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Molecular Detection and Characterization of *Mycoplasma pneumoniae* Among Patients Hospitalized With Community-Acquired Pneumonia in the United States

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Background. *Mycoplasma pneumoniae* is a common cause of community-acquired pneumonia (CAP). The molecular characteristics of *M pneumoniae* detected in patients hospitalized with CAP in the United States are poorly described.

Methods. We performed molecular characterization of *M pneumoniae* in nasopharyngeal/oropharyngeal swabs from children and adults hospitalized with CAP in the Centers for Disease Control and Prevention Etiology of Pneumonia in the Community (EPIC) study, including P1 typing, multilocus variable-number tandem-repeat analysis (MLVA), and macrolide susceptibility genotyping.

Results. Of 216 *M pneumoniae* polymerase chain reaction-positive specimens, 40 (18.5%) were obtained from adults and 176 (81.5%) from children. P1 type distribution differed between adults (64% type 1 and 36% type 2) and children (84% type 1, 13% type 2, and 3% variant) ($P < .05$) and among sites ($P < .01$). Significant differences in the proportions of MLVA types 4/5/7/2 and 3/5/6/2 were also observed by age group ($P < .01$) and site ($P < .01$). A macrolide-resistant genotype was identified in 7 (3.5%) specimens, 5 of which were from patients who had recently received macrolide therapy. No significant differences in clinical characteristics were identified among patients with various strain types or between macrolide-resistant and -sensitive *M pneumoniae* infections.

Conclusions. The P1 type 1 genotype and MLVA type 4/5/7/2 predominated, but there were differences between children and adults and among sites. Macrolide resistance was rare. Differences in strain types did not appear to be associated with differences in clinical outcomes. Whole genome sequencing of *M pneumoniae* may help identify better ways to characterize strains.

Keywords. community-acquired pneumonia; macrolide resistance; molecular epidemiology; *Mycoplasma pneumoniae*.

Mycoplasma pneumoniae is a common cause of respiratory infections, including community-acquired pneumonia (CAP). However, the disease burden is diffi-

cult to estimate due to limitations of diagnostic assays and lack of systematic surveillance [1, 2]. Real-time polymerase chain reaction (PCR) for *M pneumoniae* detection is preferred due to the improved sensitivity and specificity compared with culture and serology, yet routine PCR testing in the clinical setting remains uncommon [2].

Molecular characterization of circulating *M pneumoniae* strains in the United States is particularly limited. Classification as type 1, type 2, or variant genotypes based on sequence variation in the gene encoding the immunogenic P1 surface protein has been the standard

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method for differentiating *M pneumoniae* [1–3]. More recently, the development of a multilocus variable-number tandem-repeat analysis (MLVA) typing scheme has allowed more precise categorization of strains based on the variable copy number of tandemly repeated sequences at multiple stable genetic loci [4, 5]. However, the clinical utility of P1 and MLVA typing is uncertain because no genotype has been associated with increased virulence or epidemic potential.

Advanced molecular methods are also useful for identifying genetic mutations conferring macrolide resistance in *M pneumoniae*, a growing global public health concern [6, 7]. Some studies suggest that individuals infected with macrolide-resistant *M pneumoniae* experience a longer febrile period, more persistent cough, and extended antibiotic therapy compared with persons infected with macrolide-sensitive strains [8–13], although the impact of macrolide resistance on patient outcome remains uncertain. In Asia, over 90% of isolates are resistant to macrolides [14, 15]. Resistance has also emerged in the United States over the last 15 years [7, 16, 17], but the prevalence is unknown.

We characterized *M pneumoniae* detections from a cohort of adults and children hospitalized with radiographically confirmed CAP prospectively enrolled in the Centers for Disease Control and Prevention (CDC) Etiology of Pneumonia in the Community (EPIC) study.

METHODS

Study Population, Case Definitions, and Clinical Specimens

Children (<18 years old) and adults were enrolled in the EPIC study from January 2010 to June 2012 at 8 hospitals in Chicago, Illinois, Memphis, Tennessee, Nashville, Tennessee, and Salt Lake City, Utah [18]. Adults were enrolled in Chicago and Nashville; children were enrolled in Nashville, Memphis, and Salt Lake City. Informed consent was obtained before enrollment. The study protocol was approved by the institutional review boards at each institution and the CDC. Individuals admitted to a study hospital with evidence of acute respiratory infection and radiographic confirmation of pneumonia were included: patients who were recently hospitalized or severely immunocompromised were excluded [18]. Combined nasopharyngeal/oropharyngeal (NP/OP) swabs were obtained from all patients for molecular detection of respiratory viruses and atypical bacteria, including *M pneumoniae* [18]. Testing for *M pneumoniae* was performed using an individual real-time PCR assay designed to detect the community-acquired respiratory distress syndrome (CARDS) toxin gene and validated by the CDC Pneumonia Response and Surveillance Laboratory (PRSL) [19]. For this analysis, cases were defined as enrolled patients meeting the final CAP case definition [18] with an *M pneumoniae*-positive PCR result (crossing threshold [Ct] value <40) from a NP/OP specimen collected within 72 hours of admission. Additional respiratory and blood specimens were also collected from adults

and children (and urine for adults only) for bacterial and viral testing as previously described (Supplementary Material) [18].

Specimen Processing

After initial testing at study sites, NP/OP specimens were stored at $\leq -70^{\circ}\text{C}$ and shipped to the CDC for long-term storage. All *M pneumoniae* PCR-positive specimens were transferred to PRSL for additional molecular testing. Total nucleic acid (TNA) was extracted using the MagNA Pure Compact System with Total Nucleic Acid Isolation Kit I (Roche Applied Science) according to manufacturer's instructions. All *M pneumoniae*-positive specimens were tested at CDC using a validated multiplex PCR assay for detection of *M pneumoniae* (CARDS toxin gene), *Chlamydomphila pneumoniae*, *Legionella* spp, and human RNaseP (internal control) to confirm the initial positive *M pneumoniae* PCR result obtained at the study site [20]; no Ct value cutoff was used for confirmatory testing. Various testing methodologies, including culture, PCR, antigen detection, and serology, were performed for detection of other bacteria and viruses in other specimen types collected from these patients per the EPIC study diagnostic algorithm as previously described (Supplementary Material).

Culture

Culture was attempted on all *M pneumoniae* PCR-positive specimens using SP4 medium (Remel) as previously described to obtain isolates for testing [21]. Nucleic acid was extracted from liquid culture, and recovery of *M pneumoniae* was confirmed by individual singleplex real-time PCR assay [19].

P1 Subtyping

Genotyping of the P1 adhesin gene was attempted for all isolates recovered from culture using real-time PCR with high-resolution melt (HRM) as previously described [22]. Isolates were classified as type 1, type 2, or variant genotypes based on comparison of the HRM profile to reference strains M129 (type 1) and FH (type 2) included in each run [3, 22]. Because P1 typing of primary specimen extracts is unreliable, this assay was performed only on culture isolates using a normalized concentration of nucleic acid.

Multilocus Variable-Number Tandem-Repeat Analysis

Multilocus variable-number tandem-repeat analysis was performed on nucleic acid extracts from all primary specimens and isolates as previously described [4, 5]. Multilocus variable-number tandem-repeat analysis types were reported using the modified 4 variable-number tandem-repeat (VNTR) loci method; the Mpn1 locus was excluded due to documented instability [4, 23].

Macrolide Susceptibility

Macrolide susceptibility testing was performed on all *M pneumoniae* PCR-positive specimens and corresponding culture

Table 1. Characteristics of Patients With *Mycoplasma pneumoniae*-Positive NP/OP Specimens^a

Characteristic	Total (n = 216), n (%)	Adults (n = 40), n (%)	Children (n = 176), n (%)
Site			
Chicago	30 (13.9)	30 (75.0)	N/A ^a
Memphis	59 (27.3)	N/A	59 (33.5)
Nashville	47 (21.8)	10 (25.0)	37 (21.0)
Salt Lake City	80 (37.0)	N/A	80 (45.5)
Age Group			
0–23 months	17 (7.9)	–	17 (9.7)
2–4 years	30 (13.9)	–	30 (17.0)
5–9 years	67 (31.0)	–	67 (38.1)
10–17 years	62 (28.7)	–	62 (35.2)
18–49 years	25 (11.6)	25 (62.5)	–
50–64 years	7 (3.2)	7 (17.5)	–
65–79 years	6 (2.8)	6 (15.0)	–
≥80 years	2 (0.9)	2 (5.0)	–
Gender			
Male	124 (57.4)	19 (47.5)	105 (59.7)
Female	92 (42.6)	21 (52.5)	71 (40.3)
Race/Ethnicity			
Non-Hispanic White	131 (60.6)	22 (55.0)	109 (61.9)
Non-Hispanic Black	41 (19)	10 (25.0)	31 (17.6)
Hispanic	36 (16.7)	8 (20)	28 (15.9)
Other	8 (3.7)	0 (0)	8 (4.5)

Abbreviations: N/A, not applicable; NP/OP, nasopharyngeal/oropharyngeal.

^a Adults were enrolled at Chicago and Nashville. Children were enrolled at Nashville, Memphis, and Salt Lake City.

isolates by genotyping of the 23S rRNA gene using a real-time PCR assay with HRM analysis as previously described [7, 17]. This method allows detection of an A to G transition at position 2063 or 2064 within the 23S rRNA gene, the 2 mutations most commonly associated with macrolide resistance in *M pneumoniae* [7, 10, 24]. High-resolution melt profiles were compared with sensitive and resistant reference strains included in each run and previously confirmed by sequencing analysis and minimum inhibitory concentration determination [7]. Sequencing analysis was performed on all macrolide-resistant isolates to identify the specific single-base mutation (A2063G or A2064G) in the 23S rRNA gene [7]. Prior exposure to macrolide antibiotics, defined as azithromycin or clarithromycin received 1–13 days before enrollment, was determined by patient interviews and medical chart abstraction.

Analysis

The proportions of macrolide-resistant *M pneumoniae*, P1 subtypes, and MLVA types were compared between age groups (adults vs children), enrollment city, and select clinical characteristics

Table 2. Molecular Characteristics of *Mycoplasma pneumoniae* by Age Group

Characteristic	Total (n = 216), n (%)	Adults (n = 40), n (%)	Children (n = 176), n (%)	P Value ^a
Macrolide profile^b				
Sensitive	195 (96.5)	32 (97.0)	163 (96.4)	1.0
Resistant	7 (3.5)	1 (3.0)	6 (3.6)	
P1 genotype^c				
Type 1	142 (81.1)	18 (64.3)	124 (84.4)	.02
Type 2	29 (16.6)	10 (35.7)	19 (12.9)	
Variant	4 (2.3)	0 (0)	4 (2.7)	
MLVA type^d				
4/5/7/2	149 (71.6)	18 (48.6)	131 (76.6)	<.01
3/5/6/2	33 (15.9)	13 (35.1)	20 (11.7)	
Other	26 (12.5)	6 (16.2)	20 (11.7)	
3/4/6/2	1 (0.5)	1 (2.7)	0 (0)	
3/6/6/2	9 (4.3)	2 (5.4)	7 (4.1)	
4/0/7/2	2 (1.0)	0 (0)	2 (1.2)	
4/5/6/2	2 (1.0)	1 (2.7)	1 (0.6)	
4/5/7/0	5 (2.4)	1 (2.7)	4 (2.3)	
4/6/7/2	6 (2.9)	1 (2.7)	5 (2.9)	
5/5/7/0	1 (0.5)	0 (0)	1 (0.6)	

Abbreviations: MLVA, multilocus variable-number tandem-repeat analysis.

^a The χ^2 test or Fisher's exact test as appropriate comparing children with adults.

^b Macrolide profile could not be determined for 14 (6.5%) of 216 specimens, including 7 (17.5%) of adults and 7 (4%) of children, due to poor amplification of target sequence from primary specimen and/or lack of isolate recovery.

^c P1 genotype was determined for culture isolates only (n = 175).

^d MLVA type could not be determined for 8 (3.7%) of 216 specimens, including 3 (7.5%) of adults and 5 (2.8%) of children, due to poor amplification of target sequence from primary specimen and/or lack of isolate recovery. Other types shown were grouped for statistical comparison with the predominant types 4/5/7/2 and 3/5/6/2.

using χ^2 or Fisher's exact test as appropriate. All analyses were conducted using SAS version 9.3 (Cary, NC); $P < .05$ was considered significant.

RESULTS

Characteristics of Patients With *Mycoplasma pneumoniae* Community-Acquired Pneumonia

Among 225 *M pneumoniae* PCR-positive specimens received from the study hospitals, 9 (4%) were negative for *M pneumoniae* upon repeat real-time PCR testing at the CDC and were excluded (Supplementary Figure 1). *Mycoplasma pneumoniae* isolates were recovered by culture from 175 (81%) of the 216 confirmed PCR-positive specimens (Supplementary Figure 1). Among 216 patients having an *M pneumoniae* PCR-positive NP/OP specimen, 24.5% had another bacterial or viral pathogen detected, including 7.5% of adults and 28.5% of children

Table 3. Molecular Characteristics of *Mycoplasma pneumoniae* by Site

Characteristic	Chicago (n = 30), n (%)	Memphis (n = 59), n (%)	Nashville (n = 47), n (%)	Salt Lake City (n = 80), n (%)	P Value ^a
Macrolide profile ^b	n = 23	n = 59	n = 46	n = 74	.3
Sensitive	23 (100)	56 (94.9)	43 (93.5)	73 (98.6)	
Resistant	0 (0)	3 (5.1)	3 (6.5)	1 (1.4)	
P1 genotype ^c	n = 20	n = 56	n = 33	n = 66	<.01
Type 1	12 (60.0)	42 (75.0)	26 (78.8)	62 (93.9)	
Type 2	8 (40.0)	11 (19.6)	6 (18.2)	4 (6.1)	
Variant	0 (0)	3 (5.6)	1 (3.0)	0 (0)	
MLVA type ^d	n = 28	n = 57	n = 44	n = 79	<.01
4/5/7/2	13 (46.4)	43 (75.4)	30 (68.2)	63 (79.7)	
3/5/6/2	10 (35.7)	10 (17.5)	9 (20.5)	4 (5.1)	
Other	5 (17.9)	4 (7.0)	5 (11.4)	12 (17.7)	
3/4/6/2	1 (3.6)	0 (0)	0 (0)	0 (0)	
3/6/6/2	2 (7.1)	4 (7.0)	3 (6.8)	0 (0)	
4/0/7/2	0 (0)	0 (0)	0 (0)	2 (2.5)	
4/5/6/2	0 (0)	0 (0)	2 (4.5)	0 (0)	
4/5/7/0	1 (3.6)	0 (0)	0 (0)	4 (5.1)	
4/6/7/2	1 (3.6)	0 (0)	0 (0)	5 (6.3)	
5/5/7/0	0 (0)	0 (0)	0 (0)	1 (1.3)	

Abbreviations: MLVA, multilocus variable-number tandem-repeat analysis.

^a The χ^2 test or Fisher's exact test comparing all 4 cities.

^b Macrolide profile could not be determined for 14 (6.5%) of 216 specimens due to poor amplification of target sequence from primary specimen and/or lack of isolate recovery.

^c P1 genotype was determined for isolates only (n = 175).

^d MLVA type could not be determined for 8 (3.7%) of 216 specimens due to poor amplification of target sequence from primary specimen and/or lack of isolate recovery. Other types shown were grouped for statistical comparison with the predominant types 4/5/7/2 and 3/5/6/2.

(Supplementary Table 1). *Chlamydia pneumoniae* and *Legionella* spp were not detected in any specimens upon confirmatory PCR testing at the CDC.

Of the 216 *M pneumoniae* PCR-positive specimens included in this analysis, 40 (18.5%) were obtained from adults and 176 (81.5%) were from children (Table 1). Among these patients, 4 (10.0%) of 40 adults and 19 (10.8%) of 176 children required intensive care unit (ICU) admission; there were no deaths. No adults and 3 (1.7%) of 176 children required invasive mechanical ventilation. Median length of stay was 2.5 days for adults (interquartile range [IQR], 1.5–4) and 2.0 days (IQR, 2.5–4) for children (data not shown).

P1 Genotyping and Multilocus Variable-Number Tandem-Repeat Analysis

The 2 main P1 genotypes of *M pneumoniae*, types 1 and 2, accounted for 81.1% and 16.6% of total cultured isolates (n = 175), respectively (Table 2). Type 1 accounted for the majority of detections across all ages, including 64.3% of detections in adults and 84.4% in children, whereas type 2 accounted for 35.7% in adults and 12.9% in children ($P < .01$). Four variant strains (2.3%) were also identified, all in pediatric specimens.

Multilocus variable-number tandem-repeat analysis type could not be determined for 8 (3.7%) of 216 specimens due to poor amplification of the target sequence from the primary specimen and lack of isolate recovery (Supplementary Figure 1). Nine distinct MLVA types were identified among the remaining 208 *M pneumoniae* PCR-positive specimens (Tables 2 and 3). The majority (71.6%) of detections were type 4/5/7/2, including 48.6% of adults and 76.6% of children. Type 3/5/6/2 was the second most commonly identified MLVA type in both adults (35.1%) and children (11.7%). There were no significant differences in ICU admission, invasive mechanical ventilation, length of stay, or the proportion of other bacterial or viral pathogen detections among P1 genotypes or MLVA types (Supplementary Tables 1 and 2).

The distribution of P1 genotypes and MLVA types differed significantly between adults and children ($P < .05$; Table 2) and among sites ($P < .01$, Table 3). Multilocus variable-number tandem-repeat analysis type 4/5/7/2 predominated at each site across the entire study period except in Chicago (adults only) where type 3/5/6/2 was also common (Figure 1 and Table 3). The proportions of MLVA types 4/5/7/2, 3/5/6/2, and other types were similar in adults from Nashville and Chicago (Table 3 and Supplementary Figure 2); P1 type distribution was also

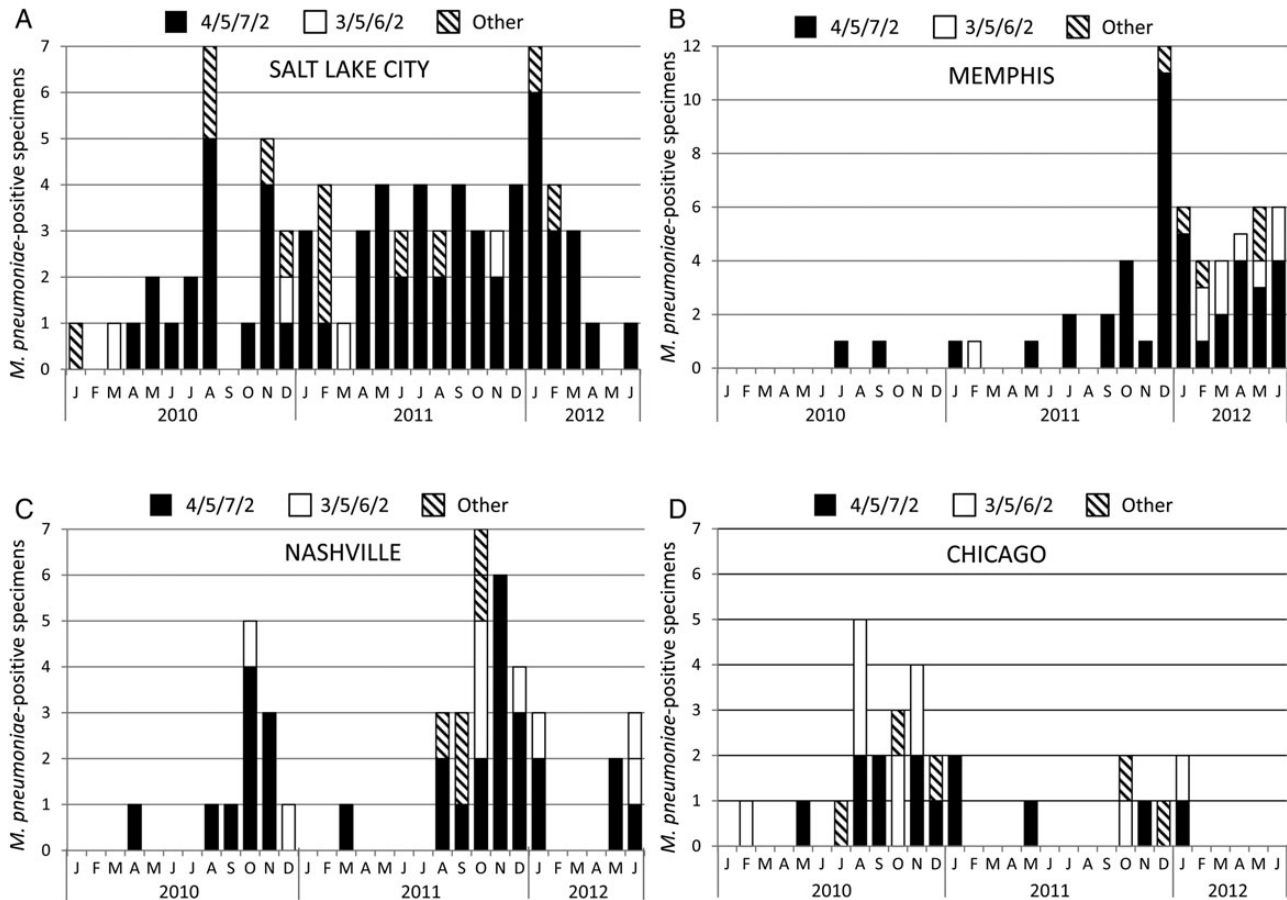


Figure 1. Number of *Mycoplasma pneumoniae* detections of the 2 predominant multilocus variable-number tandem-repeat analysis (MLVA) types, 4/5/7/2 (black bars) and 3/5/6/2 (white bars), and other types (hashed bars) from children in Salt Lake City (A) and Memphis (B), children and adults in Nashville (C), and adults in Chicago (D) over the study period. Other category includes the following: 3/6/6/2, 3/4/6/2, 4/0/7/2, 4/5/6/2, 4/5/7/0, 4/6/7/2, and 5/5/7/0. Note that the y-axis scale for Memphis (B) differs from the other graphs. Detections of each MLVA type in adults and children in Nashville are shown separately in [Supplementary Figure 2](#).

similar among adults at these 2 sites (Chicago: 60% type 1 and 40% type 2; Nashville: 75% type 1 and 25% type 2). Likewise, the proportions of P1 types (80% type 1, 16% type 2, 4% variant) and MLVA types ([Supplementary Figure 2](#)) among children enrolled in Nashville were similar to those observed at the other pediatric enrollment sites (Table 3). In Nashville, the only site enrolling both adults and children, there were no significant differences in the proportions of MLVA types or P1 types between age groups. Two MLVA types, 4/0/7/2 ($n = 2$) and 5/5/7/0 ($n = 1$), were identified only in children, whereas type 3/4/6/2 was identified in a single adult specimen (Table 2). Three novel types were identified, including 4/5/7/0 and 5/5/7/0, which lacked the Mpn16 locus, and 4/0/7/2, which lacked the Mpn14 locus. Multilocus variable-number tandem-repeat analysis results were confirmed by repeating the test with a normalized concentration of TNA from the culture isolate to ensure that the lack of amplification of an individual locus was not due to limited nucleic acid in the primary specimen.

Macrolide Susceptibility

The macrolide susceptibility genotype could not be determined for 14 (6.5%) of the 216 *M pneumoniae* PCR-positive specimens, including 7 (17.5%) adults and 7 (4%) children, due to poor amplification of the target sequence from the primary specimen and inability to culture the organism ([Supplementary Figure 1](#)). Of the remaining 202 specimens, 195 (96.5%) were sensitive to macrolides and 7 (3.5%) were resistant (Table 2). The proportions of predominant MLVA types were similar among resistant isolates (86% type 4/5/7/2 and 14% type 3/6/6/2) and all specimens (72% type 4/5/7/2 and 16% type 3/6/6/2) (Tables 2 and 4). Sequencing analysis revealed the presence of the A2063G mutation in 6 isolates and A2064G in 1 isolate.

The proportion of resistant *M pneumoniae* did not differ significantly between adults (3.0%) and children (3.6%). Characteristics of the 7 macrolide-resistant isolates are shown in Table 4. At least 1 resistant isolate was identified at all 3 pediatric sites (Table 3). Five (71%) of 7 resistant isolates were

Table 4. Characteristics of Macrolide-Resistant *Mycoplasma pneumoniae* (n = 7)

Site	n (%)
Chicago	0 (0)
Memphis	3 (43)
Nashville	3 (43)
Salt Lake City	1 (14)
Specimen collection year	
2010	1 (14.3)
2011	5 (71.4)
2012	1 (14.3)
Patient age	
0–23 months	1 (14)
2–4 years	0 (0)
5–9 years	2 (29)
10–17 years	3 (43)
18–49 years	1 (14)
≥50 years	0 (0)
Timing of macrolide relative to specimen collection	
Before	5 (71%)
After ^a	2 (29%)
23S rRNA genotype	
A2063G	6 (86)
A2064G	1 (14)
P1 genotype	
Type 1	6 (86)
Type 2	0 (0)
Variant	1 (14)
MLVA type	
3/6/6/2	1 (14)
4/5/7/2	6 (86)

Abbreviations: MLVA, multilocus variable-number tandem-repeat analysis.

^a Includes macrolide received on same day as specimen collection (n = 2).

recovered from patients who received a macrolide for the current illness before specimen collection compared with 44 (23%) of 195 susceptible specimens ($P < .01$). Among patients with resistant *M pneumoniae* isolates, none required ICU admission or invasive mechanical ventilation, and median length of stay was 3.0 days (IQR, 1–6) compared with 2.0 days (IQR, 2–4) for patients with macrolide-sensitive *M pneumoniae* (Supplementary Table 2).

DISCUSSION

This study is a comprehensive molecular analysis of *M pneumoniae*-associated CAP in both children and adults at multiple sites within the United States. P1 type 1 and MLVA type 4/5/7/2 were most common in our study, which is consistent with recent reports from the United States and other regions of the world [17, 23]. A single MLVA type, 4/5/7/2, predominated in

children, whereas 2 MLVA types, 4/5/7/2 and 3/5/6/2, were common in adults. In addition, in this study of hospitalized US adults and children using a strict definition of CAP, *M pneumoniae* macrolide genotypic resistance was low. No significant differences in clinical characteristics were identified among patients with varying strain types or between macrolide-resistant and sensitive *M pneumoniae* infections.

This study occurred simultaneously with a reported increase in *M pneumoniae* transmission in Europe and Asia during 2010–2012 [23, 25]. Four *M pneumoniae* outbreaks in the United States and several sporadic cases or clusters were investigated by CDC in 2013, which was an increase from previous years [17]. Increased detection of *M pneumoniae* in the United States could be partially explained by broader implementation of molecular diagnostics in clinical settings [26, 27]. However, reports of increased detections on multiple continents suggest that 2010–2013 represented a period of increased *M pneumoniae* transmission, which occur regularly every 3 to 7 years [1, 28]. Although this periodicity of *M pneumoniae* has been attributed to continual re-emergence of the less prevalent P1 type due to waning immunity within the population over time [28], numerous recent reports have described co-circulation of multiple P1 and MLVA types within a population or during an outbreak [5, 17, 29, 30]. Co-circulation of the 2 main P1 types was also observed in each city in the current study. Implementation of a systematic surveillance program incorporating molecular characterization may be useful for determining whether changes in *M pneumoniae* disease burden correlate with changes in distribution of strain types within the population.

Using a modified typing method based on 4 VNTR loci, 15 unique MLVA types have been described to date [23]. The most prevalent MLVA types among EPIC specimens were 4/5/7/2 and 3/5/6/2, which is consistent with recent analyses of circulating MLVA types within the United States and internationally [4, 5, 23, 25, 31–33]. Significant differences were identified in the proportions of MLVA types and P1 types between adults and children as well as among sites: MLVA type 4/5/7/2 was predominant in all age groups and at all sites, but 3/5/6/2 was more common in adults than children. However, in Nashville, the only site enrolling both adults and children, there were no significant differences in the proportions of MLVA types or P1 types between age groups. Thus, the observed differences in distribution of strain types may be attributed to geography, patient age, or a combination of factors. Although co-detections of other bacterial or viral or pathogens were observed, mostly in children, no differences in presence of co-detections were observed based on P1 genotype or MLVA type.

A correlation between the 2 main typing methods has previously been demonstrated such that P1 type can be predicted by MLVA profile; type 4/5/7/2 strains are P1 type 1, whereas type 3/5/6/2 strains reliably have the P1 type 2 genotype [4, 17, 34]. This correlation was also observed among *M pneumoniae*

PCR-positive specimens in the current study (data not shown). Therefore, MLVA typing may have greater utility because it affords the ability to further distinguish strains while still predicting P1 type. Several previously unreported MLVA types lacking either the Mpn14 or Mpn16 locus were discovered in this analysis. Further sequence analysis of these strains is needed to identify whether the entire locus is deleted or otherwise modified and what implications this may have for pathogenesis, transmission, or disease outcomes. Furthermore, no significant differences in clinical characteristics were observed among this cohort of patients based on strain type. Our results indicate that current typing methods are insufficient to meaningfully differentiate *M pneumoniae* because the majority of strains can be categorized into only a few main types, none of which have been identified as clinically informative. Whole genome sequencing of *M pneumoniae* may result in improved methods for strain characterization.

Macrolide resistance was identified in only 3.5% of *M pneumoniae* PCR-positive specimens in this study. This prevalence is consistent with the previously reported prevalence of $\leq 10\%$ seen during surveillance studies and outbreak investigations in the United States, although few reports are available for comparison [6, 16, 35]. Previous reports were limited in sample size and geographic coverage, were based on clinical testing practices, and included both hospitalized patients and those with mild disease [16, 17]. Our prospective, population-based analysis included 4 geographically distinct cities enrolling adults, children, or both age groups, and thus it represents the most comprehensive examination of macrolide resistance among hospitalized patients with *M pneumoniae* CAP in the United States to date.

The proportions of macrolide-resistant *M pneumoniae* reported in the United States are generally consistent with those observed from routine surveillance in northern Europe, varying between 3% and 10% [25, 29, 36]. In contrast, macrolide resistance dramatically increased in Japan and China during the past decade to over 90% in some reports, underscoring the potential for rapid emergence of antimicrobial resistance within *M pneumoniae* [11, 14, 15, 37]. Efforts to reduce unnecessary antibiotic prescribing and inappropriate antibiotic selection for respiratory infections could help prevent emergence of widespread macrolide resistance in North America [38]. Although only 7 resistant isolates were identified, macrolide resistance was associated with recent receipt of a macrolide before study enrollment, supporting the theory that a resistant subpopulation may develop or expand during the course of macrolide therapy within an individual patient [13, 39, 40]. The proportions of predominant MLVA types were similar between resistant and susceptible isolates, suggesting that resistance is not more likely to develop in a specific MLVA type. Further laboratory and epidemiological studies are needed to understand the effect of macrolide exposure and potential mechanisms for selection or development of resistant *M pneumoniae* in response to macrolide treatment.

There are several limitations to this analysis. Inadequate amplification of target regions resulting in inconclusive MLVA and macrolide genotyping was most likely due to low quantity of pathogen-specific nucleic acid in the primary specimen as suggested by high Ct values upon initial specimen testing (Supplementary Figure 1). Although this was a multicenter study, the microbiological characteristics of *M pneumoniae*, including the prevalence of macrolide resistance, may differ in other regions of the United States not represented in our study. Furthermore, enrollment of adults and children occurred only at 1 of the 4 sites, precluding definitive attribution of significant differences in strain type distribution to either age or geography. Macrolide susceptibility testing was performed on *M pneumoniae*-positive specimens collected within 72 hours of admission. Thus, resistance mutations induced as a result of subsequent treatment were not detected.

This study provides a deeper understanding of *M pneumoniae* biology, molecular epidemiology, and macrolide resistance among patients hospitalized with CAP. Further investigation is also warranted to understand the biological and epidemiological reasons that may explain the differences in distribution of *M pneumoniae* types between adult and pediatric populations. Investigation of *M pneumoniae* using next-generation sequencing may (1) provide further characterization and insight into the evolution of *M pneumoniae* within the human host population and (2) afford the opportunity to improve upon current typing methods. Further studies are warranted to understand the full spectrum of *M pneumoniae* illness, monitor the emergence of antibiotic resistance, and define specific microbial determinants of pathogenesis.

Supplementary Material

Supplementary material is available online at *Open Forum Infectious Diseases* (<http://OpenForumInfectiousDiseases.oxfordjournals.org/>).

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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