Investigations of *Mycoplasma pneumoniae* Infections in the United States: Trends in Molecular Typing and Macrolide Resistance from 2006 to 2013

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*Mycoplasma pneumoniae* is a leading cause of respiratory infections, including community-acquired pneumonia (CAP). Currently, pathogen-specific testing is not routinely performed in the primary care setting, and the United States lacks a systematic surveillance program for *M. pneumoniae*. Documentation of individual cases and clusters typically occurs only when severe illness and/or failure to improve with empirical antibiotic therapy is observed. Outbreaks, some lasting for extended periods and involving a large number of cases, occur regularly. However, many more likely go unrecognized due to the lack of diagnostic testing and structured reporting. We reviewed data from 17 investigations of cases, small clusters, and outbreaks of *M. pneumoniae* infections that were supported by the Centers for Disease Control and Prevention (CDC) between 2006 and 2013. We examined 199 *M. pneumoniae*-positive specimens collected during this time period in order to identify trends in antimicrobial resistance and circulating types. Overall, macrolide resistance was identified in approximately 10% of *M. pneumoniae* infections occurring during this time period. Typing of strains revealed cocirculation of multiple multilocus variable-number tandem-repeat analysis (MLVA) and P1 types throughout this period, including diversity in types detected within individual outbreaks. Three MLVA types (4572, 3562, and 3662) accounted for 97% of the infections during the study period. A systematic surveillance program is necessary to understand the burden of *M. pneumoniae* disease in the United States, facilitate case and outbreak identification, and inform appropriate therapeutic and infection control strategies.

Methods for molecular typing of *M. pneumoniae* strains have been developed, including discrimination of two major types and variants based on the sequence of the P1 adhesion molecule gene and identification of multiple types using multilocus variable-number tandem-repeat analysis (MLVA) to examine four or five polymorphic loci. Despite development of these sophisticated typing methods, characterization of *M. pneumoniae* strains is even rarer than detection and is only performed by a few specialized laboratories in the United States. As a result, the majority of information on circulating *M. pneumoniae* strains, including the prevalence of macrolide susceptibility, originates from investigations of outbreaks and individual cases or clusters of severe disease. Here, we review the sporadic cases, small clusters, and outbreaks of *M. pneumoniae* infection that were investigated by the Centers for Disease Control and Prevention (CDC) between 2006 and 2013 and examine trends in strain types and macrolide resistance during this period.

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TABLE 1 Characteristics of the CDC-assisted investigations of M. pneumoniae infections, 2006–2013

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) of investigations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CDC-assisted investigations</td>
<td>17</td>
</tr>
<tr>
<td>Sporadic cases</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>Cluster*</td>
<td>3 (17.6)</td>
</tr>
<tr>
<td>Outbreak</td>
<td>10 (58.8)</td>
</tr>
<tr>
<td>Epi-Aid response</td>
<td>3 (18)</td>
</tr>
<tr>
<td>Associated with educational institution</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Evidence of household transmissionb</td>
<td>5 (29)</td>
</tr>
<tr>
<td>Extrapulmonary involvementc</td>
<td>2 (12)</td>
</tr>
<tr>
<td>Macrolide resistance detectedd</td>
<td>8 (47)</td>
</tr>
</tbody>
</table>

* Two to three cases with an epidemiological connection.

b Not evaluated in all investigations.

c Documented in at least one patient.

d Identified by molecular analysis.

MATERIALS AND METHODS

Respiratory specimens (n = 199), including nasopharyngeal (NP) and/or oropharyngeal (OP) swabs (n = 135), sputum (n = 1), bronchoalveolar lavage (BAL) fluid (n = 8), and respiratory aspirates (nasal, NP, or OP) (n = 55) submitted to the Pneumonia Response and Surveillance Laboratory (PRSL) at the CDC between 2006 and 2013 were included in this analysis. When multiple specimens from the same patient were available, only one was included in the analysis. Each investigation was classified based on the number of M. pneumoniae-positive specimens as either an individual case (n = 1), a cluster (n ≥ 3), or an outbreak (n > 3).

All respiratory specimens were tested using either a singleplex quantitative PCR (qPCR) assay for the specific detection of M. pneumoniae (17) and/or a Clinical Laboratory Improvement Amendments (CLIA)-approved multiplex assay for detection of M. pneumoniae, Chlamydia pneumoniae, Legionella spp., and human nucleic acid (18). Sputa specimens were treated with dithiothreitol (DTT) for 30 min prior to extraction. Total nucleic acid was extracted from each specimen using the MagNA Pure Compact or MagNA Pure LC instrument (Roche Applied Science, Indianapolis, IN) with a total nucleic acid isolation kit L. Cultures were attempted for all M. pneumoniae-positive specimens as previously described (19). The recovered isolates were confirmed using a singleplex qPCR assay for M. pneumoniae (17).

MLVA was performed on all PCR-positive specimens having a sufficient volume of original material and all isolates using previously described methods (20). MLVA types were reported using four loci (Mpn13, Mpn14, Mpn15, and Mpn16) as previously suggested (20–22). High-resolution melt (HRM) analysis for identification of the macrolide susceptibility genetic profile was performed with a slight modification of the original procedure (9). The HRM profiles for clinical specimens were compared to those of the macrolide-resistant and macrolide-sensitive reference strains which were included in each run. The reference strains had been previously verified by sequencing and determination of MICs. P1 typing was performed using HRM analysis as previously described (23). The isolates were classified as type 1, type 2, or variant based upon comparison of melting profiles to that of the type 1 reference strain (M129) and the type 2 reference strain (FH) included in each run.

RESULTS

Characteristics of recent investigations. Between 2006 and 2013, the CDC PRSL assisted in 17 investigations of M. pneumoniae infections, including individual cases (n = 4), small clusters (n = 3), and outbreaks (n = 10) in 14 different states (Table 1). The number of investigations in a single year ranged from zero to eight during this time period with the highest number of investigations (n = 8, including four outbreaks) occurring in 2013. Of the 10 outbreaks during this period, seven were associated with a school, school district, or university. Patient ages were available for 149 (75%) specimens; the average patient age was 13 years (range, 0 to 50 years). Extrapulmonary manifestations, including encephalitis and dermatological complications (Stevens-Johnson syndrome [SJS]) occurred in two outbreaks. Three outbreaks involved official requests for epidemiological assistance (Epi-Aid) by the CDC, which included retrospective and prospective case findings and investigation of household contacts. The number of M. pneumoniae-positive specimens identified in each outbreak investigation varied widely (range, 4 to 72). More specimens were collected, and more positive specimens were identified during Epi-Aid investigations than for other outbreaks.

Macrolide resistance. Macrolide-resistant M. pneumoniae strains were detected in at least one patient specimen during eight (47%) of 17 investigations, including 5 (50%) of 10 outbreaks (Table 1). The proportion of macrolide-resistant M. pneumoniae strains detected in qPCR-positive specimens varied between individual outbreaks and among nonoutbreak specimens (sporadic cases and clusters) over the time period studied (Fig. 1). Macrolide-resistant M. pneumoniae strains were identified in 0 to 23% of specimens in a given outbreak (Fig. 1A). Across all outbreaks, the average proportion of macrolide-resistant M. pneumoniae specimens detected was 7%, whereas 29% of specimens not associated with an outbreak were identified as resistant (Fig. 1A). Overall, 10% of M. pneumoniae-positive specimens tested at the CDC during this time period displayed genetic profiles consistent with resistance to macrolides (Fig. 1). Variations in macrolide-resistant specimens over time generally reflected the pattern observed by examining individual outbreaks in order of occurrence (Fig. 1).

Molecular typing. P1 typing, historically used to differentiate strains based on the genetic sequence encoding the P1 adhesion molecule, was performed only on isolates. During the 8-year study period, 57% of M. pneumoniae isolates were identified as type 1 and 34% as type 2 (Fig. 2A). Nine percent of isolates were determined to be variants of the type 2 strain, differing slightly in melting profile from that of either reference strain. The proportion of each type varied over the study period (Fig. 2B). Type 1 isolates accounted for the majority of M. pneumoniae isolates in 2006, but dropped to 50% of isolates in 2013. Type 2 and variant strains each increased between 2011 and 2013.

The primary MLVA types identified during the study period were 4572 (54%), 3562 (32%), and 3662 (11%) (Fig. 3A). MLVA types identified in recovered isolates matched the types identified from primary specimens in each case (data not shown). Other rare types, including 3572 (n = 1), 4472 (n = 1), and 4672 (n = 3) accounted for only 3% of all specimens tested and were observed in 2013 only. Multiple MLVA types were detected during 8 (80%) of 10 outbreak investigations. A single MLVA type was observed in two outbreaks. Six different MLVA types were observed in a single outbreak investigation; this outbreak involved the highest number of M. pneumoniae-positive specimens (n = 72). MLVA types 4572 and 3562 each were the predominant MLVA pattern identified in five outbreaks. The pattern of MLVA type distribution over the study period approximately mirrored the P1 typing distribution, consistent with a previously observed correlation between P1 and MLVA types (22).

Notably, multiple cocirculating types, as determined by P1 and
MLVA typing, were identified in 8 (80%) of 10 outbreaks. The two outbreaks in which only a single P1 or MLVA type was identified consisted of only two or three *M. pneumoniae*-positive specimens each. Furthermore, the 19 macrolide-resistant isolates were identified as different P1 types, including type 1 (n = 13), type 2 (n = 4), and type 2 variant (n = 2). The resistant isolates were similarly distributed among the predominant MLVA profiles, including 4572 (n = 13), 3562 (n = 4), and 3662 (n = 2). The distributions of P1 types (Fig. 4A) and MLVA types (Fig. 4B) between resistant and sensitive *M. pneumoniae* strains were similar. There were no apparent differences in the distributions of MLVA types in sporadic cases and clusters compared to those in outbreaks (data not shown).

**DISCUSSION**

In the absence of national surveillance for *M. pneumoniae*, some information can be gleaned from a comprehensive examination of recent investigations of *M. pneumoniae* illness. We examined *M. pneumoniae* specimens from 17 investigations of individual cases, small disease clusters, and outbreaks of various sizes that occurred in the United States between 2006 and 2013 in which the CDC led or assisted in a laboratory and/or epidemiological investigation. The CDC became involved in investigating individual cases or small clusters when the etiology could not be identified in the clinical or state public health laboratory or when a patient failed to improve despite appropriate antibiotic therapy. In some cases, the CDC assisted in identifying *M. pneumoniae* as the causative agent, while in other outbreaks, the CDC provided confirmatory laboratory testing and further characterization. More than 40% of outbreaks were associated with an educational institution, which reflects the fact that *M. pneumoniae* is a leading cause of CAP in children and adolescents (24). Transmission of *M. pneumoniae* infections between family members is common (8, 25, 26); multiple cases within a household were documented in approximately one-third of the clusters or outbreaks in this investigation, although evidence of household transmission was not evaluated in all of the clusters and outbreaks.

A macrolide-resistant *M. pneumoniae* strain was identified in at least one patient specimen in approximately half of the investigations included in the current study. We identified an overall rate of macrolide resistance of 10% among specimens collected be-

![FIG 1](http://jcm.asm.org/) Macrolide resistance in *M. pneumoniae* specimens in the United States, 2006 to 2013. (A) Proportions of macrolide-resistant and sensitive *M. pneumoniae* strains identified in individual outbreaks. The proportions of sensitive and resistant strains in all outbreak specimens (n = 156), sporadic cases and clusters (n = 20), and all specimens (n = 176) are also shown. Macrolide susceptibility could not be determined for 23 (12%) of 199 specimens due to the lack of an available specimen or inadequate amplification of the target region. (B) Proportion of macrolide-resistant *M. pneumoniae* infections identified over the study period. Nineteen resistant strains (10%) were identified during this time period. The number of *M. pneumoniae*-positive specimens with macrolide susceptibility results is shown for each year; no positive specimens were available from 2008 or 2010.

![FIG 2](http://jcm.asm.org/) P1 typing of *M. pneumoniae* isolates, 2006 to 2013. (A) Proportion of each P1 type identified among all isolates (n = 139). (B) Proportions of P1 types during each year of the study period. The number of *M. pneumoniae* isolates for each year is shown; no isolates were available from 2008 or 2010.
tween 2006 and 2013. Substantial variations in the rates of macrolide resistance in individual outbreaks were noted, ranging from 0 to 23%. The proportion of infections in which macrolide resistance was identified was higher in individual cases and clusters than in outbreaks. The most likely reason for this observation is that requests for assistance from the CDC to investigate individual cases and clusters are more likely when patients fail to improve despite antibiotic treatment and/or when the clinician suspects antibiotic resistance. The introduction of commercially available tests to determine macrolide susceptibility in the primary care setting might facilitate identification of macrolide-resistant \textit{M. pneumoniae} infections and help prevent further transmission by informing antibiotic therapy decisions.

Studies describing the rates of macrolide resistance in \textit{M. pneumoniae} strains in the United States are limited. Yamada and colleagues previously reported a rate of 8.2% macrolide-resistant \textit{M. pneumoniae} among specimens collected between 2010 and 2012 (10). This is similar to the prevalence of resistance observed in the current study. However, the rate of resistance in an individual outbreak can be substantially higher. For instance, 23% of the \textit{M. pneumoniae} specimens had macrolide-resistant strains.

FIG 3 MLVA typing of \textit{M. pneumoniae} specimens and/or isolates, 2006 to 2013. Results for four loci (Mpn13 to Mpn16) are reported. (A) Proportion of each MLVA type identified among specimens/isolates (n = 170). MLVA type could not be determined for 29 (15%) of 199 specimens due to the lack of an available specimen/isolate or inadequate amplification of all four loci. Other types detected include 3572 (n = 1), 4472 (n = 1), and 4672 (n = 3). (B) Proportions of MLVA types during each year of the study period. The number of \textit{M. pneumoniae}-positive specimens with available MLVA results is shown for each year; no positive specimens were available from 2008 or 2010.

FIG 4 Distribution of strain types in macrolide-resistant (inner circle) and macrolide-sensitive (outer circle) \textit{M. pneumoniae} specimens. (A) P1 types in sensitive (n = 122) and resistant (n = 16) \textit{M. pneumoniae} isolates (n = 138). (B) MLVA types in sensitive (n = 151) and resistant (n = 19) \textit{M. pneumoniae}-positive specimens (n = 170).
pneumoniae-positive specimens were identified as resistant to macrolides in a single outbreak in the current study. This may truly indicate a higher proportion of resistant specimens in this particular outbreak or simply be a function of testing a limited number of patient specimens. In recent years, several reports have documented low levels of macrolide resistance (<3%) in M. pneumoniae infections across Europe, including Denmark, France, and Germany, during an active epidemic period (27–29). A recent report by Ferguson and colleagues documented the first macrolide-resistant M. pneumoniae infections in the United Kingdom in 6 of 32 (19%) patients studied (30). The rates of macrolide resistance observed in the United States and Europe contrast dramatically with those in reports from Asia where 80 to 90% of M. pneumoniae infections are resistant (6, 7). Improved surveillance and monitoring are necessary to manage the threat of emerging resistance to the first-line therapy for M. pneumoniae infection in the United States.

The current analysis of 8 years of M. pneumoniae infections in the United States reveals trends in the circulating strain types. Notably, we observed cocirculation of both type 1 and 2 strains during this time period. Multiple MLVA types were detected in 80% of discrete outbreaks. The greatest diversity in MLVA types in a single outbreak setting was observed in the investigation having the highest number of specimens. The appearance of clonality in the remaining two outbreaks was likely due to testing of too few specimens to obtain an accurate representation of the diversity of circulating strains, as has been suggested previously (20, 22). Cocirculation of P1 types in both endemic and epidemic settings has been documented (22, 31). Three MLVA types (4572, 3562, and 3662) accounted for 97% of the M. pneumoniae infections in this study. In 2013, several other MLVA types were infrequently detected; this small increase in strain diversity may simply reflect a more accurate picture of the repertoire of circulating strains due to the testing of a higher number of specimens in that particular year or may indicate the emergence of an increasingly diverse repertoire of M. pneumoniae strains in the community. The same three types identified most frequently during this period were also identified by Sun and colleagues as the predominant MLVA types identified most frequently during this period. The predominance of type 1 or 2 strains in the population has historically been documented, and the cyclic pattern of type-specific dominance was attributed to the development of temporary immunity to one type, yielding an opportunity for the alternate type to reemerge (34). The reasons for the more recently observed cocirculation of type strains in the global population and even within a defined outbreak are unclear, and further investigation is warranted to explain this phenomenon. Although current strain typing methods provide some insight into the epidemiology of M. pneumoniae, these methods only allow for classification of strains into a few unique types. Implementation of whole-genome sequencing might provide vastly more information and improve our ability to compare and classify M. pneumoniae strains and to identify bacterial factors which may contribute to severe disease and poor patient outcomes.

There are several limitations to this study. First, the data described here are limited to investigations in which the CDC was invited to participate. There was substantial variation in the number of specimens tested in individual outbreaks. For example, the three Epi-Aid responses were conducted for larger outbreaks and included both retrospective and prospective case findings, resulting in the collection of a large number of specimens and, therefore, identification of more M. pneumoniae-positive specimens than for other outbreaks. This retrospective analysis likely includes only a fraction of the actual infections attributable to this pathogen. The lack of a comprehensive and systematic approach prevents estimation of incidence of individual cases or outbreaks and could conceal geographic or temporal trends in circulating types or macrolide resistance both endemically and during localized outbreaks. Second, this study is limited to 8 years, which may be long enough to observe changes in circulating types, but spanning of a longer time period would be necessary in order to identify broader trends occurring over time and to identify a classical cyclic pattern (34).

Establishment of a systematic surveillance program for M. pneumoniae will be critical for providing the necessary data to fully understand the biology and epidemiology of this organism in the United States. The potential for macrolide resistance to grow to levels observed in other parts of the world is also particularly concerning, especially considering the widespread administration of azithromycin in the United States. Monitoring M. pneumoniae and the prevalence of macrolide resistance is critical for providing appropriate therapy, recognizing outbreaks, and identifying the emergence of hypervirulent or drug-resistant strains.

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