AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EPIDEMIOLOGY AND SURVEILLANCE

Macrolide Resistance in *Mycoplasma pneumoniae*, Midwestern United States, 2014 to 2021

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ABSTRACT The epidemiology of macrolide resistance in *Mycoplasma* (*Mycoplasmoides*) *pneumoniae* in the United States is incompletely described. Using a PCR assay targeting common mutations associated with macrolide resistance in *M. pneumoniae* (23S rRNA gene, A2063G/A2064G), the frequency of macrolide resistance was estimated to be 10% based on analysis of 114 samples tested from January 2014 to September 2021 at Mayo Clinic Laboratories. Seasonality data showed the highest rates of *M. pneumoniae* infection in the fall/early winter.

KEYWORDS *Mycoplasma pneumoniae*, antibiotic resistance, macrolides, pneumonia, surveillance studies

Mycoplasma (*Mycoplasmoides*) *pneumoniae* causes upper respiratory tract infection, alongside an estimated 20% to 40% of community-acquired pneumonia cases in the general population (1, 2). While an estimated 2 million cases of *M. pneumoniae* infection occur annually in the United States (3), the true extent of this infection is incompletely defined due to mild symptoms in a majority of infections, similarity of *M. pneumoniae*-associated symptoms to those of other respiratory pathogens (including viruses), and lack of widespread testing, reporting, and surveillance (4). Although historically serologic testing has been the main approach to *M. pneumoniae* diagnosis, nucleic acid amplification tests are now the preferred tests (5–7).

Macrolides are a common treatment for respiratory tract infections and a recommended treatment for *M. pneumoniae*. However, macrolide resistance in *M. pneumoniae* has been increasing for over 2 decades and is reported to be as high as 90% in some areas in Japan and China and 30% in areas in Europe (5). Worldwide studies of the prevalence of macrolide-resistant *M. pneumoniae* were amalgamated by Waites et al. in a study published in 2017 (5); however, there was limited data on macrolide-resistant *M. pneumoniae* prevalence in the United States. In 2019, Waites et al. reported macrolide resistance surveillance data between 2015 and 2018 from 8 states; the overall prevalence of macrolide-resistant *M. pneumoniae* was 8% (27/360 specimens) and was highest in southern and eastern portions of the United States (15 to 21%) (4). Additionally, Lanata et al. reported the prevalence of macrolide resistance in *M. pneumoniae* as 3% (14/498) based on *M. pneumoniae*-positive samples collected in central Ohio from 2015 to 2019 (8). The current study aimed to supplement United States prevalence data by assessing *M. pneumoniae* macrolide resistance in a convenience sample of specimens that tested positive for *M. pneumoniae* at Mayo Clinic Laboratories in Rochester, Minnesota. In addition, the performance of a PCR assay designed to detect macrolide resistance in *M. pneumoniae* by targeting the 23S rRNA gene was compared to a previously described *M. pneumoniae* assay targeting *ptsI* (9). Finally, seasonality of *M. pneumoniae* PCR test positivity was assessed.

From January 2014 to September 2021, 27,645 patient samples were tested for *M. pneumoniae* at Mayo Clinic Laboratories using a PCR assay targeting *ptsI* (9). Overall assay positivity was 2% (410/27,645) (Fig. 1). The highest rates of *M. pneumoniae* infection generally

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The authors declare a conflict of interest. Dr. Patel reports grants from Merck, ContraFect, TenNor Therapeutics Limited and Shionogi. Dr. Patel is a consultant to Curetis, Specific Technologies, Next Gen Diagnostics, PathoQuest, Selux Diagnostics, 1928 Diagnostics, PhAST, and Qvella; monies are paid to Mayo Clinic. Dr. Patel is also a consultant to Netflix. In addition, Dr. Patel has a patent on *Bordetella pertussis/parapertussis* PCR issued, a patent on a device/method for sonication with royalties paid by Samsung to Mayo Clinic, and a patent on an anti-biofilm substance issued. Dr. Patel receives an editor's stipend from IDSA, and honoraria from the NBME, Up-to-Date and the Infectious Diseases Board Review Course. Scott A. Cunningham reports receiving an honorarium from the Antibacterial Resistance Leadership Group.

Received 29 December 2021
Returned for modification 3 February 2022
Accepted 26 February 2022
Published 21 March 2022

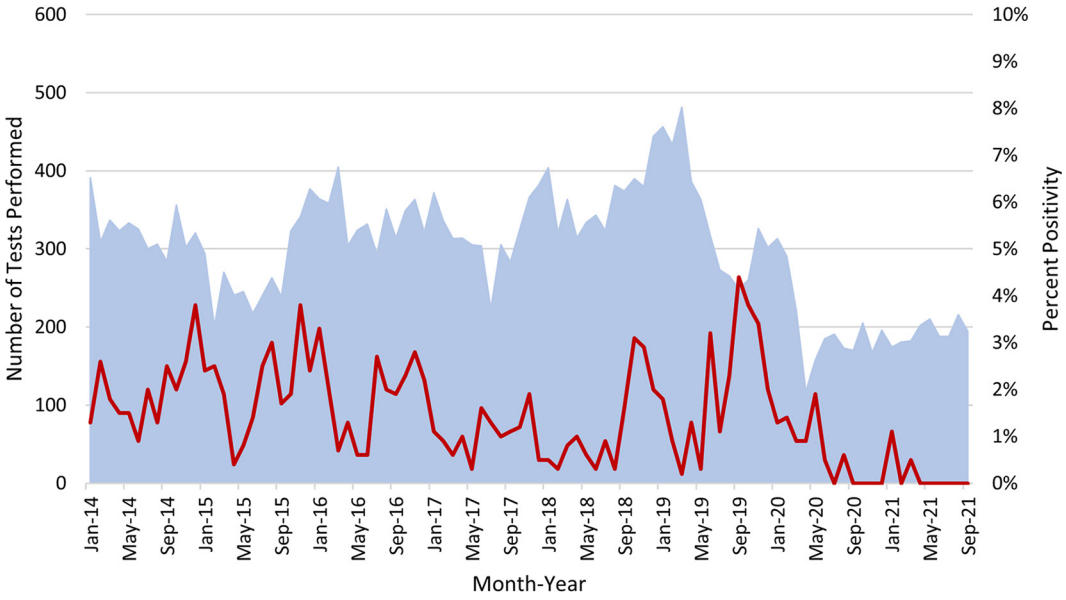

FIG 1 *M. pneumoniae ptsI* PCR tests performed from January 2014 through November 2021 showing total numbers of monthly tests performed (blue background) and monthly percent positivity (red line). There was typically a rise in positivity in late fall/winter, except for COVID-19 pandemic times and, to some extent, the 2017–2018 season. Between February 2017 and August 2018, the overall positivity rate was 1% (52/6,238). Peak annual positivity rates were observed in December 2014 (4%), November 2015 (4%), January 2016 (3%), June 2017 (2%), October 2018 (3%), and September 2019 (4%). Macrolide resistant isolates were found in 2014 (n = 1), 2015 (n = 3), 2016 (n = 3), and 2019 (n = 4), but notably, not all specimens testing positive for *M. pneumoniae* were assessed for macrolide resistance.

occurred in the fall/early winter, with peak positivity rates of 3 to 4% between September and January (apart from 2017 to 2018). After April 2020, overall testing and percent positivity rates were lower than those in antecedent times due to the COVID-19 pandemic. Of the 410 positive specimens, 142 (respiratory swabs [n = 92], lower respiratory samples [n = 47], pleural fluids [n = 2], and cerebrospinal fluid [n = 1]) were archived and available for further study.

Macrolide resistance in *M. pneumoniae* corresponds to mutations in the 23S rRNA gene, of which A2063G and A2064G are the most common and confer high-level macrolide resistance (5). A real-time PCR assay using fluorescent resonance energy transfer (FRET) hybridization probes targeting the 23S rRNA gene was designed to detect *M. pneumoniae* and predict macrolide resistance (by assessing A2063G and A2064G) (Table 1) (10). Specimens were extracted on a MagNA Pure 96 (Roche Diagnostics, Indianapolis, IN) and

TABLE 1 Primer and probe sequences used for the real-time PCR assay targeting the *M. pneumoniae* 23S rRNA gene[a]

Sequence type	Sequence
Primers	
Forward (F1-Mpn)	5′-GAAGGAGGTTAGCGCAA-3′
Reverse (R2Mpn)	5′-TTCTCTACATGATAATGTCC-3′
Probes	
FL3	5′-CGGGACGGAAAGACCCCGTG-FL-3′
LC3[b]	5′-LC610-AGCTTTACTGTAGC+T+TAA+TA+T+TGA-PO4-3′
Recovery template probes	
FL	5′-GGTGCCGTTCACTTCCCGAATAAC-FL-3′
LC	5′-LC670-CGGATATTTTTGATCTGACCGAAGCG-PO4-3′

[a]A 262-bp region of the 23S rRNA gene of *M. pneumoniae* (121917 to 122178 of GenBank accession number U00089) was targeted using primers and fluorescence resonance energy transfer hybridization probes (set no. 4903; 10× concentration; TIB MolBio, Aldelphia, NJ). Target donor and acceptor probes were synthesized by TIB MolBiol (LCMPRP no. 4903) and labeled with fluorescein and LightCycler Red 610, respectively. A recovery template was added to the master mix to monitor for PCR inhibition. The recovery template was amplified with the same primers used to amplify *M. pneumoniae* with the amplification region internal to the primers replaced with sequence complementary to the recovery template probes. Recovery template donor and acceptor probes were synthesized by TIB MolBiol and labeled with fluorescein and LightCycler Red 670, respectively.
[b]+, locked nucleic acid.

TABLE 2 Comparison of results of PCR assays targeting the *ptsI* and 23S rRNA gene targets

M. pneumoniae assay parameter	*ptsI* gene target		McNemar's test *P* value
	Positive	Negative	
23S rRNA gene target (no.)			
Positive	114	0	0.00006
Negative	15[a]	13	
Total	129	13	
Positive agreement (%)	88		
Negative agreement (%)	100		

[a]Includes 12 respiratory swabs (nasopharyngeal, throat, and nasal), 1 bronchoalveolar lavage fluid sample, 1 pleural/pericardial fluid sample, and 1 sputum sample.

amplified on a LightCycler 480 (Roche); melting temperature analysis was used to predict macrolide resistance. The wild-type genotype displays a melting temperature of 66.5 ± 2°C, whereas in the presence of A2063G or A2064G, the melting temperature is 60.5 ± 2°C. All PCR-positive samples were subjected to bidirectional Sanger sequencing through reamplification of extracted product for macrolide resistance genotype confirmation. A recovery template was used to monitor for potential PCR inhibition.

A previously described assay targeting *ptsI* (9) was compared to the assay targeting the 23S rRNA gene (Table 2). Notably, 13 previously positive archived samples tested negative by both PCR assays, suggesting sample degradation during prolonged storage. A total of 129 samples tested positive using the *ptsI* assay, of which only 114 were positive using the 23S rRNA gene assay; all 15 discrepant samples had crossing threshold (Ct) values of >35 cycles via *ptsI* gene detection. No PCR inhibition was detected.

Macrolide resistance prediction for all samples detected by the 23S rRNA gene PCR assay was assessed through melting temperature analysis and confirmed using bidirectional Sanger sequencing. Of the 114 samples tested, 11 (10%) were predicted and confirmed to have A2063G ($n = 9$) or A2064G ($n = 2$) mutations. Geographical location of positive samples by state is illustrated in Fig. 2, with demographic data presented in Table 3. All predicted mac-

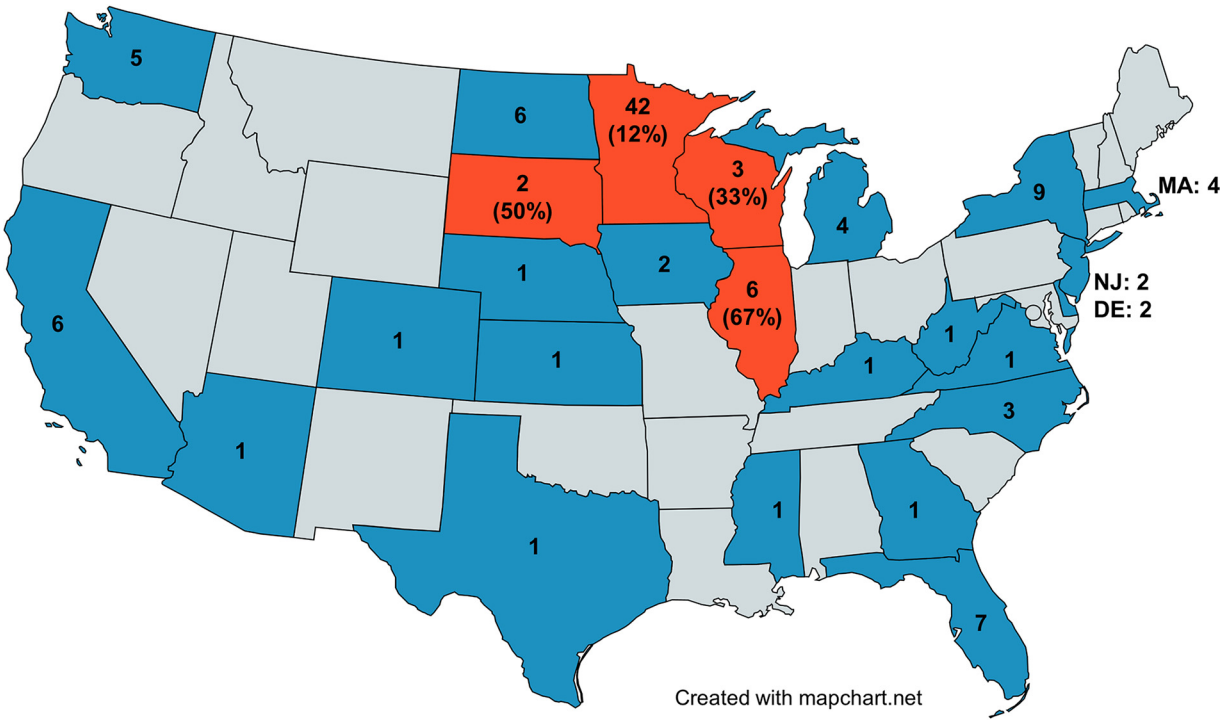

FIG 2 Geographic locations of patients testing positive for *M. pneumoniae*. Shown is the total number of positive tests by state. States shown in gray had no positive results; states shown in dark blue or orange had positive results. Macrolide-resistant *M. pneumoniae* was detected in states shown in orange, with the percentage of macrolide-resistant *M. pneumoniae* listed below the specimen numbers.

TABLE 3 Patient demographics by sex and age, alongside specimen type, according to macrolide resistance for those testing positive by both the *ptsI* and 23S rRNA gene targets

Demographic and population	No. (%) of patients infected with:		Fisher exact test *P* value
	Macrolide-resistant *M. pneumoniae* (*n* = 11)	Macrolide-susceptible *M. pneumoniae*[a] (*n* = 103)	
Sex			
Female	5 (45)	43 (42)	1.0
Male	6 (55)	59 (58)	
Age (in yr)			
≤18 (*n* = 73)	6 (55)	67 (65)	0.5
≥19 (*n* = 41)	5 (45)	36 (35)	
Mean ± SD (range) age, yr	25 ± 18 (8–63)	22 ± 18 (2–78)	0.3[b]
Specimen type			
Respiratory swabs (nasopharyngeal, nasal, throat)	6 (8)	74 (92)	0.1
Bronchoalveolar lavage fluids/bronchial washings	2 (13)	15 (87)	
Sputum/tracheal secretions	2 (8)	24 (92)	
Cerebrospinal fluid	1 (100)	0 (0)	

[a]Sex of one patient was unknown.
[b]Wilcoxon rank sum test.

rolide-resistant specimens were from the Midwest (Minnesota [*n* = 5], Illinois [*n* = 4], South Dakota [*n* = 1], Wisconsin [*n* = 1]), as were most *M. pneumoniae*-positive specimens.

Despite study limitations of sample selection for convenience (and not being uniformly spread across the United States or time), lack of culture and phenotypic susceptibility testing, and small sample size (many previously positive specimens were unavailable for testing), there are three findings of this study. First, the overall test positivity for *M. pneumoniae* was highest in the fall/early winter and positivity rates fell during the COVID-19 pandemic, consistent with findings from prior studies (3, 5, 11). Second, although it is possible to accurately detect macrolide resistance in *M. pneumoniae* using a real-time PCR approach, the described 23S rRNA gene assay is less likely to qualitatively detect *M. pneumoniae* in clinical specimens than the previously described assay targeting *pts1* (Table 2). This suggests that the former should not be used as a standalone assay for *M. pneumoniae* detection but instead performed on samples testing positive using an assay that more often tends to detect *M. pneumoniae*. Third, the prevalence of macrolide-resistant *M. pneumoniae* was 10% overall (and 12% in Minnesota, the state with the largest number of samples tested). If macrolides are used to treat *M. pneumoniae*, testing for macrolide susceptibility should be considered; in addition, continued surveillance for macrolide-resistant *M. pneumoniae* should be performed the United States.

ACKNOWLEDGMENTS

We thank the Bacteriology Laboratory at Mayo Clinic, Rochester, MN, for testing and collecting *M. pneumoniae*-positive patient samples and Matthew Wolf and Seanne Buckwalter for assistance with 23S rRNA gene assay design.

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