Detection and Characterization of *Mycoplasma pneumoniae* during an Outbreak of Respiratory Illness at a University

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An outbreak at a university in Georgia was identified after 83 cases of probable pneumonia were reported among students. Respiratory specimens were obtained from 21 students for the outbreak investigation. The TaqMan array card (TAC), a quantitative PCR (qPCR)-based multipathogen detection technology, was used to initially identify *Mycoplasma pneumoniae* as the causative agent in this outbreak. TAC demonstrated 100% diagnostic specificity and sensitivity compared to those of the multiplex qPCR assay for this agent. All *M. pneumoniae* specimens \((n = 12)\) and isolates \((n = 10)\) were found through genetic analysis to be susceptible to macrolide antibiotics. The strain diversity of *M. pneumoniae* associated with this outbreak setting was identified using a variety of molecular typing procedures, resulting in two P1 genotypes \((60\% \text{ and } 40\%\) ) and seven different multilocus variable-number tandem-repeat analysis (MLVA) profiles. Continued molecular typing of this organism, particularly during outbreaks, may enhance the current understanding of the epidemiology of *M. pneumoniae* and ultimately lead to a more effective public health response.

*Mycoplasma pneumoniae* infections account for 20 to 40% of community-acquired pneumonia (CAP) cases in certain populations \((1, 2)\). Infection is often characterized by relatively long incubation periods and a wide spectrum of clinical symptoms and disease manifestations \((3)\). Infections may occur in the upper and lower respiratory tracts and in some cases can lead to extrapulmonary manifestations without obvious respiratory disease \((4)\). Illness is frequently mild and self-limiting, but it can result in pneumonia in an estimated 30% of cases and may occasionally lead to severe complications requiring hospitalization \((2)\). The true incidence of *M. pneumoniae* infection in the community remains unknown because patients are often treated empirically, and the clinical syndrome overlaps with other pneumonia etiologies. The limited availability of reliable diagnostic tests further complicates the accurate determination of disease burden.

Molecular assays, such as quantitative PCR (qPCR), have demonstrated utility in outbreak settings due to their high sensitivities and specificities and their relatively short time to results \((<1 \text{ day})\) compared to those of other diagnostics, such as serology and culture \((5)\). Multipathogen qPCR diagnostics are a more recently developed tool that can be extremely helpful in unknown respiratory outbreak scenarios where a variety of etiologies may be suspected as the cause \((6)\). The TaqMan array card (TAC), a microfluidic platform designed for the simultaneous detection of up to 48 pathogen targets using singleplex qPCRs, has been used in respiratory disease outbreak investigations \((6)\).

The current report describes the laboratory detection and characterization of *M. pneumoniae* during an outbreak investigation conducted by the Georgia Department of Public Health with assistance from the Centers for Disease Control and Prevention (CDC) and the Fulton County Department of Health and Wellness. Molecular diagnostics, including a multiplex qPCR assay and the TAC, were used to identify *M. pneumoniae* as the etiology of the outbreak. Molecular typing assays were also performed on specimens and isolates to provide greater characterization and epidemiological information.

**MATERIALS AND METHODS**

**Case identification, specimen collection, and nucleic acid extraction.** The details of the epidemiological investigation and assessment of outbreak communications efforts were reported previously \((7)\). Active surveillance for probable pneumonia cases, including retrospective chart review, began at the University Health Services clinic on 17 October 2012. Probable pneumonia cases were categorized as radiologically or clinically diagnosed pneumonia, occurring in a university student, with onset between September and December 2012. Nasal or nasopharyngeal (NP) and/or oropharyngeal (OP) swab specimens were obtained from the students identified as probable pneumonia cases or presenting with unexplained respiratory symptoms and who agreed to be tested. One follow-up swab was collected approximately 5 weeks after the initial swab was documented as a laboratory-confirmed *M. pneumoniae* case. NP/OP swabs were placed in 2 ml of universal transport medium (UTM) and transported at 4°C to the Pneumonia Response and Surveillance Laboratory at the CDC. Total nucleic acid (TNA) was extracted from 200 μl of UTM from each swab specimen and eluted into 100 μl using a MagNA Pure compact total nucleic acid isolation kit (Roche Applied Science) in accordance with the manufacturer instructions.

**Use of the TaqMan array card for molecular detection of respiratory pathogens.** All patient specimens and a single follow-up swab specimen \((n = 22)\) were tested using the TAC. The first shipment of specimens \((n = 22)\) were tested using the TAC.
4) submitted to the CDC were screened using the TAC in order to identify the etiology of the outbreak; testing was continued on subsequent specimens in order to evaluate possible codetections and to compare the TAC to the laboratory standard qPCR assay for \textit{M. pneumoniae} detection. Briefly, 50 \( \mu \)l specimen TNA was combined with 50 \( \mu \)l of 1 \( \times \) qScript XLT one-step reverse transcription (RT)-qPCR ToughMix, Low ROX (Quanta Biosciences, Gaithersburg, MD) for the reaction mixture. A no-template control (NTC) and a positive control consisting of combined RNA transcripts, generated as previously described (8), were included on each TAC along with up to six patient specimens. Reaction mixtures were loaded into the individual portals, and the card was centrifuged twice at 336 \( \times \) g for 1 min and sealed to close the reaction wells. All TACs (\( n = 4 \) cards) were run on an Applied Biosystems Viia7 real-time PCR instrument (Life Technologies) under the following cycling conditions: 45°C for 10 min, 94°C for 10 min, 45 cycles of 94°C for 30 s, and 60°C for 60 s. The assays included on the TAC were specific for \textit{Bordetella pertussis}, \textit{Chlamydia pneumoniae}, \textit{Mycoplasma pneumoniae}, \textit{Streptococcus pyogenes}, \textit{Streptococcus pneumoniae}, \textit{Haemophilus influenzae} (all serotypes, including nontypeable), \textit{Legionella pneumophila}, \textit{Legionella} spp., human metapneumovirus, adenovirus, coronavirus types 1 to 4, human enterovirus, rhinovirus, human parainfluenza virus types 1 to 4, respiratory syncytial virus, influenza A virus, influenza B virus, and influenza C virus.

Multiplex real-time PCR for the detection of atypical bacterial respiratory pathogens. A multiplex qPCR assay was used to test the specimens (\( n = 22 \)) in triplicate reactions for the presence of \textit{M. pneumoniae}, \textit{C. pneumoniae}, and \textit{Legionella} spp., as previously described (9). The multiplex qPCR assay was used as the primary testing modality following etiological identification because of its proven reliability and validation and its compliance with Clinical Laboratory Improvement Amendments (CLIA) standards (9).

Culturing and confirmation of isolates. Isolations were carried out for all qPCR-positive swab specimens (\( n = 12 \)), as previously discussed (5). No blind passages were performed. Briefly, specimens were cultured in SP4 medium, and those displaying a color change after incubation at 37°C in 5\% CO\(_2\) were extracted for total nucleic acid as described above. The recovery of \textit{M. pneumoniae} was confirmed by singleplex qPCR as previously reported (9, 10), with slight modifications. Briefly, the 25-\( \mu \)l reaction volume contained 12.5 \( \mu \)l PerfeCTa Multiplex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD), the appropriate volumes of each primer and probe, nuclease-free water, and 5 \( \mu \)l TNA. The PCR was run on an Applied Biosystems 7500 real-time PCR system using the following cycling conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Macrolide susceptibility analysis. Macrolide sensitivity profiles were generated for the 12 \textit{M. pneumoniae}-positive primary specimens and 10 isolates using real-time PCR followed by high-resolution melt (HRM) analysis using LUX chemistry for primary specimens and intercalating dye (SYBR GreenER; Invitrogen) chemistry for isolates, as previously described (11). All specimens and corresponding isolates were tested in triplicate on the Rotor-Gene Q 6000 system (Qiagen).

P1 genotyping. P1 typing profiles were generated for the 10 \textit{M. pneumoniae} isolates with real-time PCR followed by HRM analysis using SYBR GreenER chemistry, as previously described (12). All isolates were tested in triplicate on the Rotor-Gene Q 6000 system (Qiagen) and were classified based upon comparison of melting profiles to the type 1 reference strain (M129) and the type 2 reference strain (FH).

MLVA typing. PCR amplification of the five variable-number tandem-repeat (VNTR) loci (Mpn1, Mpn13, Mpn14, Mpn15, and Mpn16) selected for \textit{M. pneumoniae} multilocus variable-number tandem-repeat analysis (MLVA) typing was carried out using extracted nucleic acid from the 12 primary specimens and the 10 isolates, as previously described (13). For the PCR amplification of primary specimen extracts, the number of amplification cycles was increased to 45. The MLVA nomenclature used is based on the numeric combination of tandem repeats for strain typing, as proposed previously (14). MLVA types were further evaluated using the four-loci (Mpn13 to Mpn16) typing scheme, as previously described (15).

RESULTS

Detection of \textit{M. pneumoniae} by TAC and multiplex qPCR. Eighty-three cases of probable pneumonia were identified between September and December 2012 from the student population, which ranged in age from 18 to 30 years. The patients reported symptoms of cough, fever, shortness of breath, body aches, headache, sore throat, nasal congestion, and/or rash. Sixty-one (73\%) probable pneumonia cases were radiologically confirmed by a physician. NP and/or OP swab specimens were obtained from 19 probable pneumonia cases and 2 students with unexplained respiratory symptoms. The first four swab specimens collected and tested with the TAC were all \textit{M. pneumoniae} positive. \textit{H. influenzae} was also detected in two of the four swab specimens but was eliminated as the causative agent due to its common occurrence in asymptomatic carriage. Once \textit{M. pneumoniae} was identified as the etiology, the multiplex qPCR assay was utilized as the primary testing method. Twelve (60\%) of 21 specimens were positive for \textit{M. pneumoniae}, with crossing threshold (\( C_T \)) values ranging from 21.3 to 35.6 in the multiplex qPCR assay. The single follow-up swab collected was negative for \textit{M. pneumoniae} 5 weeks after the initial positive swab.

Following multiplex qPCR testing of the 21 specimens and the single follow-up swab, TAC testing was resumed for the remaining 18 swab specimens in order to compare the performance of the two detection methods and to examine codetected respiratory pathogens present in \textit{M. pneumoniae}-positive and -negative specimens. TAC testing identified \textit{M. pneumoniae} in the same 12 of 21 specimens, demonstrating 100\% diagnostic sensitivity and specificity compared to those of the multiplex assay (as the reference standard). The \( C_T \) value differences between the TAC and the multiplex qPCR ranged from <1 cycle to 3 cycles. Among the 12 specimens identified as having \textit{M. pneumoniae} by the TAC, the \( C_T \) values ranged from 20.14 to 34.72, with 67\% of these containing codetections of other agents. The other respiratory pathogens identified in the patient specimens included \textit{H. influenzae} (\( n = 11 \)), \textit{S. pneumoniae} (\( n = 4 \)), \textit{S. pyogenes} (\( n = 3 \)), rhinovirus (\( n = 2 \)), adenovirus (\( n = 1 \)), coronavirus type 3 (identified in the follow-up swab specimen) (\( n = 1 \)), and influenza A virus (\( n = 1 \)) (Fig. 1).

Macrolide susceptibility profiles. Patient specimens from laboratory-confirmed \textit{M. pneumoniae} cases (\( n = 12 \)) and recovered isolates (\( n = 10 \)) were evaluated for the presence of the genotype associated with macrolide susceptibility (11). One patient specimen failed to amplify, most likely due to insufficient \textit{M. pneumoniae} nucleic acid in the extract. HRM profiles consistent with macrolide susceptibility were observed in the remaining specimens (\( n = 11 \)) and all the clinical isolates.

P1 and MLVA typing. The major adhesion molecule P1 subtype was determined for all the clinical isolates. The 10 isolates were distributed approximately equally into the two distinct groups, type 1 (\( n = 6 \), 60\%) and type 2 (\( n = 4 \), 40\%). In addition to P1 typing, MLVA was performed on all \textit{M. pneumoniae}-positive primary specimens (\( n = 12 \)) and recovered isolates (\( n = 10 \)) (Table 1). Analysis of the combination of tandem repeats at the five VNTR loci revealed seven distinct MLVA types in the original specimens (Fig. 2A) and five types among the isolates. There were no discrepancies in MLVA types when comparing the primary
specimens to the corresponding isolates. No novel MLVA types were identified. In addition, mixed MLVA types (4-6/3/6/6/2, 4-5/3/6/6/2) were observed in two primary specimens. The primary specimen with the mixed MLVA type 4-6/3/6/6/2 generated an isolate with an identical mixed MLVA type. No isolate was recovered from the mixed MLVA type 4-5/3/6/6/2. The mixed types were the results of tandem-repeat variation at the Mpn1 locus, known to be inherently unstable (14,15). The removal of the variable Mpn1 locus from the typing scheme and a reevaluation of the MLVA types using four loci (Mpn13 to Mpn16), as proposed by Sun et al. (15), revealed only three unique MLVA types, 3/5/6/2, 3/6/6/2, and 4/5/7/2 (Fig. 2B).

DISCUSSION

In this outbreak investigation, two diagnostic strategies, the TAC and multiplex qPCR, were used for identification. The TAC is useful for outbreaks in which the etiology is initially unknown, like this one, because it enables simultaneous testing for multiple pathogens within a specimen. The interpretation of the findings can be complex, however. The mere presence of a microorganism does not necessarily equate to the etiology of the illness, since many potential respiratory pathogens are carried asymptomatically within the population. In the current investigation, the TAC was successfully used to rapidly identify M. pneumoniae as the outbreak etiology upon specimen receipt by the CDC. Excellent concordance of results was observed for the TAC and the CLIA-approved multiplex qPCR assay for the detection of M. pneumoniae in this outbreak. TAC testing also identified both H. influenzae, S. pyogenes, and S. pneumoniae among M. pneumoniae-positive and -negative specimens (Fig. 1). This is not surprising, since the asymptomatic carriage of S. pneumoniae, S. pyogenes, and H. influenzae is relatively common (8, 25, 26, 27). In addition, some cases of pneumonia diagnosed among university students during the outbreak may have been caused by these other pathogens, although most of the pneumonia cases were likely caused by M. pneumoniae. Further studies would be necessary to fully understand the clinical and epidemiological significance of co-detected agents, especially during M. pneumoniae outbreaks.

The successful implementation of diagnostic methods used to determine etiology is dependent upon timely recognition of a potential outbreak and collection of appropriate specimens. This M. pneumoniae outbreak was identified after a significant number of probable pneumonia cases were reported among students at the University Health Services clinic. Active surveillance for probable pneumonia and specimen collection for diagnostic testing began almost 2 months after the first case was identified (7). This delay resulted in a limited number of specimens available for the laboratory confirmation of an M. pneumoniae infection. The collection delay, and the restriction of surveillance to only probable pneumonia cases, likely caused a significant underestimation of the true number of students who were affected during this outbreak. Most students seeking medical attention were prescribed azithromycin or doxycycline for the empirical treatment of bacterial respiratory infection (7). While these antibiotics are appropriate for the treatment of M. pneumoniae infection, four cases of viral infections (adenovirus, rhinovirus, influenza virus A, and coronavirus type 3) were identified from M. pneumoniae-negative specimens during this investigation. This further demonstrates the utility of multipathogen testing approaches, even during outbreaks where a primary etiology has been identified, and it underscores the potential to improve targeting of the most appropriate

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<tr>
<th>Isolate P1 type</th>
<th>Specimen MLVA type</th>
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<tbody>
<tr>
<td>1</td>
<td>5/4/5/7/2</td>
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* Not applicable; no isolate was obtained for specimen MLVA types 4-5/3/6/6/2 and 5/3/5/6/2.
in strains within the United States. Various rates of macrolide resistance have been reported during recent domestic outbreaks and sporadic cases. Wolff et al. reported 8% rate in the central United States from 2007 to 2010 (11, 17). In Rhode Island, while Yamada et al. found just higher than 27% macrolide resistance in isolates recovered during a 2007 outbreak. In addition, during a 2011 outbreak in West Virginia, 2 (9%) of 23 M. pneumoniae isolates were macrolide resistant (18). All M. pneumoniae isolates were macrolide susceptible; however, only 14% of the cases were confirmed by laboratory testing. Therefore, it is possible that macrolide-resistant M. pneumoniae was simply not detected due to limited sampling. Further, as in all outbreaks, early recognition followed by proper and timely specimen collection and shipment to a qualified testing laboratory are paramount for accurately identifying and characterizing the etiology.

Further molecular characterization, including P1 typing and MLVA, was performed on all specimens and isolates in the current outbreak investigation. M. pneumoniae can be classified into one of two main genomic groups (type 1 and type 2) based on sequence variation within the gene encoding the major adhesion molecule P1. In this outbreak, we observed type 1 and type 2 isolates in similar proportions. Heterogeneity in circulating P1 types has been reported in two of eight documented M. pneumoniae outbreak investigations in the United States since 1995 (14). Various international reports have also shown heterogeneity in P1 types circulating within the population (19–22). In contrast, the identification of either P1 type alone has also been reported during outbreaks (14). However, this appearance of clonality among specimens may simply be attributable to a limited number of specimens being collected during these investigations. Comprehensive analyses of recent outbreaks together may reveal trends in circulating M. pneumoniae types that are masked by the limited specimen collection in individual outbreak scenarios. Previous studies have reported alternating dominance of P1 types in 3- to 7-year cycles (23, 24), a phenomenon that may be driven by the development of temporary immunity to the dominant type within a population. In support of this observation, no type 1 clinical isolates were recovered from 1995 to 2007 (n = 60) in any CDC-investigated U.S. outbreak. However, since that time, 71% of M. pneumoniae isolates recovered during domestic outbreaks (n = 35) have been identified as type 1 (14). Similarly, increases in the proportions of type 1 strains in other regions of the world were recently reported (21, 22).

The current study also used the highly discriminatory MLVA typing method, and seven MLVA types were identified in this outbreak. Differentiation was mostly limited to variability of tandem repeats at the Mpn1 locus, which has been previously described (14, 15). The instability of the Mpn1 locus was again demonstrated in this M. pneumoniae outbreak by the identification of mixed MLVA types (4-6/3/6/6/2 and 4-5/3/6/6/2) in two specimens resulting from tandem-repeat variation within Mpn1. The lack of stability suggests that Mpn1 is not a good candidate locus for comparison of M. pneumoniae and has been recently proposed to be eliminated from this typing scheme (15). Eliminating Mpn1 from the current analysis reveals only three distinct MLVA types, two of which account for 92% of the M. pneumoniae isolates in this investigation. The predominance of types 4/5/7/2 and 3/6/6/2 observed in this outbreak is consistent with recent reports in which 71 to 84% of domestic or international M. pneumoniae isolates were either of these two MLVA types (13, 14). In addition, these modified MLVA types correlate with P1 typing results. All type 1 isolates from this outbreak were MLVA type 4/5/7/2, while all type 2 isolates were MLVA type 3/6/6/2. As a result, the observed increase in P1 type 1 strains among domestic M. pneumoniae outbreaks since 2007 is similarly reflected by an increase in MLVA type 4/5/7/2 (14). These analyses suggest that the exclusion of Mpn1 may result in a more accurate determination of the relatedness of strains and help better define patterns of circulating M. pneumoniae in an outbreak investigation and within the population.

Broader implementation of molecular typing, including macrolide resistance, may lead to an improved understanding of the diversity, distribution, and pathogenesis of M. pneumoniae and, ultimately, a more effective public health response. A reduction in the time between outbreak recognition and initiation of active surveillance with prioritized specimen collection will lead to improved patient treatment and provide a more accurate accounting.

FIG 2 Distribution of MLVA types in this outbreak: comparison of MLVA typing based on five loci (A) or four loci (B).
of disease burden caused by this organism. By developing point-of-care diagnostics, increasing public awareness, and actively strengthening the collaboration between providers, health care facilities and public health agencies, *M. pneumoniae* infection can be more appropriately managed and directly addressed for better patient care and outbreak control.

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REFERENCES


