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Review Article

The clinical and molecular epidemiology of *Mycoplasma pneumoniae* infections

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Abstract: *Mycoplasma pneumoniae (M. pneumoniae)* is a major pathogen causing respiratory tract infection in adults and children. It is one of the most common causes of community-acquired pneumonia. This review provides a general overview of the clinical and molecular epidemiology of *M. pneumoniae* infection, which will give a better understanding of the disease.

Keywords: *Mycoplasma pneumoniae*, clinical epidemiology, molecular epidemiology

Mycoplasma pneumoniae (*M. pneumoniae*) is the main etiology of respiratory tract infections in adults and children with high incidence, especially among children >5-year-old [1]. *M. pneumoniae* can cause upper and lower respiratory tract infections and is one of the most common pathogens of community-acquired pneumonia (CAP) [2]. It can also lead to extrapulmonary complications and even to be life-threatening [3, 4]. In recent years, with the development of molecular biotechnology, the role of *M. pneumoniae* in epidemiology has attracted more attention. Herein, we present an up-to-date review of the current knowledge of the clinical and molecular epidemiology of *M. pneumoniae* infection.

Clinical epidemiology of *M. pneumoniae* infection

M. pneumoniae is a leading pathogen of human respiratory tract infections and can also cause extrapulmonary manifestations [4]. Among adults, *M. pneumoniae* is one of the major pathogens of CAP. According to a survey by Community-acquired Pneumonia Organization (CAPO), *M. pneumoniae* accounted for 11-15% of all CAP pathogens and was the most common respiratory tract pathogen along with *Streptococcus pneumoniae* [5, 6]. Among children, *M. pneumoniae* is a primary pathogen in the respiratory tract and accounts for 30-40% of all CAP pathogens [7, 8]. *M. pneumoniae* infection might be periodic with an interval of about 3-7 years [9, 10]. Moreover, *M. pneumoniae* potentially breaks out in populations in a closed or semi-closed environment. The long-term asymptomatic carriers of *M. pneumoniae* among healthy individuals are also a recent concern among researchers. In this section, we summarize the clinical-epidemiological characteristics, outbreaks, and asymptomatic carriers of *M. pneumoniae*.

Overview of clinical epidemiological characteristics of M. pneumoniae

M. pneumoniae is a common respiratory tract pathogen. It occurs sporadically or hypoendemically in a population in a region irregularly. A regional epidemic peak appears every 3-7 years [9, 10]. Although *M. pneumoniae* infection exhibits a prolonged latency period (1-3 weeks), the infectivity is not strong, and hence, it can be hypoendemic in the population for a long-term and break out in a closed or semi-closed environment. The onset of *M. pneumoniae* infection is considered to be related to temperature, humidity, and geographical position [11, 12]; thus, the epidemic season varies from area-to-area. In the USA, the high incidence of the infection occurs in late summer or early autumn

[13]. However, the high incidence emerges in autumn and winter in north China [14] and autumn in south China [8]. Although *M. pneumoniae* can invade the upper and lower respiratory tracts, there are no reliable epidemiological data showing *M. pneumoniae* is related to upper respiratory tract infections to date. Still, it is the second most frequent pathogen of CAP just coming after *S. pneumoniae* [6]. Based on the epidemiological investigations of CAP in 1962 and 1975, Foy et al. [10] found that *M. pneumoniae* accounted for about 15-20% of CAP. However, a recent epidemiological study [1] showed that *M. pneumoniae* accounted for 8% of the pathogens in hospitalized CAP patients aged 0-18 years and ranked top among the bacterial pathogens. Considering the prevalence rate is much higher than hospitalization rate, the proportion of *M. pneumoniae* in CAP pathogens is considerably high. *M. pneumoniae* infection can occur in every age group as well as cause neonatal congenital pneumonia [15]. Although the number of reports on *M. pneumoniae* pneumonia in infants has increased in recent years [16, 17], a large sample-based statistical data is still lacking and school-age children and teenagers are still the main populations of *M. pneumonia* infections. The latest pathogenic epidemiology of CAP demonstrated that the proportions of *M. pneumoniae* in children > or <5 years were 19% and <3%, respectively, among CAP hospitalization pathogens [1]. To date, gender bias was not found in these reports on *M. pneumoniae* infection.

Epidemiology of clinical manifestations of M. pneumoniae infection

Clinical symptoms of *M. pneumoniae* infection in the respiratory tract mainly include fever and flu-like symptoms (nasal obstruction, runny nose, sore muscle, and hypodynamia), sore throat, cough, and chest pain. As the upper respiratory tract infections are rarely taken seriously, and *M. pneumoniae* infection is self-limited, identifying any characteristic symptoms and signs highly related to *M. pneumoniae* upper respiratory tract infections is rather challenging. Therefore, we can only analyze *M. pneumoniae* pneumonia symptoms one by one. Fever is one of the most common clinical manifestations of *M. pneumoniae* infections. Goto et al. [18] reported that 29.9% adult *M. pneumoniae* pneumonia patients may not have fever. However, pediatric studies revealed that fever is the most common symptom of *M. pneumoniae* pneumonia. In a survey in Taiwan, only 1 child <5 years had no fever among 127 *M. pneumoniae* pneumonia patients aged 3-10 years [19]. Cough is also another one of the most common clinical symptoms of *M. pneumoniae* pneumonia. Originally, it is a non-productive cough, similar to pertussis; however, after 3-4 days, it gradually converts to a productive cough. Thus, symptoms sometimes are important indications for distinguishing the upper and lower respiratory tract infections. Generally, *M. pneumoniae*-derived upper respiratory infections do not present specific manifestations, but mainly presented with upper airway hyperemia, flu-like manifestations, and pharyngitis. The occurrence of cough mostly indicates that *M. pneumoniae* invaded the tracheal or bronchi mucosa. The onset of wheeze, dyspnea or hypoxia occurs, often lead to a diagnosis of severe *M. pneumoniae* pneumonia that is often accompanied by pleural effusion. Chest X-ray is critical for diagnosing *M. pneumoniae* pneumonia. Only non-specific symptoms, such as fever and cough, are observed in early *M. pneumoniae* pneumonia, and no obvious rale was detected in early lung auscultation, which easily result in the ignorance of early *M. pneumoniae* pneumonia. However, changes in the X-ray often appear early. Therefore, in order to diagnose *M. pneumoniae* pneumonia earlier, patients at a high-incidence age and those with constant fever, dry cough, and chest pain should undergo chest X-ray scan if the symptoms last for >3 days. The chest imaging manifestations of *M. pneumoniae* pneumonia present in three forms [20-23]: (1) lobar pneumonia: lobar lung parenchyma is involved, characterized by consolidation in lobes controlled by lobar or segmental bronchi; (2) lobular pneumonia: patchy or nodular shadows are observed around bronchi, the boundary of pulmonary vascular shadow is blurred, and there are patchy shadows on the route of pulmonary vascular shadow; (3) interstitial pneumonia: there are diffused shadows in the whole lung field, which are homogeneously patchy, and mostly accompanied by infiltration shadows around the pulmonary vessels. Generally, lobar pneumonia is considered as the most common manifestation with incidence rate of 17-76.5% [24, 25]. Moreover, the simul-

taneous involvement of multiple lobes is a major clinical feature of severe *M. pneumoniae* pneumonia [26]. Gao et al. reported that the imaging manifestation of lobar pneumonia was predominantly found in pre-school-aged children (4-6-year-old) with *M. pneumoniae* pneumonia and was more closely associated with severe clinical manifestations (e.g. higher rates of fever, pleural effusion and extrapulmonary manifestations) [27].

In addition, extrapulmonary complications is another important feature of *M. pneumoniae* infection. About 25-50% *M. pneumoniae* pneumonia patients have extrapulmonary complications; however, these data are often underestimated, as several patients with extrapulmonary manifestations do not have any respiratory tract manifestations. The pathogenesis of extrapulmonary complications caused by *M. pneumoniae* infection occurs in three forms: (1) *M. pneumoniae* arrives at an extrapulmonary organ through blood circulation and causes local inflammation; (2) *M. pneumoniae* stimulates auto-immune response by inducing cytokines, inflammatory mediators, or immune molecules production rather than entering an extrapulmonary organ; (3) Vasculitis leads to thrombosis in an extrapulmonary organ [3, 28]. Extrapulmonary complications can involve any system of the body, especially the skin and nervous system. Skin lesions are extremely common and account for 25% of all *M. pneumoniae* infections and are usually mild. A severe skin lesion is nodular erythema, with an incidence rate of about 3-11% [29]. Furthermore, serious dermatological manifestations of *M. pneumoniae* infection has also been reported, for instance, Stevens-Johnson syndrome [30]. The incidence of neurological complications is about 6-7%, which includes encephalitis, meningitis, cranial nerve palsy, Guillain-Barre syndrome, cerebral embolism, and mental disorders [31, 32]. Moreover, the involvement of other systems, including cardiovascular system [33-35], digestive system [35, 36], urogenital system [37, 38], blood system [39], and musculoskeletal system [40], has also been reported, despite a low rate of incidence.

Asymptomatic carrier of M. pneumoniae

When *M. pneumoniae* invades the respiratory tract, it adheres to the epithelial cells of the respiratory tract through adhesive protein complexes, which mainly consist of P1 and P30 proteins, and then triggers a series of symptoms and manifestations in the respiratory tract. An array of specific serum antibodies (e.g. anti-P1 adhesion antibody) produced by host affect the adhesion and sliding of *M. pneumoniae* on the epithelial cells of the respiratory tract, which reduce the adhesion and eventually remove *M. pneumoniae* [41]. Unlike pneumococcus and other intracellular bacteria, *M. pneumoniae* depends on its close adhesion to host cells to obtain nutrients and maintain metabolism. Therefore, whether there is a long-term carrier of *M. pneumoniae* state is still controversial. Some Dutch groups investigated 726 patients, aged 3 months-16 years, admitted to a children's hospital in Rotterdam from 2008-2011. Among them, 405 surgical patients were classified into the asymptomatic group, while 321 patients presented with respiratory symptoms were classified into the symptomatic group. *M. pneumoniae* DNA tests showed that the carrier rate (16.2% and 21.2% respectively, P=0.11) did not differ significantly between these two groups [42]. Therefore, we concluded that the asymptomatic carrier of *M. pneumoniae* exists and the proportion was not low. The current diagnostic methods of *M. pneumoniae* detection cannot distinguish the asymptomatic carrier from symptomatic infection. Inversely, a study in Greenland recruited 54 children with otitis media and 201 healthy children as controls. The study tested the nasopharyngeal specimens and found that the bacteria carrier rates were >90% in both groups. Of note, the *M. pneumoniae* positive rates were 0 [43]. Similarly, from 2005-2007, Kumar et al. assimilated the results of common acute respiratory tract pathogen tests from 129 asymptomatic surgical patients from a Children's Hospital in Wisconsin and found that bacteria carrier rate was up to 28%, while the *M. pneumoniae* positive rate was 0 [44]. Why did the results of these studies differ? This concern was addressed previously: *M. pneumoniae* infection can be manifested as upper and lower respiratory tract infection or occult infection. The carrier state of laryngopharyngeal bacteria was typically maintained for 6-7 weeks. However, the carrier state in some individuals may persist for 4-7 months, and eventually turn negative [10, 45]. The studies mentioned above [42] only tracked 21 asymptomatic patients

and 22 symptomatic patients for a month. Most of them (15/21 and 19/22) turned negative and were not followed up. Only 1 symptomatic patient turned negative 4 months later in the follow-up. The high positive rate among asymptomatic children may due to a recent outbreak in the same population. This finding was not contradictory to the results presented by Foy et al. [10] 20 years ago. The study showed that in immunecompetent children, the pathogen can be carried for a specific period after *M. pneumoniae* infection (generally <8 weeks). The host produces a specific antibody and gradually removes the pathogen. Thus, only in very few cases, the pathogen would last up to 4 months. Nilsson et al. studied 53 patients with *M. pneumoniae* respiratory tract infections and showed that after anti-*M. pneumoniae* treatment and full production of specific serum antibody, the median time for carriage of *M. pneumoniae* DNA was 7 weeks after disease onset (range 2 days-7 months) [45]. Hitherto, there is no long-term study (>1 year) suggesting that *M. pneumoniae* can be carried for prolonged duration in the respiratory tract of immunocompetent hosts. Since *M. pneumoniae* has different serotypes and the titer of specific *M. pneumoniae* antibody declines over a period, repeated *M. pneumoniae* infections are plausible. Therefore, the immunocompetent hosts with positive nasopharyngeal *M. pneumoniae* should be traced for the history of respiratory tract infection within 2 months. Also, for patients with long-term positive nasopharyngeal *M. pneumoniae*, apart from repeated *M. pneumoniae* infection, the occurrence of immunodeficiency, ciliary dysfunction, and other basic diseases should be monitored.

Outbreaks of M. pneumoniae infection

Although *M. pneumoniae* is sporadic in most cases, outbreak cases are often reported. These outbreaks mostly occur in a closed or semi-closed environment, such as kindergartens, primary and secondary schools, universities, military camps, old-age nursing facilities, and hospitals [46-50]. In outbreak units, each age group is involved, including *M. pneumoniae* high-incidence age (preschool and school-age children) in primary and secondary schools and non-*M. pneumoniae* high-incidence age (adults) in military camps and old-age nursing facilities. During the breakout, the infection rate is 40-45% [46, 49, 51]. The incidences of pneumonia among adults and children are different: the incidence among adults is about 25% [51], while that among children is >50% [49, 51]. These differences can be related to different strains; however, the immature immune functions of children may be an extremely important reason. The duration of outbreaks is from 12 days to 4 months [46, 49, 50], which is highly correlated to the early detection of symptoms and timely quarantine and treatment. In most cases, due to the managers' negligence, timely quarantine is not taken. As a result, the epidemic period is greatly prolonged. The different strains and populations lead to differential consequences. In brief, most patients may present with upper and lower respiratory tract infections, however, severe extrapulmonary complications may occur during the outbreaks of *M. pneumoniae* [50] and even mortality may occur in some populations with basic diseases, such as old-age nursing facilities [46, 50]. Risk factors including smoking, hypogammaglobulinemia, and close contact are frequently reported for the outbreaks of *M. pneumoniae* [49, 51]. Therefore, most of the outbreaks have occurred in a closed or semi-closed environment, indicating that close contact in a closed environment is a major risk factor for the spread of *M. pneumoniae*.

Molecular epidemiology of *M. pneumoniae* infection

Molecular epidemiology is a science that measures the distribution of biological markers using advanced molecular biotechnology, clarifies the causes of diseases and relevant pathogenic processes at a molecular or genetic level, combined with the epidemiological field research methods as well as explores the strategies for preventing diseases and promoting health. Several molecular classification methods of *M. pneumoniae*, including P1 genotyping, multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) [52], multilocus sequence typing (MLST) [53], and single nucleotide polymorphism (SNP) minisequencing [54] are currently utilized.

P1 genotyping

As *M. pneumoniae* survives by adhering to the ciliated epithelial cells in the human respiratory tract, its adhesion function would be highly

related to its virulence. This adhesion function is mainly executed by protein complexes with P1 protein as the core. Therefore, *M. pneumoniae* molecular classification research is mainly based on *P1* gene and its polymorphism. The P1 protein contains repetitive DNA fragments of RepMP2/3 and RepMP4. The analysis of nucleotide diversity results showed that RepMP4 was more likely to be genetically diverse than RepMP2/3 [55]. Depending on these different repetitive sequences, *M. pneumoniae* was divided into various molecular subtypes. Presently, the restricted fragment length polymorphisms (RFLP) is a common and simple method based on the principle of amplifying the *P1* gene of *M. pneumoniae* using specific primers and gel electrophoresis after restriction enzyme digestion. Type I and II classifications, most widely used in current clinical applications, are based on different fragment sizes of the products. Standard strains *M. pneumoniae*-M129 and *M. pneumoniae*-FH often used in scientific research belong to Types I and II, respectively [56].

P1 genotyping can also be performed using pulse-field gel electrophoresis (PFGE), wherein *M. pneumoniae* DNA is subject to endonuclease cleavage by *Apa I* restriction enzyme. Subsequently, agarose gel electrophoresis is performed for genotyping according to the different fragment sizes and band positions of the products. *M. pneumoniae* P1-I strain can produce three sizes of fragments: 242 bp, 125 bp, and 82 bp; however, P1-II strain cannot produce these three fragments after digestion. Moreover, PFGE can divide the original P1-II type into 2 subtypes. *M. pneumoniae*-FH strain belongs to P1-IIa. P1 genotyping not only differs in P1 gene sequence but previous studies [57, 58] also demonstrated that different P1 subtypes vary in invasiveness and community-acquired respiratory distress syndrome (CARDS).

Furthermore, some investigators [59, 60] speculated that the host produces specific antibodies according to different P1 subtypes, which underlies molecular epidemiology of *M. pneumoniae* reinfections. Some studies showed that a regional *M. pneumoniae* peak every 3 to 7 years is based on the alternative prevalence between the two subtypes [61-63]. The other studies [64, 65] showed that the alternative prevalent characteristic of P1 subtype strains is not significant, thereby further large-scale multi-center studies are needed. In accordance with the view of most experts, Dumke et al. [66] found an intermittent epidemic peak of *M. pneumoniae* was caused by at least 4 kinds of variant epidemic strains with different molecular subtypes.

MLVA

MLVA is a molecular classification technology that utilizes the characteristics of VNTR in genomes. It is characterized by simple, rapid, high-throughput, and strong discrimination. Presently, it has been widely used in molecular classification of a variety of bacteria. VNTR has different numbers of tandem repeats depending on different *M. pneumoniae* strain types, and hence, can be used for epidemiological surveys and monitoring of *M. pneumoniae*. According to the reported whole genome sequences of *M. pneumoniae* strains, Cousin-Allery detected the VNTR loci in their genomes and found 5 loci (including MPn1, MPn13, MPn14, MPn15 and MPn16) with stable polymorphisms [67]. MLVA classification is based on these 5 loci (MLVA-5) that are amplified using multiple PCRs. Combined with capillary electrophoresis, the number of repeats of VNTR loci can be measured accurately for classification. In addition, this classification method has is highly discriminative and can be used in a small range of epidemiological surveys [68, 69]. Some studies showed that [70] MP*n*1 is not stable enough in clinical isolates and laboratory standard strains, because its repetitive sequences may change during culture. Thus, the MPn13-16 method (MLVA-4) was recommended by some scientists, although the discrimination degree of classification drops inevitably [71]. Therefore, to improve the discrimination power of MLVA towards *M. pneumoniae*, a new MLVA scheme should be developed that includes additional VNTR markers such as those identified in a recent study [72]. MLVA can be used to classify *M. pneumoniae* into >20 MLVA types, whereas *P1* genotyping classifies *M. pneumoniae* into 2 major types (Types I and II) and three P1 type II variants (Types 2a, 2b, and 2c. Compared to P1 typing, MLVA typing was more valuable in distinguishing *M. pneumoniae* during outbreaks with respect to molecular characteristics [73].

Table 1. Comparison of key features of several *M. pneumoniae* molecular classification technologies

Classification Technologies	Discrimination	Stability	Timeliness	Economy	Throughput
P1 Gene Subtyping	+	++	++	++	+
MLVA	++	+	+	++	++
MLST	++	++	+	+	+
SNP-minisequencing	++	++	++	+	++

MLST

MLST is a bacterial classification method based on nucleotide sequence determination. The PCR amplification determines the sequences of the internal fragments of multiple housekeeping genes and analyzes the variations of strains for classification. Compared to the conventional molecular biological classification method, MLST has a high discrimination power. It can identify the same bacteria to more subtypes and determine the phylogenetic relationship between different subtypes and their correlation with diseases. MLST is easy to operate, and the results can be obtained quickly to facilitate comparison between different laboratories. It has been used in epidemiological monitoring and evolution research of multiple bacteria. Previously, some investigators proposed to introduce the molecular classification method of MLST to *M. pneumoniae* classification studies. However, due to less polymorphism of *M. pneumoniae* housekeeping genes, MLST was regarded as inapplicable to *M. pneumoniae* classification [74]. Brown et al. [53] succeeded in incorporating the polymorphism of 8 housekeeping genes into MLST research of *M. pneumoniae*, which not only provided a clinical specimen-based molecular classification method with higher discrimination degree than *P1* gene subtype and MLVA but also used evolution analysis and identification of sequence types (STs) to build a dendrogram for clustering analysis and found two genetically distinct clusters. Importantly, this method was supported by the public network database (http://pubmlst.org/M. pneumoniaeneumoniae).

SNP minisequencing

SNP refers to the variations in single nucleotides in genomes, including transition, transversion, deletion, and insertion. The formed genetic markers have abundant polymorphisms. SNP mainly refers to the polymorphisms in DNA sequences caused by variations of single nucleotides at the genomic level. For organisms, SNP is a critical genetic marker and can be used for molecular classification of bacteria. In 2003, some groups attempted to classify *M. pneumoniae* using SNPs. However, since the number of *M. pneumoniae* housekeeping genes was small, the polymorphisms were not high, and the whole genome sequencing cost was high; thus, this method was considered to have low prospects [66]. With the improvement of genetic detection technology, especially the emergence of SnaPshot minisequencing-based approach, SNP minisequencing has been established. Touti et al. [54] selected SNPs of 5 housekeeping genes, *P1* gene, and 2 lipoprotein genes in *M. pneumoniae* genomes and carried out a new molecular classification of *M. pneumoniae* using SNP minisequencing. The study found that the discrimination degree of this method for *M. pneumoniae* was only surpassed by MLVA-5 but superior to classification methods of MLVA-4 and P1-RFLP. Moreover, this method, in combination with MLVA-5, can make up the instability of *M. pneumoniae*n1 in MLVA-5. Due to the relative stability of SNP, SNP minisequencing classification method was different from that of MLVA. Briefly, MLVA was applied for short-term regional epidemiological classification of *M. pneumoniae* outbreaks, while the former was applied for long-term and global epidemiological investigations.

In conclusion, with the progress of molecular biotechnology, several new *M. pneumoniae* molecular classification methods have been applied; however, the degree of discrimination, cost, and timeliness are variable (**Table 1**). P1 typing and MLVA are the most widely used in current *M. pneumoniae* molecular epidemiology [47]. In addition, molecular classification has been found to be of critical importance with respect to diagnosis, virulence, susceptibility, drug resistance, and antibiotics resistance. Therefore, it is essential to compare and analyze a large number of clinical *M. pneumoniae* strains, as well as conduct a comprehensive

analysis combined with the clinical findings to select an optimal method for clinical purpose.

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Disclosure of conflict of interest

None.

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