Emergence of Macrolide-Resistant *Mycoplasma pneumoniae* in Hong Kong Is Linked to Increasing Macrolide Resistance in Multilocus Variable-Number Tandem-Repeat Analysis Type 4-5-7-2

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Macrolide-resistant *Mycoplasma pneumoniae* (MRMP) is rapidly emerging in Asia, but information on the temporal relationship between the increase in macrolide resistance and changes in strain types is scarce. Between 2011 and 2014, *M. pneumoniae* infection was diagnosed by PCR as part of routine care in a health care region in Hong Kong. Testing was initiated by clinicians, mainly in patients with suspected *M. pneumoniae* pneumonia. Specimens positive for *M. pneumoniae* were retrospectively investigated by macrolide resistance genotyping and a four-locus (Mpn1 to -16) multilocus variable-number tandem-repeat analysis (MLVA) scheme. The overall percentage of *M. pneumoniae*-positive specimens was 17.9%, with annual rates ranging from 9.8% to 27.2%. The prevalence of MRMP had rapidly increased from 13.6% in 2011 to 30.7% in 2012, 36.6% in 2013, and 47.1% in 2014 (P < 0.038). Two major MLVA types, 4-5-7-2 and 3-5-6-2, accounted for 75% to 85% of the infections each year. MLVA types 4-5-7-2 and 3-5-6-2 predominated among macrolide-resistant and macrolide-sensitive groups, respectively. The increase in MRMP was mainly caused by increasing macrolide resistance in the prevalent MLVA type 4-5-7-2, changing from 25.0% in 2011 to 59.1% in 2012, to 89.7% in 2013, and to 100% in 2014 (P < 0.001). In conclusion, increasing MRMP in Hong Kong was linked to a single MLVA type, which was both prevalent and increasingly resistant to macrolides.

**MATERIALS AND METHODS**

**Study design.** This retrospective study was conducted in a health care region in Hong Kong comprising one university-affiliated hospital with 1,600 beds, three extended-care hospitals with a total of 1,600 beds, and one pediatric hospital with 160 beds. A diagnostic PCR assay for *M. pneumoniae* was provided as a routine service for inpatients by a clinical microbiology laboratory (7, 16). Testing was initiated by clinicians, mainly in patients with features suspected to be due to *M. pneumoniae* pneumonia (2, 17). Nasopharyngeal aspirate samples were collected in viral transport medium (18). Sputum and other respiratory specimens were collected using standard techniques (16). Patients were included if their respiratory specimens were obtained for *M. pneumoniae* testing by PCR between January 2011 and December 2014. During the study period, a total of 1,657 respiratory specimens from 1,433 patients were investigated by a real-time PCR test for the presence of *M. pneumoniae*. Overall, 257 (17.9%) patients, including 274 (16.5%) specimens, were positive for *M. pneumoniae*. The 274 *M. pneumoniae*-positive specimens comprised 264 nasopharyngeal aspirates/swabs, five pleural specimens, and five other...
respiratory specimens (sputum and bronchial aspirate). The data were analyzed by five age groups: 0 to 1 year (infants, n = 11), 2 to 11 years (children, n = 195), 12 to 17 years (teenagers, n = 33), 18 to 64 years (adults, n = 16), and ≥65 years (seniors, n = 2). The patients were diagnosed with pneumonia (n = 231), upper respiratory tract infection (n = 7), nonspecific respiratory illness (n = 9), and acute bronchiolitis (n = 1). In nine patients, no information on the syndromic diagnosis was available. Clinical features and macrolide resistance genotyping results for 101 patients were reported previously (7, 16). Nucleic acid extracts from the 257 patients with positive M. pneumoniae results were retrospectively retrieved for further testing. Only one specimen from each patient was included.

**Nucleic acid extraction.** Nucleic acid extraction was performed by using the NucliSENS easyMAG extraction system (bioMérieux, France) and stored at −80°C, as described previously (16). All testing was performed on nucleic acid extracted from the clinical specimens. Culture for M. pneumoniae was not performed.

**Real-time qPCR for detection of M. pneumoniae.** Real-time quantitative (qPCR) was conducted to detect M. pneumoniae using TaqMan universal PCR master mix (Applied Biosystems) in a StepOnePlus instrument (Applied Biosystems, Foster City, CA), as previously described (16). A series of 6 log₁₀ dilutions equivalent to 10 to 1 x 10⁶ copies per reaction mixture were prepared from a plasmid (pC-RII-TOPO vector; Invitrogen, CA) containing the corresponding target bacterial sequence to generate calibration curves; these were run in parallel with the test specimens. The detection limit of the qPCR assay was approximately 10 copies per reaction mixture (16).

**MRMP genotype detection.** Simple-Probe real-time PCR coupled to melting curve analysis (Simple-Probe PCR) was performed on the extracted nucleic acid from specimens to identify MRMP. The MRMP assay was done using the LightCycler FastStart DNA master HybProbe kit (Roche Diagnostics, Germany), according to a previously published protocol (16). The detection limit of Simple-Probe PCR for both wild-type and mutants was 10⁴ copies per reaction. A randomly chosen subset of the specimens was subjected to Sanger sequencing for confirmation.

**MLVA typing.** Previously published primers were used to amplify four variable-number tandem-repeat (VNTR) loci (Mpn13 to -16) (12). Our initial testing showed that nonspecific bands were commonly observed if all loci were amplified together in one multiplex reaction. After optimization, good results were obtained through amplification of the loci in two duplex reactions, with one for Mpn13 and Mpn15, and one for Mpn14 and Mpn16. The PCR was performed using 2 µl of nucleic acid and 15 µl of 2× Qiagen multiplex master mixes, and the concentrations of each primer were 0.2 µM for Mpn13 and Mpn15, 0.4 µM for Mpn14, and 0.08 µM for Mpn16. The total reaction mixture volume was made up to 30 µl with nuclease-free water. A Veriti 96-well thermal cycler (Applied Biosystems) was used for amplification. The cycling conditions were a denaturation step of 15 min at 95°C, and an amplification step of 40 cycles of 30 s at 95°C, 30 s at 62°C, and 45 s at 72°C. The products were then pooled into one tube for product size determination in one lane by capillary electrophoresis using an ABI 3130 genetic analyzer (Applied Biosystems), and the data were analyzed using the GeneMapper software (version 4.0; Applied Biosystems). The primers were fluorescently labeled at the 5′ end with VIC (green, Mpn13 and Mpn15), NED (yellow, Mpn14), or 6-carboxyfluorescein (6-FAM) (blue, Mpn16) (Applied Biosystems). The fluorescent labels for the targets together with the expected product sizes of each locus allowed the amplicons for all four loci to be sized in one reaction mixture. The number of repeats for each locus was calculated according to the PCR fragment size. The MLVA type was designated by the numeric combination of the number of tandem repeats at four loci (Mpn13 to -16), as suggested previously (13, 15). The number of repeats was rounded up to an integer value (9, 12). The number of repeats in each locus (1 to 2 specimens for each product size) was confirmed by Sanger sequencing.

**Statistical analysis.** Statistical analysis was performed using SPSS Statistics version 23 for Windows. Chi-square tests were used to compare categorical variables. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Prevalence of M. pneumoniae.** The percentages of test-positive patients per month from 2011 to 2014 are shown in Fig. 1. Higher positive rates (>1 standard deviation above average for the entire
period) were observed in 2012 (April, June, July, and September) and 2013 (May to July). The percentages of test-positive patients by year were 9.8% in 2011, 27.2% in 2012, 24.3% in 2013, and 11.4% in 2014 ($P < 0.001$).

The *M. pneumoniae*-positive rate was highest among children age 2 to 11 years (33.2%) and teenagers age 12 to 17 years (30.6%), and then in infants age 0 to 1 year (7.6%); it was lowest in adults age 18 to 64 years (4.0%) and seniors age ≥65 years (1.0%) ($P < 0.001$). The positive rate was higher in females than in males (21.8% versus 14.5%, respectively; $P < 0.001$).

**Prevalence of macrolide-resistant genotype.** The *M. pneumoniae*-positive specimens for 16 patients were of an insufficient amount and not investigated further. Macrolide resistance genotyping was successfully carried out on all specimens from the remaining 241 patients. Simple-Probe real-time PCR coupled to melting curve analysis identified 34.9% (84/241) of the unique patient specimens as having the MRMP genotype. The A2063G transition was the only mutation identified. A subset of 88 specimens, including 61 with the macrolide-sensitive *M. pneumoniae* (MSMP) genotype and 27 with the MRMP genotype, were further analyzed by Sanger sequencing. The results were 100% concordant with the melting curve analysis. The annual prevalence of MRMP among all *M. pneumoniae*-positive patients had significantly increased from 13.6% (3/22) in 2011 to 30.7% (23/75) in 2012, 36.6% (34/93) in 2013, and 47.1% (24/51) in 2014 ($P = 0.038$). The prevalence of the MRMP genotype was highest among children (age 0 to 1 year, 30.0%; age 2 to 11 years, 36.1%; age 12 to 17 years, 39.7%); it was in adults (age 18 to 64 years, 20.0%, age ≥65 years, 0%), but the difference was not statistically significant ($P = 0.122$). The MRMP prevalence rates among males (33.7%) and females (35.7%) were similar ($P = 0.742$).

**Temporal changes in macrolide resistance rate and MLVA types.** Specimens from the 241 patients with sufficient DNA extracts were further investigated by MLVA typing, and successful results were obtained for 205 (85.1%) patients. The number of repeats in the four loci were 3 to 5 for Mpn13, 4 to 6 for Mpn14, 6 to 7 for Mpn15, and 2 to 3 for Mpn16, giving seven distinct MLVA types. The major types were 3-5-6-2 (44.4%), 4-5-7-2 (36.6%), and 4-5-7-3 (14.1%). Other rare types, including 3-6-6-2 ($n = 4$), 5-5-7-2 ($n = 3$), 4-6-7-3 ($n = 2$), and 4-4-7-3 ($n = 1$), accounted for only 4.9% of the total.

During the 4-year period, types 4-5-7-2 and 3-5-6-2 were predominant (Fig. 2). Together, the two types comprised 75% to 85% of all positive specimens in each year. The proportion of type 4-5-7-2 had an increasing trend from 29% in 2011 to 34% in 2012, 36% in 2013, and 43% in 2014, but the difference was not statistically significant ($P = 0.686$). Of the seven MLVA types, five and four MLVA types were found among specimens with the macrolide-sensitive *M. pneumoniae* (MSMP) and MRMP genotypes, respectively (Fig. 3A). The two major MLVA types (3-5-6-2 and 4-5-7-2) occurred in the MRMP and MSMP groups at different frequencies. The prevalence of MLVA type 4-5-7-2 was substantially higher in the MRMP group than in the MSMP group (89.6% versus 10.9%, respectively; $P < 0.001$). In contrast, MLVA type 3-5-6-2 was more prevalent in the MSMP group than in the MRMP group (64.5% versus 3.0%, respectively; $P < 0.001$). The other five MLVA types were found at low frequencies in the MRMP group (for types 3-5-7-2 and 4-6-7-3) and the MSMP group (for types 4-5-7-3, 4-4-7-3, and M3-6-6-2). Stratification by year revealed that the macrolide resistance rate of MLVA type 4-5-7-2 had significantly increased from 25.0% in 2011 to 100% in 2014 (Fig. 3B) ($P < 0.001$).

**DISCUSSION**

In this study, an increase in the rate of *M. pneumoniae* infection was noted in 2012 and 2013, suggesting that there was an epidemic outbreak during this period. During the entire period, the changes in the annual cycle of positive rates were irregular, with peaks in early summer (in 2012), mid-summer (in 2013), and early autumn (in 2014). This is in line with reports describing more *M. pneumoniae* infections with increased relative humidity and ambient temperature (19, 20). In our neighborhood areas, recent epidemic outbreaks of *M. pneumoniae* infections were also noted in South Korea from 2010 to 2011, in Japan from 2011 to 2012, and in Beijing and Shanghai, China, in 2012 (4, 21, 22). Notably, a substantial increase in the prevalence of MRMP infection was noted in the areas during or shortly after those epidemics (3, 4, 21–23). Our data revealed that the MRMP infection rate had increased by >3-fold from 13.6% in 2011 to 47.1% in 2014. Reported rates of MRMP infection range from 62.9% in South Korea (23) and >80% to 90% in China and Japan (3, 8), compared to ≤10% in Europe and the United States (1, 15). The relationship between *M. pneumoniae* epidemics and MRMP emergence is likely to be complex, involving selection pressure from the widespread administration of macrolides (23).

Two MLVA types (3-5-6-2 [44.4%] and 4-5-7-2 [36.6%]) accounted for 81.0% of the infections during the study period. Both types occurred worldwide and were among the predominant types found in many studies. Among international collections of *M. pneumoniae* isolates collected over decades, 14.0% to 20.8% and 50.6% to 55.1% were of MLVA types 3-5-6-2 and 4-5-7-2, respectively (9, 13). During the periods with a high proportion of *M. pneumoniae*-positive specimens, the major genotypes did not change (Fig. 2A). This suggests that the increase in diagnosis of *M. pneumoniae* infections is likely a result of increased transmission of cocirculating MLVA types rather than the introduction of new types into the community.

This study found that the increase in MRMP was predominantly a result of increasing resistance in MLVA type 4-5-7-2. The
Macrolide resistance rate of this type had drastically increased from 25% in 2011 to 100% in 2014. All other MLVA types, including the prevalent 3–5–6–2 type, remained largely macrolide sensitive. Qu et al. (22) previously reported the same significant correlation between macrolide resistance and susceptibility with types 4–5–7–2 and 3–5–6–2, respectively. In the two international populations described by Dégrange et al. (9) and Benitez et al. (13), four (33.3%) of 12 and nine (90%) of 10 MRMP isolates, respectively, had MLVA type 4-5-7-2. In the United States, 13 (68.4%) of 19 MRMP isolates identified during CDC-assisted investigations across the country between 2006 and 2013 belonged to MLVA type 4-5-7-2 (15). In China, this MLVA type accounted for >90% of the MRMP isolates identified in Beijing from 2010 to 2013 (12, 22, 24). Of the 21 distinct types that can be distinguished by the amended four-locus MLVA scheme (5, 6, 9–15, 22, 24–26) (see Table S1 in the supplemental material), MRMP has been detected in 12 types. Among the published reports, the prevalences of the other 11 types among MRMP were low, and their occurrences were sporadic (5, 6, 9, 12–15, 22, 24, 26).

As far as we know, this is the first report to demonstrate a link between changes in MRMP prevalence and increasing resistance within a single MLVA type. The inclusion of consecutive specimens from a 4-year period and a relatively large sample size are the strengths of this study. Given that this retrospective analysis examined specimens from inpatients only, of whom the majority were diagnosed with pneumonia, the findings may not be representative of mild *M. pneumoniae* infections in the community. *M. pneumoniae* is a genetically conserved organism. A pairwise comparison of the four published *M. pneumoniae* genomes (strains M29, M129, 309, and FH) revealed that the difference between strains of different MLVA types (4-5-7-2 versus 3-5-6-2) was 0.3% to 0.5%, while the difference between strains of the same MLVA types was 0.08% (see Table S2 in the supplemental material). Therefore, whole-genome sequencing may be the ultimate approach for resolving whether there is any MRMP subclone within type 4-5-7-2.

In summary, we demonstrated a link between increasing macrolide resistance and the expansion of MRMP strains of the MLVA type 4-5-7-2 in Hong Kong during 2011 to 2014. It is worrying that type 4–5–7–2 may be associated with more severe disease (22). Increasing public awareness, enhancing access to rapid diagnostics, and improving surveillance for *M. pneumoniae* infection and macrolide resistance are necessary to inform case and outbreak management and to understand the burden of disease.

**REFERENCES**


