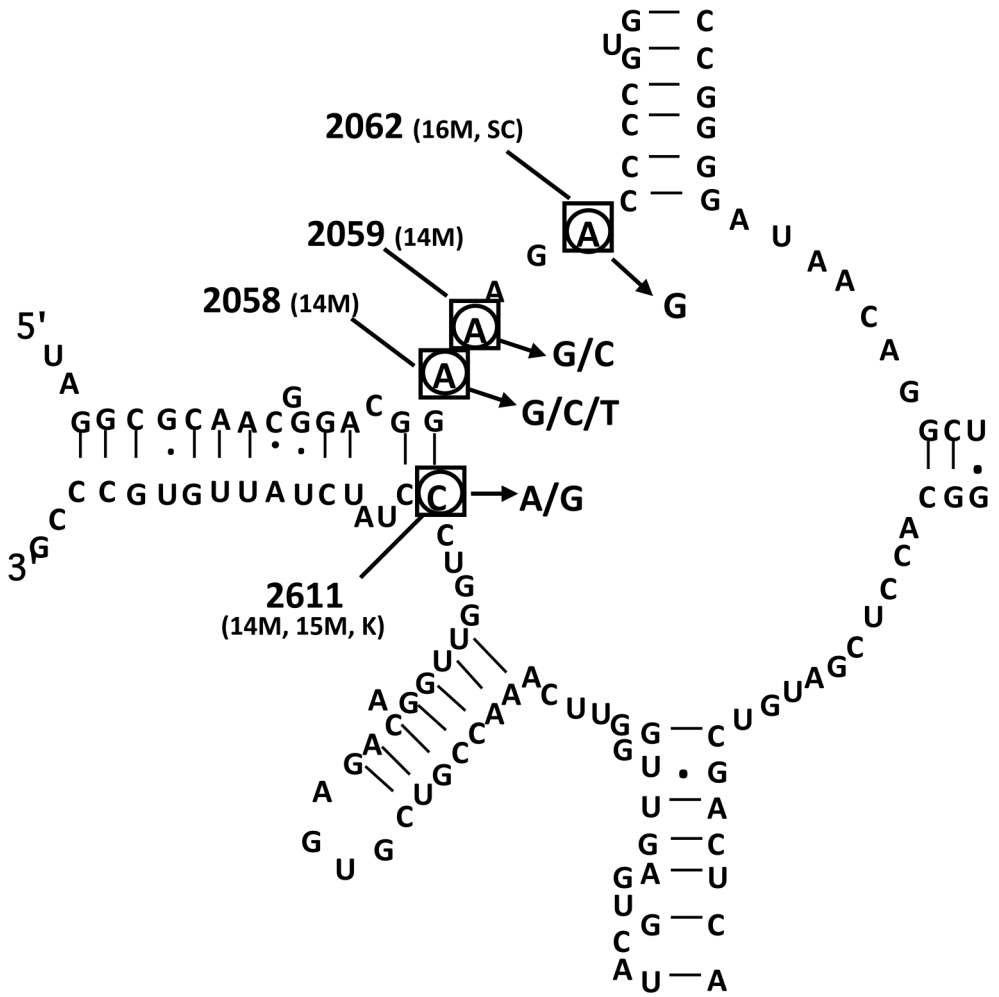

FIGURE 1 | Peptidyltransferase loop of domain V of 23S rRNA of *Mycoplasma pneumoniae* (*Escherichia coli* numbering) with nucleotides found mutated in *in vitro*-selected strains and in clinical isolates of macrolide-resistant *M. pneumoniae*. Adapted from Bébéar et al. (2011). Squared nucleotides indicate positions mutated in *in vitro*-selected macrolide resistant mutants. Antibiotics used for *in vitro* selection are in parentheses (14M, 14-membered macrolides; 15M, 15-membered macrolides; 16M, 16-membered macrolides; SC, streptogramin combinations; K, ketolides). Circled nucleotides indicate positions mutated in clinical macrolide resistant isolates.

better MICs, comparable to those of MLSK, with MIC_{90} of 0.015 and 0.006 μg/ml, respectively (Pucci et al., 2011; Sader et al., 2012).

MECHANISMS OF *M. PNEUMONIAE* ACQUIRED RESISTANCE AND RESISTANCE MOLECULAR DETECTION METHODS

In *M. pneumoniae*, only antimicrobial target modifications by acquired mutations have been associated with antibiotic resistance (Bébéar and Pereyre, 2005). The high mutation rates and the small amount of genetic information dedicated to DNA repair in mycoplasmas (Rocha and Blanchard, 2002) may be associated with this single mode of antibiotic resistance. Resistance through mutation was reported in *in vitro*-selected mutants for all three classes of antibiotics used to treat *M. pneumoniae* whereas to date, resistance in clinical isolates was only reported for the MLSK antibiotic class.

Acquired Resistance to Macrolides and Related Antibiotics

Macrolide resistance in the *M. pneumoniae* species, which harbors only one ribosomal operon, is defined by mutations in the ribosomal target of the antibiotic, i.e., the 23S rRNA and the ribosomal proteins L4 and L22 (Bébéar and Pereyre, 2005; Bébéar et al., 2011). The A2058G (*Escherichia coli* numbering) transition in the peptidyltransferase loop of domain V of 23S rRNA is the most common mutation that is associated with macrolide resistance (**Figure 1**, **Table 2**). Other substitutions have been reported at position 2058 (A2058C,

A2058T), at position 2059 (A2059G, A2059C), at position 2062 (A2062G) and at position 2611 (C2611G, C2611A). No mutation has been detected in domain II of 23S rRNA. Mutations in conserved regions of ribosomal L4 and L22 proteins such as single amino acid change, insertion and deletion of amino acids have also been associated with low-level macrolide resistance in *in vitro* selected mutants (Pereyre et al., 2004a). Rare mutations have been reported *in vivo* in ribosomal proteins L4 and L22 but were not associated with significant increased MICs of macrolides (Cao et al., 2010). Comparison of sequencing results with antimicrobial susceptibility testing confirmed that mutations A2058G and A2059G led to a high level resistance to 14- and 15-membered macrolides and lincosamides (Xin et al., 2009; Cao et al., 2010; Akaike et al., 2012; Zhao et al., 2013b; Table 1). Whereas 16-membered macrolides were highly affected by the A2059G substitution, the A2058G mutation was associated with an intermediate level of resistance to these antibiotics. Mutations at position 2611 were associated with low-level of resistance to MLSK. Interestingly, the streptogramin combinations, quinupristin-dalfopristin and pristinamycin, and the ketolide solithromycin (CEM-101) retained activity on resistant mutants harboring mutations at position 2058, 2059, and 2611 (Pereyre et al., 2007; Waites et al., 2009; **Table 1**). However, an *in vitro* mutant selection study showed that the A2062G transition was associated with significant increased MICs of these two streptogramin combinations (Pereyre et al., 2004a).

Cross-resistance was not observed between MLSK and other antibiotic families commonly used against *M. pneumoniae* because isolates with macrolide resistance-associated mutations remain susceptible to tetracyclines and fluoroquinolones (**Table 1**).

Several molecular methods applicable directly on respiratory specimens were developed to detect macrolide resistance and to circumvent the fastidious, insensitive and time-consuming isolation of *M. pneumoniae* from clinical samples. Apart from the conventional amplification and sequencing of the hot spots of the 23S rRNA gene, macrolide resistance determination was achieved by PCR-restriction fragment lengh polymorphism (Matsuoka et al., 2004), real-time PCR and melting curve analysis (Peuchant et al., 2009), pyrosequencing (Spuesens et al., 2010, 2012) and real-time PCR and high resolution melt (HRM) analysis (Wolff et al., 2008). A nested-PCR combined with single-strand conformation polymorphism and capillary electrophoresis (Lin et al., 2010) and a singe nucleotide polymorphism (SNP) PCR assay (Ji et al., 2014) were also developed to detect macrolide-resistant mutants directly from clinical specimens. Most of these in-house approaches allow resistance screening in *M. pneumoniae*-positive respiratory tract samples but the clinical sensitivity i.e., the proportion of *M. pneumoniae*-positive specimens capable of being resistance typed varies according to methods, ranging between 72.6 and 80.2% in the studies where it was calculated (Wolff et al., 2008; Peuchant et al., 2009; Spuesens et al., 2012). The need to perform such tests differs according to the prevalence of macrolide resistance in each country. In countries where the percentage of macrolide resistance is over 10%, it could be recommended that all *M. pneumoniae* detection be followed up with an assay capable of detecting macrolide resistance-associated mutations. This strategy would allow a non-macrolide treatment to be promptly started in the event that a macrolide-resistant genotype is detected in an individual patient. In contrast, in countries where macrolide resistance remains below 10%, this kind of test could be performed only in case of treatment failure.

Currently, this strategy is hampered by the lack of commercially available sensitive kits that detect macrolide resistance-associated mutations. However, such kits are currently in development and may soon become available. They will be useful for routine diagnostics in microbiology laboratories.

Acquired Resistance to Tetracyclines and Fluoroquinolones

To date, no tetracycline or fluoroquinolone resistance has been reported in *M. pneumoniae* clinical isolates. However, resistant strains have been selected *in vitro* for both classes of drugs. Target mutations were identified in the 16S rRNA gene of tetracycline-resistant mutants selected with subinhibitory concentrations of doxycycline. Mutations were associated with reduced susceptibility to tetracycline, doxycycline and minocycline with MICs remaining below $\leq$2 μg/ml (Degrange et al., 2008). Mutations within conserved regions of the *gyrA*, *gyrB*, *parC,* and *parE* genes referred to as the quinolone resistance-determining regions were reported for fluoroquinolone-resistant mutants selected with different fluoroquinolones and were associated with MICs of ciprofloxacin, levofloxacin and moxifloxacin up to 32, 16, and 4 μg/ml, respectively (Gruson et al., 2005). Mutations rates were low for levofloxacin and moxifloxacin, ranging from 1.3×10^{-6} to 7×10^{-9} (Gruson et al., 2005).

PREVALENCE OF MACROLIDE RESISTANCE IN *M. PNEUMONIAE*

Recent rates of macrolide resistance in *M. pneumoniae* clinical isolates in countries in which publications have been released since the last review (Bébéar et al., 2011) are presented in **Table 2**. Prior to the year 2000, very few *M. pneumoniae* clinical isolates were resistant to macrolides. Rare strains resistant to erythromycin were reported in the literature between 1968 and 1999 in Japan, Israel, Finland, USA and France (Niitu et al., 1970; Stopler and Branski, 1986; Critchley et al., 2002; Pereyre et al., 2007). By contrast, several Japanese studies have reported a significant and constant increase in macrolide resistance rates since 2000, reaching 30% in 2006, around 60% in 2009 and up to 89% in 2010–2011 (Morozumi et al., 2008; Okada et al., 2012; Matsuda et al., 2013). However, regional differences in rates of macrolide-resistant *M. pneumoniae* were recently reported in Japan, for example in Hokkaido island, where rates ranged from 0 to 100% according to regions (Ishiguro et al., 2015). The situation is worse in China where a dozen of articles have reported a prevalence of macrolide resistance

TABLE 2 | Prevalence of macrolide resistance in *M. pneumoniae* clinical isolates (continents and countries are presented in alphabetical order).

Country	Year	% of macrolide resistance (number of resistant strains or *M. pneumoniae*-positive specimens/total strains or specimens tested)	23S rRNA mutations (%)	References
AMERICA				
Canada (Ontario)	2010–2012	12.1% (11/91)	A2058G (91%) A2059G (18%)	Eshaghi et al., 2013
USA (14 states)	2006–2013	10.8% (19/176)	ND	Diaz et al., 2015b
USA (St. Louis, Missouri)	2010–2012	8.2% (4/49)	A2058G (100%)	Yamada et al., 2012
USA (3 states)	2010–2012	3.5% (7/202)	A2058G (85.7%) A2059G (14.3%)	Diaz et al., 2015a
USA (6 states)	2012–2014	13.2% (12/91)	A2058G (100%)	Zheng et al., 2015
ASIA				
China (Beijing)	2003–2006	92% (46/50)	A2058G (86.9%) A2058C (2.2%) A2059G (10.9%)	Xin et al., 2009
China (Shanghai)	2005–2009	90.1% (137/152)	ND	Liu et al., 2012
China (Beijing)	2008–2009	69% (46/67)	A2058G (89.1%) A2059G (8.7%) A2058T (2.2%)	Cao et al., 2010
China (Shanghai)	2008–2009	90% (90/100)	A2058G (98%) A2058T (1%) A2059G (1%)	Liu et al., 2010
China (Beijing)	2008–2011	88.1% (177/201)	A2058G (96.6%) A2059G (2.8%) A2059T (0.6%)	Zhao et al., 2013a
China (Beijing)	2008–2012	90.7% (280/309)	A2058G (97.1%) A2059G (2.5%) A2058T (0.4%)	Zhao et al., 2013b
China (Beijing)	2009	91% (58/64)	A2058G (98.3%) A2058T (1.7%)	Lin et al., 2010
China (Beijing)	2010–2012	90.8% (59/65)	A2058G (100%)	Sun et al., 2013
China (Beijing, Dongcheng, Xicheng)	2011	95% (38/40)	A2058G (97%) A2059G (3%)	Zhao et al., 2011
China (Zhejiang province)	2012–2014	100% (71/71)	A2058G (100%)	Zhou et al., 2015
China (Beijing)	2013	98.5% (128/130)	A2058G (100%)	Yan et al., 2014
Hong-Kong	2011 2012 2013 2014	13.6% (3/22) 30.7% (23/75) 36.6% (34/93) 47.1% (24/51)	A2058G (100%)	Ho et al., 2015
Japan (65 institutions)	2008 2009	56% (9/16) 69% (9/13)	A2058G (95.9%)* A2058T (3.2%)	Kawai et al., 2013

(Continued)

TABLE 2 | Continued

Country	Year	% of macrolide resistance (number of resistant strains or *M. pneumoniae*-positive specimens/total strains or specimens tested)	23S rRNA mutations (%)	References
	2010 2011 2012	71% (79/110) 63% (176/281) 82% (288/349)	A2059G (0.5%) A2058C (0.2%) C2611G (0.2%)	
Japan (Fukuoka prefecture)	2010–2011	89.2% (58/65)	A2058G (53%) A2058T (47%)	Matsuda et al., 2013
Japan (5 institutions)	2011	87.1% (176/202)	A2058G (90.9%) A2058T (6.2%) A2059G (2.3%) A2058C (0.6%)	Okada et al., 2012
South Korea	2003 2006 2010 2011	2.9% (1/34) 14.7% (10/68)	A2058G (% ND) A2059G (% ND) 47.2% (25/53) 62.9% (44/70)	Hong et al., 2013
Taiwan	2010–2011	23.3% (14/60)	A2058G (100%)	Wu et al., 2013
EUROPE				
Denmark	2010–2011	1.6% (6/365)	ND	Uldum et al., 2012
England and Wales	2010	0% (0/24)	-	Chalker et al., 2011
England and Wales	2011–2012	0% (0/12)	-	Chalker et al., 2012
England	2014–2015	9.3 (4/43)	A2058G (100%)	Brown et al., 2015
France	2005–2007	9.8% (5/51)	A2058G (60%) A2059G (20%) C2611G (20%)	Peuchant et al., 2009
France	2007–2010	3.4% (1/29)	A2059G	Pereyre et al., 2012
France	2011	8.3% (6/72)	A2058G (67%) A2059G (16.5%) A2062G (16.5%)	Pereyre et al., 2013
Germany	2003–2008	1.2% (2/167)	A2058G A2058C	Dumke et al., 2010
Germany	2009–2012	3.6% (3/84)	A2058G (100%)	Dumke et al., 2013
Germany	2011–2012	3.1% (3/96)	A2058G (100%)	Dumke et al., 2015
Italy	2010	26% (11/43)	A2058G (63.6%) A2059G (36.4%)	Chironna et al., 2011
Slovenia	2006–2014	1% (7/783)	A2058G (100%)	Kogoj et al., 2015
Switzerland	2011–2013	2% (1/50)	A2058G	Meyer Sauteur et al., 2014
MIDDLE EAST				
Israel	2010	30% (9/30)	A2058G (100%)	Averbuch et al., 2011
Israel	2010	22% (9/41)	A2058G (100%)	Pereyre et al., 2012
OCEANIA				
Australia (Sydney)	2008–2012	3.3% (1/30)	A2059G	Xue et al., 2014

ND, not determined.

**Percentages calculated among the 561 resistant isolates collected over the 5 years.*

between 90 and 100% since 2003. Other Asian countries seem less affected with resistance rates of 62.9, 47.1, and 23.3% in South Korea, Hong-Kong and Taiwan, respectively (**Table 2**). It should be noted that most reports regarding macrolide resistance relate on hospitalized patients. It cannot be excluded that the macrolide resistant rate in *M. pneumoniae* may be higher in hospitalized patients in whom the resistant population may be concentrated than in outpatients. However, comprehensive studies on outpatients are not easily achievable because many *M. pneumoniae* infections such as mild tracheobronchitis are often undiagnosed.

The high macrolide resistance rates in these countries are certainly associated with antibiotic selective pressure because of extensive macrolide use. This is supported by the highest macrolide resistance rates being reported in countries with extensive macrolide use such as Japan (Okada et al., 2012). In addition, macrolide resistance was often associated with recent receipt of macrolides, suggesting that a resistant subpopulation may develop or expand during the course of macrolide therapy within an individual patient (Averbuch et al., 2011; Cardinale et al., 2011; Chironna et al., 2011; Hantz et al., 2012; Dumke et al., 2014). Acquisition of resistance has first been documented in patients receiving macrolides (Averbuch et al., 2011; Cardinale et al., 2011) then confirmed using typing methods such as adhesin P1 typing and multi-locus variable-number tandem-repeat analysis (MLVA) in patients receiving macrolides (Hantz et al., 2012; Dumke et al., 2014).

In North America, Europe, and Australia, rates of macrolide resistance dramatically contrast with those in reports from Asia. In the USA and Canada, rates have been recently reported between 3.5 and 13.2% (**Table 2**). In Europe, rates have remained below 10% except in Italy were a rate of 26% was observed on a small number of *M. pneumoniae*-positive specimens collected during an outbreak (Chironna et al., 2011).

All over the world, the A2058G transition largely predominates over the A2059G substitution and mutations at position 2611 and 2062 are rare (**Table 2**). Nevertheless, the rarely reported A2058T transversion was found in 47% of macrolide-resistant *M. pneumoniae* strains infecting children during an outbreak in Fukuoka, Japan (Matsuda et al., 2013). Despite the high proportion of the A2058G transition, no association was reported between MLVA types and macrolide resistance in several studies (Dégrange et al., 2009; Benitez et al., 2012; Liu et al., 2012; Zhao et al., 2013a,b; Dumke et al., 2015; Diaz et al., 2015a,b) indicating that macrolide resistance is a result of the spread of multiple resistant clones. A possible correlation was reported in Jerusalem, Israel, between the MLVA type Z (7-4-5-7-2) and the A2058G-associated macrolide resistance but the number of cases was limited (Pereyre et al., 2012). Recently, an association between macrolide resistant *M. pneumoniae* isolates and the MLVA type 4-5-7-2 was suggested in China and Hong-Kong (Ho et al., 2015; Yan et al., 2015). However the prevalence of this MLVA type was high in these countries and the deletion of the unstable MPN1 marker from the MLVA method (Chalker et al., 2015) led to a too weakly discriminant typing method to draw accurate conclusions.

CLINICAL RELEVANCE OF *M. PNEUMONIAE* MACROLIDE RESISTANCE

Regarding clinical presentation, no difference was observed between patients infected by macrolide-resistant and macrolide-sensitive *M. pneumoniae*. Clinical symptoms, pneumonia severity, laboratory results, radiographic findings and prognostic factors were similar regardless of the *M. pneumoniae* susceptibility to macrolides (Matsubara et al., 2009; Cardinale et al., 2013; Miyashita et al., 2013; Wu et al., 2013; Diaz et al., 2015a). Most infections with macrolide-resistant *M. pneumoniae* have been reported in children because *M. pneumoniae* infections are more frequent in this population. Nevertheless, several adults have also been evaluated (Cao et al., 2010; Ferguson et al., 2013; Ho et al., 2015; Diaz et al., 2015a). To date, no difference has been found in disease manifestations between children and adults infected by macrolide-resistant *M. pneumoniae*.

As expected, the efficacy of macrolide treatment was shown to be lower in patients infected with macrolide-resistant isolates than in patients infected with macrolide-sensitive isolates. Despite macrolide administration, the duration of fever and cough, the duration of hospitalization and antibiotic administration were significantly longer in patients with macrolide-resistant *M. pneumoniae* infections. Moreover, the persistence of symptoms led to change of antibiotic prescription more often (Suzuki et al., 2006; Morozumi et al., 2008; Matsubara et al., 2009; Cardinale et al., 2013; Wu et al., 2013; Zhou et al., 2014). However, the clinical relevance of macrolide resistance in patients was usually limited to prolonging symptoms of the disease and not increasing the risk of complications. Only a single study has reported that the incidence of extrapulmonary complications was higher in children with macrolide-resistant isolates and that the radiological findings were more serious (Zhou et al., 2014).

TREATMENT OF *M. PNEUMONIAE* RESPIRATORY INFECTIONS

Macrolides and related antibiotics are the first-line treatment of *M. pneumoniae* respiratory tract infections mainly because of their low MIC against the bacteria, their low toxicity and the absence of contraindication in young children. The agent of first choice differs from country to country according to different published guidelines and owing to the fact that not all agents are available in all countries (Mandell et al., 2007; Bradley et al., 2011; Harris et al., 2011; Woodhead et al., 2011; Waites and Bébéar, 2013). The newer macrolides are now often the preferred agents with a 7-to-14 day course of oral clarithromycin or a 5-day course of oral azithromycin for treatment of community-acquired pneumonia due to *M. pneumoniae* (Waites and Bébéar, 2013). An appropriate antimicrobial therapy usually shortens the symptomatic period of *M. pneumoniae* infections, and hastens radiological resolution and recovery. However, using real-time PCR, it has been shown that the median time for carriage of *M. pneumoniae* DNA was

7 weeks after disease onset and that an adequate antibiotic treatment did not shorten the period of persistence of *M. pneumoniae* DNA in patient specimens (Nilsson et al., 2008). No treatment recommendation is available for extrapulmonary manifestations. In a few published case reports, macrolides and fluoroquinolones, mainly levofloxacin, have successfully been used (Scapini et al., 2008; Atkinson et al., 2011; Esposito et al., 2011; Meyer Sauteur et al., 2012; Godron et al., 2013).

In cases of macrolide-resistant *M. pneumoniae* strains, alternative antibiotic treatment can be required, including tetracyclines such as doxycycline and minocycline, or fluoroquinolones, primarily levofloxacin, even though fluoroquinolones and tetracyclines are contraindicated in all children and in children <8 year-old, respectively. Treatment lengths usually range between 7 and 14 days. As expected, fluoroquinolone and tetracycline regimens were shown to be more effective than macrolide regimens in patients infected by macrolide-resistant *M. pneumoniae* (Kawai et al., 2013; Miyashita et al., 2013). However, macrolides appear clinically effective in some patients infected by macrolide-resistant strains (Suzuki et al., 2006; Matsubara et al., 2009; Cardinale et al., 2013). This observation can be explained by the fact that *M. pneumoniae* infections are often self-limited diseases and that the anti-inflammatory effects of macrolides may improve clinical symptoms.

In Europe, Oceania, and America, where the prevalence of macrolide-resistant strains remains low, macrolides are the drug of choice in children with *M. pneumoniae* respiratory infections. Nevertheless, in these continents, clinicians should be vigilant for macrolide treatment failure and consider using alternative drugs if symptoms persist or if there are signs of clinical deteriorations. In countries in which the prevalence of macrolide-resistant strains is high, the replacement of macrolides as the first choice treatment by tetracyclines or fluoroquinolones was considered. However, surprisingly, in Japan, according to the 2013 recommendations of the Japanese Pediatric Society, macrolides remain the first-line treatment despite macrolide resistance rates over 80%. In this country, the efficacy of macrolides has to be evaluated by defervescence 48–72 h following the administration of these antimicrobials. In pneumonia cases in which the initial macrolide therapy resulted in failure, administration of alternative antimicrobial treatment, either respiratory fluoroquinolones or tetracyclines, must be considered. In contrast to Europe and to the United States, oral tosufloxacin, a fluoroquinolone antibiotic, was approved in Japan for pediatric use as a second line treatment in patients with community-acquired pneumonia. Indeed, in one study performed for the registration application of tosufloxacin in Japan, the occurrence of joint paint was only 0.85% (2/235) and there was no magnetic resonance imaging abnormal finding on joints (data given by Dr T. Oishi, Japan). Another study on 83 pediatric patients with *M. pneumoniae* pneumonia treated with tosufloxacin reported that side effects included mild diarrhea, but that no patients had joint symptoms (Sakata, 2012). Although, tosufloxacin was les effective than minocycline or doxycycline in achieving defervescence within 24 h and in decreasing the DNA load of *M. pneumoniae* (Okada et al., 2012; Kawai et al., 2013), its use is accepted in children under 8-year old. In countries where tosufloxacin is not available, other available respiratory fluoroquinolones might be chosen in severe cases despite contraindication. In children over 8-year old and adults, minocycline can be used as second-line treatment.

Although, no tetracycline or fluoroquinolone resistance has been reported in clinical isolates to date, resistant strains have been selected *in vitro* for both classes of drugs with target mutations identified in mutants (Gruson et al., 2005; Degrange et al., 2008). Thus, the risk of emergence of resistance in clinical isolates exists, especially for fluoroquinolones, if these antibiotics are inappropriately used. It should be noted that clinical resistance to fluoroquinolones has already been reported already in *Mycoplasma genitalium*, a urogenital mycoplasma phylogenetically close to *M. pneumoniae*, in which macrolide resistance mechanisms are similar to that of *M. pneumoniae* (Couldwell et al., 2013; Bissessor et al., 2015).

Consequently to macrolide resistance in *M. pneumoniae*, reevaluation of existing classes using and investigation of new classes of antimicrobials may be required to get additional treatment alternative beyond tetracyclines and fluoroquinolones, especially in children under 8 year-old. Randomized therapeutic trials will be necessary to establish guidelines regarding the most appropriate molecule, dose and length of treatment to use against the resistant strains. In the future, it will also be interesting to evaluate the activity of streptogramin combinations, such as oral pristinamycin, which has been shown to retain activity against 23S rRNA *M. pneumoniae* in *in vitro* mutants and in a few clinical isolates (Pereyre et al., 2004a, 2007). Indeed, pristinamycin was reported to be active on a few cases of genital infections by macrolide-resistant fluoroquinolone-resistant *M. genitalium* isolates (Bissessor et al., 2015). Although, additional studies on a large number of strains are required, pristinamycin could become an alternative antibiotic treatment in countries where this antibiotic is available (Bebear, 2012).

CONCLUSION

Nowadays, *M. pneumoniae* macrolide resistance rates are extremely high in Asia and remain moderate in Europe and North America. Macrolide resistance detection using accurate molecular methods should be considered in all *M. pneumoniae*-positive specimens since it has both a direct application in clinical practice and an epidemiological surveillance interest. At the individual level, a rapid detection of resistance-associated mutations would enable the prompt prescription of an alternative antimicrobial regimen, especially in case of persistent or recurrent *M. pneumoniae* infection. At the community level, the high prevalence of macrolide-resistant *M. pneumoniae* isolates in Asia underscore the potential for rapid emergence of macrolide resistance within *M. pneumoniae* in other parts of the world. Thus, further

epidemiological studies are needed in Europe and the USA to monitor macrolide resistance rates. Moreover, macrolide stewardship may be needed for restricting the use of these antibiotics, reduce unnecessary antibiotic prescribing, especially in countries with remaining low macrolide resistant rates. In Asia, the epidemiological surveillance of antibiotic resistance would also be of interest to early detect potential selections of fluoroquinolone- and tetracycline-resistant clinical isolates associated with the increasing use of these classes of antibiotics.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We thank Dr. Tomohiro Oishi (Department of Pediatrics, Kawasaki Medical School, Okayama, Japan) for helpful discussions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 25 February 2016
doi: 10.3389/fmicb.2016.00205

Potential Molecular Targets for Narrow-Spectrum Agents to Combat *Mycoplasma pneumoniae* Infection and Disease

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As *Mycoplasma pneumoniae* macrolide resistance grows and spreads worldwide, it is becoming more important to develop new drugs to prevent infection or limit disease. Because other mycoplasma species have acquired resistance to other classes of antibiotics, it is reasonable to presume that *M. pneumoniae* can do the same, so switching to commonly used antibiotics like fluoroquinolones will not result in forms of therapy with long-term utility. Moreover, broad-spectrum antibiotics can have serious consequences for the patient, as these drugs may have severe impacts on the natural microbiota of the individual, compromising the health of the patient either short-term or long-term. Therefore, developing narrow-spectrum antibiotics that effectively target only *M. pneumoniae* and no more than a small portion of the microbiota is likely to yield impactful, positive results that can be used perhaps indefinitely to combat *M. pneumoniae*. Development of these agents requires a deep understanding of the basic biology of *M. pneumoniae*, in many areas deeper than what is currently known. In this review, we discuss potential targets for new, narrow-spectrum agents and both the positive and negative aspects of selecting these targets, which include toxic molecules, metabolic pathways, and attachment and motility. By gathering this information together, we anticipate that it will be easier for researchers to evaluate topics of priority for study of *M. pneumoniae*.

Keywords: mycoplasma, antibiotics, toxins, metabolism, adherence

OPEN ACCESS

Edited by:
Cécile Bébéar, University of Bordeaux, France

Reviewed by:
Dinesh Sriramulu, Shres Consultancy, India
Roger Dumke, Technische Universität Dresden, Germany

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *11 January 2016*
Accepted: *08 February 2016*
Published: *25 February 2016*

Citation:
Balish MF and Distelhorst SL (2016) Potential Molecular Targets for Narrow-Spectrum Agents to Combat Mycoplasma pneumoniae Infection and Disease. Front. Microbiol. 7:205. doi: 10.3389/fmicb.2016.00205

INTRODUCTION

The use of antibiotics to treat bacterial infections is predicated on the antibiotics' ability to inhibit significant cellular processes of the bacteria, but not of the host, while avoiding inactivation by the bacteria. For cell wall-lacking mycoplasmas like *Mycoplasma pneumoniae*, a wide range of antibiotics, excluding those that target synthesis of peptidoglycan and certain metabolic pathways, is potentially useful in fighting infection. In practice, treatment of patients is largely restricted to macrolides, with tetracycline and fluoroquinolones used in some geographical regions or under some conditions (Bébéar et al., 2011; Biondi et al., 2014). Indeed, macrolides like azithromycin have historically been very effective against *M. pneumoniae*.

However, like so many other bacterial pathogens, *M. pneumoniae* has recently experienced a rapid increase in the incidence of resistance to the antibiotics commonly used to treat infections

(Principi and Esposito, 2013). At present, there are few reports of resistance of *M. pneumoniae* to antibiotics other than macrolides, and they are restricted to *in vitro* studies (Dégrange et al., 2008), but the rise of resistance of related mycoplasma species to fluoroquinolones and tetracycline (Gerchman et al., 2008; Redelinghuys et al., 2014) strongly suggests that *M. pneumoniae* is capable of developing resistance to other drugs if they become standard, widespread means of treatment. Moreover, for prevention of infection, no successful vaccine against *M. pneumoniae* has been developed. Consequently, the time is coming when alternative agents will have to be employed to prevent *M. pneumoniae* infection and to treat patients who are suffering from disease caused by this organism.

Switching to alternative, currently available antibiotics as a normal course of treatment for *M. pneumoniae* infection could be expected to provide some relief, but it is likely inevitable that the organism will develop resistance. Broad-spectrum antibiotics can bring undesirable side effects, often stemming from large-scale disruption of the host microbiome, that can cause problems whose difficulty exceeds those associated with the original infection (Modi et al., 2014). Furthermore, the selective pressure that broad-spectrum antibiotics apply to so many organisms causes resistance to develop fast and spread rapidly among different bacteria. Therefore, it is beneficial to use knowledge of the biochemistry and physiology of *M. pneumoniae* to design and develop narrow-spectrum therapeutic agents that target *M. pneumoniae* as specifically as possible. Whereas some such agents might be used to eradicate the organism, others might target *M. pneumoniae* processes that, although not essential for the life of the bacterium, exacerbate disease, and by so doing both reduce the symptoms and give the patient's immune system an advantage in clearing the infection.

The depth of understanding of the biology of *M. pneumoniae* has increased dramatically in recent years, thanks in large part to genomics, systems biology, and cell biology studies of this organism. It has become possible to consider, in a more informed way than ever, which activities of *M. pneumoniae* might provide the best targets for development of new, narrow-spectrum drugs. In this review, we will discuss the biology of *M. pneumoniae* in terms of which metabolic pathways, cellular components, and activities are likely to be suitable for future work in this area.

TARGETS

Considerable variation exists in the degrees to which potentially important therapeutic targets are understood. Toxins and toxic metabolites are in some ways the most welcoming for study because they involve a small number of proteins and often effectuate biochemical changes that are readily measurable. More complex metabolic pathways are less well-studied and warrant a greater effort. Cell-level processes like adherence, motility, and division are fairly well-characterized but the molecular basis for each of these activities is generally poorly established. In addition to the question of how well any putative inhibitor of a given activity would interfere with the life processes of an *M. pneumoniae* cell, another important consideration is how narrowly a drug would target *M. pneumoniae*. If the target is something that is found, for example, only in mycoplasmas, one would anticipate the ideal outcome of a very narrow-spectrum drug that does not interfere with other components of the host microbiota. Alternatively, if the target is broadly present in bacteria, the narrowness of the drug's action would depend on whether it can exploit structural differences in the *M. pneumoniae* version of the target. If the structure of such a target is highly conserved, then it may be difficult to develop a therapeutic agent that does not cause disruption to the host by damaging the host microbiota. However, a widely distributed target that exhibits considerable difference in sequence may be more suitable. In **Table 1**, we address these issues for each of the targets discussed in this review.

Toxins and Toxic Metabolites

Host cell damage by *M. pneumoniae* is established to occur by several routes. One, which is beyond the scope of this review, is immunopathology, wherein the organism attracts the cells of the host's immune system, causing inflammation and host cell damage. Another is the ADP-ribosylating community-acquired respiratory distress syndrome (CARDS) toxin, which, though only relatively recently identified, has come to be considered a major source of cell and tissue damage responsible for a substantial portion of the symptoms of *M. pneumoniae* infection. Damage to host cells from hydrogen peroxide and hydrogen sulfide is also potentially significant.

CARDS Toxin

One very promising candidate and target for therapeutic design for the treatment of *M. pneumoniae* infections is CARDS toxin, encoded by MPN372. This 68-kDa protein was initially identified, because of its ability to bind with high affinity to surfactant protein A, a prominent component of pulmonary surfactant (Kannan et al., 2005), but was subsequently characterized as an ADP-ribosylating toxin (Kannan and Baseman, 2006). Incubation of recombinantly produced CARDS toxin with tissue culture cells results in a major increase in ADP-ribosylation of host proteins (Kannan and Baseman, 2006). Upon entry into host cells the toxin activates the NLRP3 inflammasome via ADP-ribosylation, a mechanism likely responsible for the robust inflammation and pathology associated with *M. pneumoniae* infections (Bose et al., 2014). CARDS toxin induces extensive vacuolation in tissue culture cells, tracheal organ cultures, and model host animals in a dose-dependent manner and causes cytopathic effects and inflammatory responses similar to the histopathology and immunopathology seen during *M. pneumoniae* infections both *ex vivo* and *in vitro* (Kannan and Baseman, 2006; Hardy et al., 2009). CARDS toxin-induced vacuoles are derived from late endosomes enriched in Rab9, a host cell GTPase involved in membrane trafficking (Johnson et al., 2011). The cellular damage that results from recombinant CARDS toxin suggests that it is a major virulence factor and likely plays a large role in the pathogenesis of *M. pneumoniae*. Patients with confirmed *M. pneumoniae* infections experience

high antibody titers to CARDS toxin, likely due to the localization of a subset of the toxin to the *M. pneumoniae* membrane (Kannan and Baseman, 2006; Kannan et al., 2010; Johnson et al., 2011), suggesting the potential utility of CARDS toxin not only as a target for development of agents that inhibit its activity, but also as a vaccine component.

Community-acquired respiratory distress syndrome toxin has a modular structure, with different regions providing distinct functionality. The X-ray crystal structure of CARDS toxin reveals that the protein is composed of three domains folded in the shape of a triangle (Becker et al., 2015). Domain 1 houses the N-terminal ADP-ribosyltransferase activity; the sequence of the N-terminal region of CARDS toxin shares 27% identity with the pertussis toxin S1 subunit of *Bordetella pertussis*, which is an ADP-ribosyltransferase (Kannan et al., 2005). Domains 2 and 3 form a C-terminal tandem β-trefoil (Becker et al., 2015). The C-terminal domain, whose amino acid sequence does not resemble those of other proteins, is solely responsible for binding and internalization as well as vacuolating activity (Kannan et al., 2014). Deletion of 41 amino acids from the C-terminus of CARDS

TABLE 1 | Evaluation of potential *Mycoplasma pneumoniae* targets.

Category	Gene number	Protein name	Proposed function	Is the role or function established in *M. pneumoniae*?	Narrowness of phylogenetic distribution
Toxin	MPN372	CARDS toxin	ADP-ribosylating toxin	Yes	High
Toxic metabolites	MPN051	G3P oxidase	Hydrogen peroxide production	Yes	Medium–high
Toxic metabolites	MPN487	Cysteine desulfurase/desulfhydrase	Hydrogen sulfide production	Moderately	Medium–high
Transport	MPN415-417		Thiamine transport	No	Medium
Transport	MPN043	Glycerol facilitator	Glycerol transport	Yes	Medium–high
Transport	MPN133		Glycerol transport (accessory)	Moderately	High
Transport	MPN284		Glycerol transport (accessory)	Moderately	High
Transport	MPN421	Glycerophosphocholine transporter	Glycerophosphocholine transport	Yes	High
Transport	MPN076		Glycerophosphocholine transport (accessory)	Moderately	High
Transport	MPN077		Glycerophosphocholine transport (accessory)	Moderately	High
Anabolism	MPN336	Pantothenate kinase/nicotinate-nucleotide adenylyltransferase	CoA synthesis	No	Low–medium
Anabolism	MPN382	Dephospho-CoA kinase	CoA synthesis	No	Low
Anabolism	MPN298	Acyl carrier protein synthase	Lipid synthesis	Yes	Low
Anabolism	MPN406	Acyl carrier protein	Lipid synthesis	Yes	Low
Anabolism	MPN420	Glycerophosphocholine phosphodiesterase	Lipid synthesis and hydrogen peroxide production	Yes	Medium–high
Anabolism	MPN350	G3P acyltransferase	Lipid synthesis	No	Low
Anabolism	MPN299	1-acyl-G3P acyltransferase	Lipid synthesis	No	Low
Anabolism	MPN483	Glycosyltransferase	Polysaccharide synthesis	Moderately	High
Anabolism	MPN028	Glycosyltransferase	Polysaccharide synthesis	No	High
Anabolism	MPN075	Glycosyltransferase	Polysaccharide synthesis	No	High
Anabolism	MPN073	PRPP synthetase	Nucleotide synthesis	No	Low
Anabolism	MPN066		Nucleotide synthesis	No	Medium–high
Anabolism	MPN256	CTP synthetase	Nucleotide salvage	No	High
AO	MPN141	P1 adhesin	Adherence and motility	Moderately	High
AO	MPN142	Protein B/protein C	Adherence and motility	Moderately	High
AO	MPN626	Alternative sigma factor	Recombination of adherence and motility genes	No	High
AO	MPN453	P30 adhesin	Adherence and motility	Moderately	High
AO	MPN446	HMW1	AO core	Moderately	High
AO	MPN310	HMW2	AO core	Moderately	High
AO	MPN309	P65	AO core	No	High
AO	MPN311	P41	AO core	No	High
Cell division	MPN317	FtsZ	Cytokinesis	Moderately	Medium

toxin completely abolished binding and internalization of the protein, indicating the involvement of this region in host-cell receptor binding (Kannan et al., 2014). Thus, the data suggest that domain 3 specifically mediates CARDS toxin binding and entry to host cells (Becker et al., 2015). In addition to binding surfactant protein A, CARDS toxin also associates with the host membrane protein annexin A2, colocalizing with annexin A2 prior to internalization and remaining associated with it after internalization (Somarajan et al., 2014). The importance of this interaction was demonstrated by diminution of binding and entry of CARDS toxin into A549 cells after pretreatment of the cells with anti-annexin A2 antibodies or annexin A2-specific siRNA (Somarajan et al., 2014). Uptake occurs via a clathrin-mediated endocytic pathway in multiple mammalian cell lines (Krishnan et al., 2013; Somarajan et al., 2014). Thus, CARDS toxin, though a single molecule, has multiple distinct functionalities that could be targets for intervention.

Although CARDS toxin is not necessary for colonization or infection its production and quantity show a clear and direct relation to disease severity as indicated by the difference in pathology of different clinical strains expressing varying levels of this toxin (Techasaensiri et al., 2010). Therefore, inhibition of production, cell entry, or activity of CARDS toxin could provide a reprieve to infected patients to allow for immune clearance and significantly less cellular damage.

Toxic Metabolites

Glycerol-3-phosphate (G3P) can be used either for synthesis of lipids (see section "Metabolism of G3P") or conversion to dihydroxyacetone phosphate (DHAP), which enters the glycolytic pathway. The conversion of G3P to DHAP is significant because the enzyme that catalyzes that reaction, G3P oxidase (GlpO, encoded by MPN051), simultaneously reduces molecular oxygen to hydrogen peroxide (Hames et al., 2009; Maenpuen et al., 2015), which is cytotoxic and suggested to be important for virulence of *M. pneumoniae*. *M. pneumoniae* GlpO also uses the glycolytic intermediate glyceraldehyde-3-phosphate as a substrate with a low turnover rate (Maenpuen et al., 2015), explaining the evolution of hydrogen peroxide by *M. pneumoniae* in the absence of glycerol (Hames et al., 2009). Study of recombinantly produced *M. pneumoniae* GlpO revealed significant differences in the active site from the nominally similar mitochondrial G3P dehydrogenase (Elkhal et al., 2015), paving the way toward the use of GlpO as a therapeutic target.

Although hydrogen peroxide is cytotoxic and cell lysis *in vitro* has been attributed to this molecule, hydrogen sulfide has also been implicated in hemolysis by *M. pneumoniae*, raising the possibility that it too is a virulence factor. Hydrogen sulfide is produced from cysteine by HapE, a novel cysteine desulfurase and cysteine desulfhydrase encoded by MPN487 (Grosshennig et al., 2016). Because an enzyme with both these activities has not been described in other organisms, HapE might be a good target for development of narrow-spectrum agents. Although it is not essential *in vitro*, it might nonetheless play important roles in virulence. Further work should be done to establish the importance of HapE and hydrogen sulfide in *M. pneumoniae* pathogenesis.

Metabolism and Metabolites

Using metabolic pathway inhibitors against *M. pneumoniae* relies on identifying metabolic pathways that are both active and important, if not essential, to the organism, and distinct enough from host metabolic pathways to limit toxicity to the host. However, approaches that work in other bacteria often fail with regard to mycoplasmas. For example, *M. pneumoniae* does not synthesize folate and is therefore insensitive to sulfonamides, which target enzymes involved in its synthesis (McCormack, 1993). The reduced biosynthetic capabilities of *M. pneumoniae* and other mycoplasmas (Himmelreich et al., 1996), coincident with their evolutionarily reduced genomes, make identification of suitable pathways challenging. In short, *M. pneumoniae* is already an expert at acquiring, rather than synthesizing, metabolites, making anabolic targets few.

Transport of essential compounds, including cofactors and building blocks, could provide a reasonable set of targets for the development of therapeutic agents that inhibit *M. pneumoniae*. Many putative transporter genes have been identified, but they are largely orphan transporters whose substrates are unknown. For example, the *M. genitalium* homolog of MPN415 encodes a thiamine-binding lipoprotein, and the remaining genes in its operon, MPN416 and MPN417, encode an ABC transporter, suggesting that this transporter serves to import thiamine (Sippel et al., 2011). If so, then given that *M. pneumoniae* cannot synthesize cofactors like thiamine, this transporter could be an excellent target for interfering with *M. pneumoniae* growth, but it must first be experimentally established that thiamine import is the role of this transporter. Because of their hydrophobicity, complexity, and often their essential nature, transporters like that encoded by MPN416 and MPN417 are difficult to study, but understanding the molecular basis for the transport of essential molecules, including cofactors, amino acids, sugars, and nucleic acid precursors, should be a high priority.

The metabolic pathways of *M. pneumoniae* that seem most likely to yield productive narrow-spectrum agents are the synthesis of phospholipids and glycolipids, with G3P at a significant crossroads between membrane biochemistry and hydrogen peroxide synthesis. The poorly understood role of biofilms and extracellular polysaccharide is of considerable potential in this regard as well. Nucleotide salvage pathways, carotenoid synthesis, and catabolic pathways are also worth consideration.

Coenzyme A (CoA) Synthesis and Lipid Catabolism

Coenzyme A synthesis is an interesting potential target for inhibition because membrane biogenesis and modification, important processes in which CoA participates, are fundamental for the viability of cells in general. The value of targeting CoA biosynthesis for antibacterial effects is illustrated by the study of pantothenamides as inhibitors of pantothenate kinase (Strauss and Begley, 2002), which catalyzes the rate-limiting step in CoA synthesis in many bacteria, and the use of pyrazinamide, an anti-tuberculosis agent, which might target CoA metabolism (Zhang et al., 2014). Synthesis of CoA by *M. pneumoniae* from externally provided pantetheine, which would have to be imported through an unknown mechanism, could occur in three

steps, beginning with pantothenate kinase (Yus et al., 2009); however, pantothenate kinase activity has not been demonstrated at the biochemical level in *M. pneumoniae* and no gene is confidently annotated as such. Although MPN336, annotated as a nicotinate-nucleotide adenylyltransferase, was proposed as a potential pantetheine phosphate adenylyltransferase, catalyzing synthesis of dephospho-CoA (Yus et al., 2009), global transposon mutagenesis of *M. pneumoniae* clearly revealed the dispensability of this enzyme *in vitro* (Hutchison et al., 1999). On the other hand, there is no evidence that MPN382, which is annotated as dephospho-CoA kinase (CoaE), catalyzing the putative final step in CoA synthesis (Yus et al., 2009), is dispensable (Hutchison et al., 1999). Although this information highlights the potential for development of a CoaE inhibitor, human tissue also uses CoaE, making it important to screen any potential *M. pneumoniae* CoaE inhibitor for low inhibitory activity of its human counterpart. Furthermore, CoaE is used by many bacteria, so a therapeutic agent targeting this molecule might have a broad spectrum of activity, which might not be desirable. In any event, CoA metabolism in *M. pneumoniae* is an insufficiently investigated area that might be of considerable practical value.

Mycoplasmas can acquire fatty acids from host cells, and it is possible that they use them for synthesis of phospholipids and glycolipids (Yus et al., 2009). *M. pneumoniae* has homologs of two proteins that are likely involved in the earliest and most generalized stages of this process, acyl carrier protein synthase, AcpS, encoded by MPN298, and acyl carrier protein, AcpP, encoded by MPN406. These enzymes have both been biochemically characterized with regard to activity and substrate specificity (McAllister et al., 2006). AcpP becomes activated when AcpS catalyzes the transfer of a 4′-phosphopantetheinyl group from CoA to a serine residue on AcpP, and AcpP provides the phosphopantetheine as a cofactor for delivery of acyl groups to a nascent phospholipid or glycolipid. Both their coding genes are suggested to be essential *in vitro* (Hutchison et al., 1999). Recombinantly produced *M. pneumoniae* AcpS catalyzes the pantetheinylation of recombinantly produced AcpP, but with a markedly low affinity for CoA derivatives and, concomitantly, a slow rate of catalysis as compared with AcpS from other bacteria (McAllister et al., 2006). Indeed, it can also transfer a variety of CoA-linked substrate molecules other than phosphopantetheine, although the significance of this broad specificity and slow turnover for *M. pneumoniae* physiology is unclear. These enzymes might be interesting targets for development of novel anti-*M. pneumoniae* agents, but selecting against inhibitors of homologous human lipid synthesis proteins and, for that matter, similar proteins in commensal bacteria, would be an important consideration.

Metabolism of G3P

Although some phospholipids are acquired intact from the host or the media, the glycerol backbones of other phospholipids and glycolipids in *M. pneumoniae* are predicted to derive either from glycerol or glycerophospholipids of the host cell membrane (Yus et al., 2009). Exogenous G3P was suggested by gene annotation to be another potential source of glycerol (Himmelreich et al., 1996), in addition to free glycerol, for metabolism, but this was experimentally ruled out (Schmidl et al., 2011). However, both glycerol and glycerophospholipids are converted to G3P in the *M. pneumoniae* cell (Hames et al., 2009; Grosshennig et al., 2013). In addition, metabolism of G3P by another pathway results in production of hydrogen peroxide (see section "Toxic Metabolites"), a virulence factor of *M. pneumoniae* (Hames et al., 2009), so if the bacteria could survive therapeutic agents targeting early stages of glycerol and glycerophospholipid metabolism, they would nonetheless be impaired in their virulence, giving the host a better chance of success against the pathogen.

It is unknown whether during an infection *M. pneumoniae* relies on free glycerol, which is not abundant in the normal environment of the organism, in contrast to glycerophospholipids, which are; consequently, whether metabolism of free glycerol is physiologically significant enough to constitute a reasonable target for new drugs is unclear, making this question a priority for study. *M. pneumoniae* can take up free glycerol, as well as water, through the glycerol facilitator, GlpF, encoded by MPN043, which is essential (Hutchison et al., 1999). Growth on glycerol is negatively affected in mutants of lipoproteins encoded by MPN133 and MPN284, suggesting ancillary roles in glycerol transport for these molecules (Grosshennig et al., 2013), but these proteins are non-essential (Hutchison et al., 1999). Subsequent conversion of imported glycerol to G3P is carried out in *M. pneumoniae* by glycerol kinase, encoded by the essential gene MPN050, at the expense of a mole of ATP per mole of glycerol, making it another potential target (Hames et al., 2009), but one with a human homolog. However, even among mycoplasmas, GlpF has low sequence homology (Pritchard et al., 2014), suggesting that inhibitors specific to mycoplasmas or specifically to *M. pneumoniae* could be developed. Therefore, of the proteins involved in metabolism of free glycerol, the transporter GlpF is likely the most suitable as a novel target for interfering with glycerol uptake. Interestingly, we are unaware of homologs of GlpF having been described as targets for antibiotics.

Glycerol-3-phosphate can also be derived from breakdown of host cell membrane lipids. Although no lipases have been unambiguously identified in *M. pneumoniae*, lipase activity from other bacteria or endogenous activity of the host could provide glycerophosphocholine (GPC). GlpU, encoded by MPN421, is essential for uptake of GPC, presumably acting as a transporter (Grosshennig et al., 2013), and GlpQ, encoded by MPN420, catalyzes removal of the choline from GPC, yielding G3P (Schmidl et al., 2011). Mutants in either of these two genes exhibit greatly reduced cytotoxicity *ex vivo*, and *in vivo* one might anticipate that interference with the function of either of the two proteins by some therapeutic agent would cause *M. pneumoniae* to rely principally on free glycerol for phospholipid, glycolipid, and the vast majority of hydrogen peroxide synthesis, causing considerable impairment in both growth and virulence. The MPN076 and MPN077 genes are also involved in use of GPC, likely in terms of transport (Grosshennig et al., 2013), but their roles are unclear. A homolog of GlpQ encoded by MPN566 does not function in GPC hydrolysis, and its activity is unknown (Schmidl et al., 2011).

Membrane Lipids and Glycomoieties

The most reasonable suggested pathway for the synthesis of any phospholipids and glycolipids that are not derived directly from the host begins with G3P (Yus et al., 2009). If G3P is to be used for membrane lipid synthesis, it must receive two acyl groups via the activities of two successively acting acyltransferases. It is proposed that the first is PlsY (MPN350) and the second is PlsC (MPN299; Yus et al., 2009), but the *M. pneumoniae* enzymes have never been characterized, which is an important step toward establishing the physiological relevance of this metabolic pathway and therefore whether these enzymes, as well as any downstream of them, should be considered targets for new antibiotics. Substrate mimics that inhibit *Streptococcus pneumoniae* PlsY have been studied (Grimes et al., 2008), but because these enzymes are so widely distributed, suggesting that it may be difficult to develop narrow-spectrum agents. Uptake of cholesterol is also essential for *M. pneumoniae* (Johnson and Somerson, 1980), and is unlikely to be related to processes that occur in commensal bacteria, making it an excellent target, but there are no data describing the molecular mechanism by which this is accomplished.

Glycomoieties for use in glycolipids, protein glycosylation, or as extracellular polysaccharide are almost certainly the result of poorly defined anabolic pathways in *M. pneumoniae*. Knowledge of the diversity and function of *M. pneumoniae* polysaccharides, both free and covalently linked to other biomolecules, is limited, but the available information suggests important roles for these moieties in several *M. pneumoniae* biological processes, including glycolipid synthesis, modification of proteins, and biofilm properties (Yus et al., 2009; Simmons et al., 2013). Glycolipid biosynthesis by *M. pneumoniae* using exogenously supplied palmitic acid, ceramide, glucose, UDP-glucose, and phosphate has been demonstrated experimentally (Klement et al., 2007). Undoubtedly essential for the synthesis of these polysaccharides are three glycosyltransferases, encoded by MPN028, MPN075, and MPN483, although the activities of the first two are uncharacterized. Strains in which these genes were definitively knocked out were not isolated in a global transposon mutagenesis screen, suggesting that they are essential for *M. pneumoniae* (Hutchison et al., 1999). MPN483 encodes a promiscuous glycosyltransferase that can catalyze the processive synthesis of a variety of polysaccharides from several substrates for use as glycomoieties. When produced recombinantly in *E. coli* it can use UDP-galactose and UDP-glucose as substrates for addition to diacylglycerol, ceramide, and mono-, di-, and trisaccharide derivatives thereof (Klement et al., 2007). The specific identities and significances of its physiological product are unknown.

Significantly, *M. pneumoniae* biofilms grown *in vitro* contain considerable amounts of a polymer of unknown structure enriched in galactose and *N*-acetylglucosamine (Simmons et al., 2013). Although the roles of biofilms in *M. pneumoniae* infection have not been established yet, biofilms of other organisms contribute to virulence, resistance to antibiotics, and susceptibility to clearance by immune system processes, making *M. pneumoniae* biofilms an extremely valuable target for study, especially given the recent increase in antibiotic resistance by *M. pneumoniae* and the chronicity of *M. pneumoniae* infection. Extracellular polysaccharides, such as the one described for *M. pneumoniae*, are often essential features for the formation and integrity of these multicellular structures, and destruction or disruption of their synthesis could be significant means by which *M. pneumoniae* is rendered less virulent or at least more susceptible to other drugs. The polysaccharide that was identified in *M. pneumoniae* is particularly interesting because in a strain that makes a biofilm of reduced density, this molecule is detached from the cells that produce it, whereas a strain that makes a heavier biofilm has this polysaccharide attached to the bacteria, implicating this molecule in important aspects of biofilm integrity (Simmons et al., 2013). Beyond its composition, neither the structure of this polysaccharide nor the enzymes responsible for its synthesis and attachment to the *M. pneumoniae* cell are known. It is unlikely that the glycosyltransferase encoded by MPN483 is involved, given the presence of *N*-acetylglucosamine in it, leaving MPN028 and MPN075 as the most likely candidates for synthesis of the extracellular polysaccharide (Klement et al., 2007). Further work on the characterization and biochemical origin of this molecule is highly warranted.

The Question of Carotenoids

Synthesis of carotenoids might be another membrane-associated target, but an insufficient amount of work has been done to establish how important these molecules are and even whether *M. pneumoniae* synthesizes them or acquires them by other means. Carotenoids are membrane-associated pigment molecules with a variety of physiological roles. It is unclear exactly how they might contribute to fitness of *M. pneumoniae*, but a role in protection from photodamage is conceivable. Molecules with Raman spectra consistent with carotenoids were described in multiple *M. pneumoniae* isolates, and a set of genes encoding enzymes involved in their synthesis from the glycolytic intermediates pyruvate and glyceraldehyde-3-phosphate was proposed (Maquelin et al., 2009). However, the specific chemical identities of the final carotenoid products were not described. Maquelin et al. (2009) used analogy with *E. coli* and other organisms that synthesize carotenoids to propose a seven-enzyme pathway for *M. pneumoniae* carotenoid synthesis pathway, but only identified six genes they considered to encode likely participants in this pathway in the *M. pneumoniae* genome. The validity of this pathway has not been addressed experimentally, and some of these genes have annotations that are more consistent with other functions than the ones proposed (Maquelin et al., 2009). If, however, the biological relevance of this pathway could be confirmed and the importance of these molecules for *M. pneumoniae in vivo* could be established, the carotenoid biosynthesis pathway could be a reasonable target for development of drugs. Humans do not synthesize carotenoids, instead acquiring them principally from diet, making at least some enzymes of this pathway stand out as potential targets. For the present time, the biology of carotenoids and their synthesis and/or acquisition by *M. pneumoniae* constitute an interesting topic for further study.

Nucleoside/Nucleotide Metabolism

Mycoplasma pneumoniae cannot synthesize purines or pyrimidines (Pollack, 2002), which are required for nucleic acid synthesis as well as for anabolic processes involving UDP-and CDP-conjugated carbohydrates. Presumably, like related mycoplasma species, it acquires these molecules from the environment through the use of nucleases and transporters (Li et al., 2010; Masukagami et al., 2013). However, there is potential for interconversion of some of these molecules via nucleotide or nucleoside salvage pathways to ensure that needs are met (Pollack, 2002). It is not clear whether metabolism of these molecules is a suitable target for development of drugs specific to *M. pneumoniae,* because of both a lack of information about *in vivo* flux through various redundant routes and the broad distribution of most of the components of the salvage pathways. Nucleoside analogs that inhibit either bacterial RNA or DNA synthesis or some of the enzymes associated with metabolism of these compounds are widely discussed potential therapeutic agents in the process of development (Niu and Tan, 2015), and their use against mycoplasmas has been proposed as well (Wang et al., 2010, 2014). What remains to be seen is whether effective, narrow-spectrum agents in this category can be developed.

5-phophoribosyl-1-pyrophosphate (PRPP) is a potential source of some nucleotides, providing the sugar for addition to the free bases adenine, guanine, and uracil (Yus et al., 2009). PRPP is a product of the pentose phosphate pathway, which is abbreviated in *M. pneumoniae*, but all the activities that lead to its synthesis from five- and six-carbon sugars are accounted for, although it is unclear what enzyme contributes aldolase activity. Ribulose-5-phosphate (R5P) is the precursor of PRPP, its conversion to PRPP catalyzed by PRPP synthetase, encoded by MPN073 (Yus et al., 2009). R5P itself can derive either from the pentose phosphate pathway or, likely, from ribose-1-phosphate (R1P) yielded by the breakdown of environmental or cytoplasmic RNA. It is likely that MPN066 encodes the enzyme that interconverts R1P and R5P (Yus et al., 2009), but the identity of this protein, which is also annotated as a phosphomannomutase or phosphoglucomutase, has not been experimentally demonstrated. If PRPP can be derived through alternative routes, then a drug targeting either of the pathways might not be useful. Although it is clear that free nucleosides can support the nucleic acid needs for growth of *M. pneumoniae* (Yus et al., 2009), the relative flux through the PRPP pathway as compared with acquisition of nucleosides from nucleic acids has not been established, so even if this pathway could be inhibited, it is unclear what the impact on *M. pneumoniae* would be. The question of how these nucleosides are generated *in vivo* is therefore open, and the uncertainty concerning the activity of MPN066 is an area worth exploring experimentally in connection with this question. Conversion of adenine, guanine, and uracil to their various phosphorylated forms for incorporation into RNA and their interconversion, including into deoxynucleotides for incorporation into DNA, is catalyzed by a series of enzymes that are widely distributed throughout nature and might therefore not be ideal candidates for targets of drugs for narrow-spectrum activity against *M. pneumoniae*.

Pyrimidine metabolism is more likely to provide a good narrow-spectrum target. *M. pneumoniae* appears to lack a CTP synthase enzyme, which would convert UTP to CTP, connecting the PRPP pathway to metabolism of cytosine and thymine nucleotides. MPN256 has been proposed to encode CTP synthase (Yus et al., 2009) and others have proposed the possibility of such an activity (Pachkov et al., 2007), but the evidence is based entirely on bioinformatics approaches and not on biochemical ones. If there is indeed no such activity in *M. pneumoniae*, then the organism must use sources other than PRPP for the generation of cytosine and thymine nucleotides. CDP can be converted to deoxycytidine and thymine nucleotides (Yus et al., 2009), but the ultimate source of CDP must be DNA and RNA or free nucleosides, and not PRPP. Indeed, among pyrimidines, cytosine is sufficient to support growth of *M. pneumoniae* in minimal media (Yus et al., 2009). Drugs based on pyrimidine nucleoside analogs have been suggested as good targets for interfering with growth of *M. pneumoniae* (Wang et al., 2010, 2014), but whether these can be sufficiently specific to avoid killing off commensal microbiota is unknown. Identification and inhibition of the transporters involved in uptake of pyrimidines might also provide useful targets.

Catabolic Targets

Mycoplasma pneumoniae has genes for generation of ATP through both the arginine dihydrolase pathway and catabolism of sugars. It cannot actually metabolize arginine because of disruption to some of the genes of this pathway in all known strains (Rechnitzer et al., 2013; Xiao et al., 2015). Therefore, arginine catabolism is not a pathway that can be successfully targeted in *M. pneumoniae*. *M. pneumoniae* can utilize a number of sugars as carbon sources for growth *in vitro*, including glucose, mannose, fructose, ribose, ascorbate, glycerol, and possibly mannitol, although there is conflicting information about mannitol (Halbedel et al., 2004; Yus et al., 2009). The relative amount of growth from metabolizing each of these has been established under a defined set of conditions, with glucose and mannose outperforming the others by a considerable margin (Yus et al., 2009). Although it is not clear what the most relevant catabolic pathways *in vivo* are, they would depend upon a combination of substrate availability, affinity of each substrate for its transporter, and the rates of the rate-limiting steps in metabolism of each substrate. Nonetheless, the evolutionary conservation of these metabolic options suggests that they are all useful, and one might anticipate that they come into play at different stages of infection. For example, utilization of ribose by *M. pneumoniae* is likely to increase later in infection after host cell lysis has occurred, making nucleic acids available.

Because of the multiplicity of routes by which carbon sources can be utilized, as well as the common nature of these pathways among bacteria, including commensal ones whose elimination is undesirable, it is likely impractical to consider interfering with the uptake or early stages of metabolism of these compounds. All these metabolic pathways converge on G3P, the entry point into the energy-yielding phase of glycolysis, which is highly conserved and present in the host, and therefore a poor candidate for targeting of drugs.

Attachment Organelle

Mycoplasma pneumoniae exhibits the properties of cytadherence and gliding motility, and both are required for virulence (Balish, 2014), indicating that drugs designed to interfere with these processes could be of considerable utility in fighting or preventing *M. pneumoniae* disease. A diverse array of molecules across the surface of *M. pneumoniae* cells is involved in host cell adherence but the initial contact and attachment are mediated by a polar structure known as the terminal or attachment organelle (AO), where proteins necessary for *M. pneumoniae* adherence and gliding motility are concentrated (**Figure 1**). Although the identities of at least some of the proteins associated with adherence have been established, the process of gliding motility is less well characterized at the molecular level. The AO contains transmembrane proteins involved in attachment, motility, and perhaps other unknown functions, as well as an interior set of cytoskeletal core proteins (Balish, 2014). Analyses of the proteins of the AO have provided some insight into the spatial and temporal organization of this structure as well as functional characterization of a few of these proteins. Proteins involved in AO synthesis and function are highly specific to a subset of mycoplasma species (Balish, 2014) and therefore provide very narrow targets.

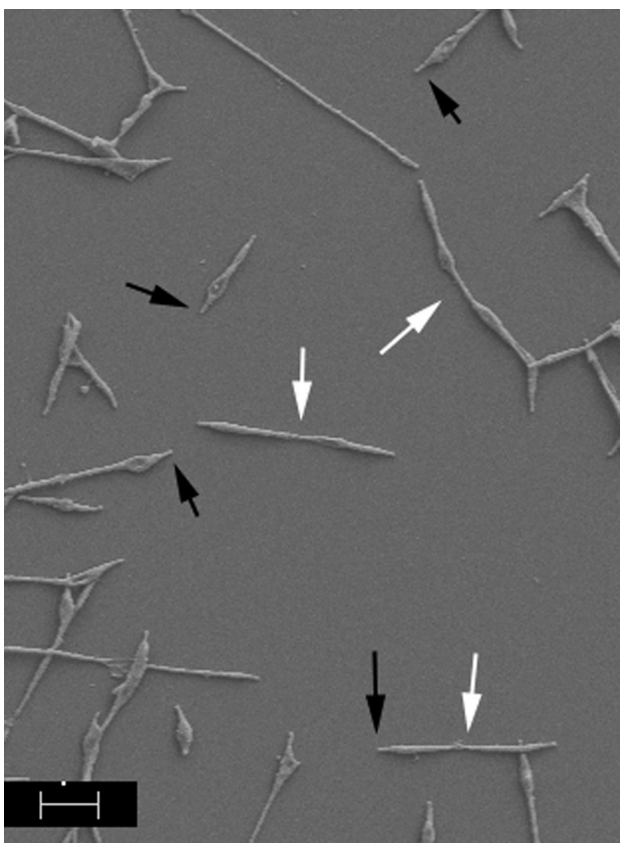

FIGURE 1 | Scanning electron image of *Mycoplasma pneumoniae* cells. Cells were prepared according to Hatchel et al. (2006). Black arrows indicate AOs; white arrows indicate dividing cells. Scale bar, 1 μm.

Substrate Molecules

The substrates for adherence and gliding motility include sialic acid-containing molecules as well as sulfated glycolipids (Loomes et al., 1984; Krivan et al., 1989; Kasai et al., 2013), suggesting that these molecules could be models for inhibitors of AO function. Although adhesins have been identified, specific interactions between these adhesins and these substrates have never been characterized. This gap in knowledge is due in large part to the absence of protein biochemistry performed on these proteins, illustrating the urgency of performing these studies. The molecular-level means by which the identified adhesins interact with host target molecules will provide targets with great therapeutic potential, but obtaining biochemically active adherence molecules is required for the necessary knowledge. Likewise, how adherence relates to motility at the molecular level is also understood only at a phenomenological level, without deep understanding of the physiological mechanisms. Because adherence and motility are essential for infection, this area of research must be a high priority.

Adhesins

One of the most significant proteins of the *M. pneumoniae* AO is the adhesin P1, encoded by MPN141. P1 is distributed across the cell surface but is concentrated at the AO (Baseman et al., 1982). The importance of P1 in host cell attachment is supported by the inhibition of cytadherence when P1 is absent in mutants or blocked by specific antibodies (Krause et al., 1982; Krause and Baseman, 1983). Although P1 is distributed across the cell, the clustering at the AO is necessary for attachment as indicated by mutants that express P1 at wild-type levels but fail to localize the protein to the AO (Baseman et al., 1982; Hahn et al., 1998; Balish et al., 2003).

Protein B (also called P90), a product of the MPN142 gene, colocalizes and copurifies with P1 (Seto and Miyata, 2003; Nakane et al., 2011). Furthermore, MPN142 is cotranscribed with the P1-encoding gene and also required for cytadherence (Krause et al., 1982; Waldo and Krause, 2006). Cleavage of the MPN142 product, which occurs via a process that is unknown, yields proteins B and C (also known as P40); protein C also colocalizes with P1 and is required for cytadherence (Krause et al., 1982; Franzoso et al., 1993; Waldo and Krause, 2006). Although protein C was not copurified with P1 (Nakane et al., 2011), proteins B, C, and P1 can be chemically cross-linked, suggesting that these proteins function together in a complex (Layh-Schmitt and Herrmann, 1994). Therefore it is likely that proteins B and C are involved in contributing to the adhesive property of P1 or that these proteins together function as a single adhesive unit. Both P1 and protein B are immunodominant, suggesting that either one, or perhaps both considered together as a polypeptide adhesin, could be a potential candidate for therapeutic development (Aubert et al., 1992). Additionally, studying the process by which the precursor of proteins B and C is proteolytically cleaved might yield another target.

Of potential significance is the fact that the MPN141 and MPN142 genes are subject to substantial sequence variation across *M. pneumoniae* isolates (Su et al., 1990; Kenri et al., 1999; Spuesens et al., 2011; Xiao et al., 2015). This variation

appears to occur as a result of recombination of related variant sequences, located throughout the chromosome, into the expression site (Kenri et al., 1999; Spuesens et al., 2009, 2011). Analogy with a presumably parallel and better characterized system in *M. genitalium* suggests that this diversity represents an antigenic variation scheme (Peterson et al., 1995; Iverson-Cabral et al., 2007; Ma et al., 2007). In *M. genitalium*, homologous recombination is stimulated by the ortholog of *M. pneumoniae* MPN626 acting as a novel sigma factor (Burgos and Totten, 2014; Torres-Puig et al., 2015), suggesting that this protein could also constitute a target for a therapeutic agent that would assist the immune system in clearing infection by blocking antigenic variation from occurring. Characterization of this recombination system in *M. pneumoniae* should therefore be a priority. At the same time, in light of this variation, any drugs that impact the functions of proteins P1, B, or C might select for strains expressing divergent sequences, indicating that any such agents should be tested against a wide variety of strains prior to deployment.

The transmembrane protein P30, encoded by MPN453, is another *M. pneumoniae* adherence protein that is also a good candidate for therapeutic development. Like P1, monoclonal antibodies against P30 inhibit cytadherence (Morrison-Plummer et al., 1986). However, unlike P1, P30 localizes exclusively at the AO (Baseman et al., 1987; Seto et al., 2001). In a mutant that lacks P30 due to a frameshift in MPN453, cells are non-motile, unable to cytadhere, and avirulent (Krause et al., 1982; Romero-Arroyo et al., 1999). Furthermore, a revertant strain, II-3R, in which a second frameshift mutation restores all but 17 amino acids, shows a near-wild-type level of hemadsorption but is almost completely non-motile (Hasselbring et al., 2005), illustrating that P30 has a specific role in gliding motility that is distinct from its role in cytadherence.

The primary structure of P30 is divisible into multiple regions. Its N-terminus, after removal of a long, atypical signal sequence, appears to be in the cytoplasm, and the C-terminus is accessible to carboxypeptidases and therefore located on the cell surface (Dallo et al., 1996; Chang et al., 2011). Mutants with progressive truncation of the P30 C-terminus exhibit decreasing levels of P30 in these mutants as well as drastically decreased gliding motility and cytadherence (Chang et al., 2011). The surface-exposed portion of P30 has an unusually high proline content, with that amino acid constituting 51 of the 125 amino acid residues in this region, and the majority of these prolines are organized into at least 13 sets of 6-amino-acid varying repeats (Dallo et al., 1990). The resulting decrease in the steady-state levels of P30 that lack a number of these repeats suggests that these prolines play a role in stabilizing P30, perhaps by enabling certain interactions or through structural integrity. Truncations in the cytoplasmic N-terminus of P30 remained stable but were unable to restore hemadsorption or gliding motility, suggesting that this region is necessary for proper function while also providing evidence that the C-terminal region has a role in P30 stability not shared with other regions of the protein (Chang et al., 2011). These results indicate the importance of both the internal and external portions of this protein for attachment and gliding motility, which may serve as an important feature in the design of therapeutic agents targeting this protein. Although an effort to create a P30 mutant strain of *M. pneumoniae* as a vaccine strain in an animal model was unsuccessful and in fact resulted in disease exacerbation when mice were infected with a virulent strain of *M. pneumoniae* (Szczepanek et al., 2012), the potential for using P30 in a subunit vaccine or of development of an agent capable of interfering with P30 function remains an option.

Internal AO Components

The specific mechanisms by which P1 and P30 are concentrated at the AO is unknown but it appears to depend heavily upon the cytoskeletal core of the AO. The core is composed of a set of cytoskeletal proteins necessary for development, structure, and proper localization of *M. pneumoniae* adhesins. These proteins, including HMW1, HMW2, HMW3, P28, P41, P200, P65, and TopJ, form a complex ordered network of interdependent interactions (Balish, 2014; Nakane et al., 2015). These proteins are necessary for proper development and function of the AO and are organized spatially (Nakane et al., 2015) and assemble in a temporal sequence (Krause and Balish, 2004). Two structural proteins that are required early in the AO assembly process, HMW1 (MPN446) and HMW2 (MPN310), have a special role in localization of P1 to the AO (Balish et al., 2003). When AO protein P65 (MPN309) is disrupted, structures containing P30 detach from *M. pneumoniae* cells, illustrating the importance of P65 for P30 localization and function (Hasselbring et al., 2012). Interestingly, loss of protein P41 (MPN311) causes the entire AO to be susceptible to release from cells during motility (Hasselbring and Krause, 2007), demonstrating the breadth of significant structural roles that AO core proteins have. Therefore, any and all AO core proteins are also potential therapeutic targets. Although the density, compactness, and cytoskeletal nature of the core make them potentially difficult for small molecules to reach, further knowledge about how these proteins assemble and interact may ultimately make it possible to design agents that target them before they become incorporated into nascent AO cores, thereby inhibiting formation of the AO.

Cell Division

Bacterial cell division is best understood in the context of cell wall biosynthesis; the absence of peptidoglycan in *M. pneumoniae* has rendered this process somewhat enigmatic. In model bacteria, the protein FtsZ forms cytoskeletal polymers at the division site, and these polymers, as components of a division machine, the divisome, coordinate rounds of iterative membrane invagination with local cell wall construction (Lutkenhaus et al., 2012). The *M. pneumoniae* genome includes a gene, MPN317, encoding a highly divergent FtsZ, whose expression levels are extremely low, at less than one mRNA per cell (Benders et al., 2005). Because cell wall synthesis is linked to FtsZ function in other bacteria, it is unclear specifically how FtsZ could contribute to efficient cell division in mycoplasmas, given the absence of peptidoglycan. Indeed, a knockout of this gene's ortholog in *M. genitalium* did not inhibit cell division but appeared to cause the cells to rely entirely on gliding motility to achieve cytokinesis (Lluch-Senar et al., 2010). Thus, the relationship of FtsZ to cell division in *M. pneumoniae* is unclear.

FtsZ has become a popular target for the development of new antibacterial agents (den Blaauwen et al., 2014). The sequence divergence of *M. pneumoniae* FtsZ, reflected in its inability to complement an *E. coli ftsZ* mutant (Osawa and Erickson, 2006), potentially makes this protein suitable as a target in terms of narrowness, since inhibitors might be specific to divergent features of the protein. However, the low expression levels of MPN317, uncertain relationship of the protein to the actual cell division process, and absence of knowledge about its role in virulence of *M. pneumoniae* raise questions about its suitability that are potentially resolvable with further study.

CONCLUSION

Despite a reduced genome and a small number of biosynthetic pathways, *M. pneumoniae* provides ample potential targets for development of narrow-spectrum agents to combat disease caused by this unusual bacterium. Some, like CARDS toxin and adhesins, are reasonably well-studied and could provide excellent substrates for inhibition by new drugs. For others, the physiological and biochemical details are lacking, but the gaps in knowledge provide numerous opportunities for research whose ultimate goal is to develop targets for fighting disease.

AUTHOR CONTRIBUTIONS

MB and SD analyzed and interpreted data and drafted the manuscript.

ACKNOWLEDGMENT

The authors thank Monica Feng and the Miami University Center for Advanced Microscopy and Imaging for contributing the image in **Figure 1**.

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