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Citation	Journal of Clinical Microbiology, 2015, v. 53 n. 11, p. 3560-3564
Issued Date	2015
URL	http://hdl.handle.net/10722/220171
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JCM01983-15 revised

Emergence of macrolide-resistant *Mycoplasma pneumoniae* in Hong Kong is linked to increasing macrolide resistance in the multilocus variable-number tandem-repeat analysis type 4-5-7-2

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Running title: MLVA type associated with macrolide-R *M. pneumoniae*

Keywords: molecular typing, Mycoplasma pneumoniae, antimicrobial resistance, pneumonia

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Word counts: Abstract 207

ABSTRACT

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 healthcare 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 loci (Mpn13-16) 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%-27.2%. Prevalence of MRMP had rapidly increased from 13.6% in 2011, 30.7% in 2012, 36.6% in 2013 to 47.1% in 2014 ($P = 0.038$). Two major MLVA types 4-5-7-2 and 3-5-6-2 accounted for 75%-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. 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, 59.1% in 2012, 89.7% in 2013 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.

INTRODUCITON

Mycoplasma pneumoniae (MP) is a common cause of community-acquired pneumonia and other respiratory tract infections (19). Community epidemics occur at intervals of 3 to 7 years. Infections develop in persons of all ages but it is primarily a disease of children and teenagers (2). When treatment is indicated, a macrolide is usually the drug of choice (2,19). However, macrolide-resistant *M. pneumoniae* (MRMP) have become increasingly prevalent worldwide and high rates (>80%) have been found in certain parts of the world (14,16,18,23). MRMP infections have been associated with persistence of symptoms, slower reduction in bacterial load, longer hospital stays, requirement for alternative therapy and higher frequency of complications (6,19,26). Strain typing is important for understanding changes in disease epidemiology and for investigation of outbreaks. In 2009, a multilocus variable-number tandem-repeat analysis (MLVA) scheme based upon five loci (Mpn1, Mpn13-16) was developed for the molecular typing of MP (8). It was initially used for investigation of isolates, but was later modified for directly typing MP in respiratory specimens (4,10,24). An amended 4 loci MLVA scheme was later proposed after studies had raised concerns on the instability of the Mpn1 locus (1,21). In clinical laboratories, culture and characterization of MP is seldom performed. Therefore, MP typing was usually carried out on isolates collected from sporadic cases and outbreaks (1,8,9), limiting the inferences that can be made about trends in MP infections. In addition, information on the temporal relationship between the increase in macrolide resistance and changes in strain types is scarce (9). Here, MLVA analysis was used to investigate the MP strain type and macrolide resistance genotype in respiratory specimens collected consecutively from patients in a healthcare region in Hong Kong over a 4-year period.

MATERIALS AND METHODS

Study design. This retrospective study was conducted in a healthcare region in Hong Kong comprising one university-affiliated hospital with 1600 beds, three extended-care hospitals with a total of 1600 beds, and one paediatric hospital with 160 beds. Diagnostic PCR assay for MP was provided as a routine service for inpatients by a clinical microbiology laboratory (5,6). Testing was initiated by clinicians, mainly in patients with features suspicious of MP pneumonia (2,15). Nasopharyngeal aspirate was collected in viral transport medium (7). Sputum and other respiratory specimens were collected using standard techniques (5). Patients were included if their respiratory specimens were obtained for MP testing by PCR between January 2011 and December 2014. During the study period, a total of 1657 respiratory specimens from 1433 patients were investigated by real-time PCR test for the presence of MP. Overall, 257 (17.9%) patients, including 274 (16.5%) specimens were positive for MP. The 274 MP-positive specimens comprised 264 nasopharyngeal aspirates/swabs, five pleural specimens and five other respiratory specimens (sputum, bronchial aspirate). The data was analysed by five age groups: 0-1 year (infants, n = 11), 2-11 years (children, n = 195), 12-17 years (teenagers, n = 33), 18-64 years (adults, n = 16), ≥65 years (seniors, n = 2). The patients were diagnosed with pneumonia (n = 231), upper respiratory tract infection (n = 7), non-specific 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 of the patients have been reported previously (5,6). Nucleic acid extracts from the 257 patients with positive MP 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 ^{o}C, as described previously (5). All testing was performed on nucleic acid extracted from the clinical specimens. Culture for MP was not performed.

Real-time qPCR for the detection of *M. pneumoniae*. Real-time quantitative (qPCR) was conducted for the detection of MP using TaqMan universal PCR master mix (Applied Biosystems) in a StepOnePlus instrument (Applied Biosystems, Foster City, CA), as previously described (5). A series of 6 $\log_{10}$ dilutions equivalent to ten to 1×10^6 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 ran in parallel with the test specimens. The detection limit of the qPCR assay was approximately ten copies per reaction mixture (5).

MRMP genotype detection. SimpleProbe real-time PCR coupled to melting curve analysis (SimpleProbe PCR) was performed on the extracted nucleic acid from specimens to identify MRMP. The MRMP assay was done by using the LightCycler FastStart DNA master HybProbe kit (Roche Diagnostics, Germany) according to a published protocol (5). The detection limit of SimpleProbe PCR for both wild-type and mutant was 10^3 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 (Mpn13-16) (24). 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, one for Mpn13 and Mpn15, and one for Mpn14 and Mpn16. The PCR was performed using 2 μl nucleic acid, 15 μl 2 × QIAGEN multiplex master mixes (QIAGEN), concentrations for each primers 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. Cycling conditions were as follows: denaturation step of 15 min at 95 °C, 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 analyser (Applied Biosystem), and the data was analysed using GeneMapper software (version 4.0, Applied Biosystems). The primers were fluorescently labelled at the 5' end with VIC (green, Mpn13 and Mpn15), NED (yellow, Mpn14) or 6-FAM (blue, Mpn16) (Applied Biosystem). The fluorescent labels for the targets together with the expected product sizes of each locus allowed sizing of the amplicons for all four loci 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 (Mpn13-16), as suggested previously (1,9). The number of repeats was rounded up to an integer value (8,24). 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 percentage of test-positive patients per month from 2011 to 2014 is shown in Fig 1. Higher positive rates (more than 1 standard deviation above average for the entire period) were observed in 2012 (April, June, July, September) and 2013 (May to July). The percentage of test-positive patients by year was 9.8% in 2011, 27.2% in 2012, 24.3% in 2013 and 11.4% in 2014 ($P < 0.001$).

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

Prevalence of macrolide-resistant genotype. The MP-positive specimens for 16 patients were of an insufficient amount and not investigated further. Macrolide-resistant genotyping could be successfully carried out on all specimens from the remaining 241 patients. SimpleProbe real-time PCR coupled to melting curve analysis identified 34.9% (84/241) of the unique patient specimens as MRMP genotype. The A2063G transition was the only mutation identified. A subset of 88 specimens, including 61 with MSMP genotype and 27 with MRMP genotype was further analysed by Sanger sequencing. The results were 100% concordant with melting curve analysis. The annual prevalence of MRMP among all MP-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 MRMP genotype was higher among children (aged 0-1 years, 30.0%; aged 2-11 years, 36.1%; aged 12-17 years, 39.7%) than in adults (aged 18-64 years, 20.0%, aged ≥65 years, 0%) but

the difference was not statistically significant (P = 0.122). MRMP prevalence 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) only accounted for 4.9% of the total.

During the four 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, 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 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%, 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% vs. 3.0%, P < 0.001). The other five MLVA types were found at low frequencies in either the MRMP group (5-5-7-2 and 4-6-7-3) or the MSMP group (4-5-7-3, 4-4-7-3 and M3-6-6-2) only. Stratification by year revealed that 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 MP infection was noted in 2012 and 2013, suggesting that there was an epidemic outbreak during this period. During the entire period, changes in the annual cycle of positive rates were irregular, with peaks in early summer (in 2012), mid-summer (in 2013) or early autumn (in 2014). This is in line with reports describing more MP infections with increased relative humidity and ambient temperature (17,22). In our neighborhood areas, recent epidemic outbreaks of MP infections were also noted in South Korea from 2010-2011, in Japan from 2011 to 2012 and in Beijing and Shanghai, China in 2012 (13,16,20). Notably, a substantial increase in the prevalence of MRMP was noted in the areas during or shortly after those epidemics (12-14,16,20). Our data revealed that the MRMP rate had increased by more than three folds from 13.6% in 2011 to 47.1% in 2014. Reported rates of MRMP range from 62.9% in South Korea (12) and >80% to 90% in China and Japan (14,26), compared to ≤10% in Europe and the United States (9,19). The relationship between MP epidemics and MRMP emergence is likely to be complex, involving selection pressure from widespread administration of macrolides (12).

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 occur worldwide and were among the predominant types in many studies. Amongst international collections of MP isolates collected over decades, 14.0%-20.8% and 50.6%-55.1% were of MLVA types 3-5-6-2 and 4-5-7-2, respectively (1,8). During the periods with a high proportion of MP-positive specimens, the major genotypes did not change (Figure 2A). This suggests that increased detection of MP infections is likely a result of increased transmission of co-circulating MLVA types, rather than introduction of new types to the community.

This study found that increase in MRMP was predominantly a result of increasing resistance in MLVA type 4-5-7-2. Macrolide resistance rate of this type had drastically

increased from 25% in 2011 to 100% in 2014. All the other MLVA types including the prevalent 3-5-6-2 type remained largely macrolide-sensitive. Qu *et al* previously reported the same significant correlation between macrolide resistance and susceptibility with type 4-5-7-2 and type 3-5-6-2, respectively (20). In the two international MP collections described by Degrange *et al* and Benitez *et al*, four (33.3%) of 12 and nine (90%) of ten MRMP isolates were of MLVA type 4-5-7-2, respectively (1,8). In the United States, 13 (68.4%) of 19 MRMP isolates identified through CDC-assisted investigations across the country between 2006 and 2013 belonged to MLVA type 4-5-7-2 (9). In China, this MLVA type accounted for >90% of the MRMP isolates identified in Beijing from 2010 to 2013 (20,24,25). Of the 21 distinct types that could be distinguished by the amended four loci MLVA scheme (1,3,4,8-11,18,20,21,23-25) (Table S1, supplementary file), MRMP has been detected in 12 types. Among the published reports, prevalence of the other 11 types among MRMP were low and occurrence was sporadic (1,8,9,11,18,20,21,23-25).

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. 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 only examined specimens from inpatients of whom the majority were diagnosed with pneumonia, the findings may not be representative of mild MP infections in the community. MP is a genetically conserved organism. Pairwise comparison of the four published MP 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%-0.5% while difference between strains of the same MLVA types was 0.08% (Table S2, supplementary file). 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 2011-2014. It is worrying that type 4-5-7-2 may be associated with more severe disease (20). Increasing public awareness, enhancing access to rapid diagnostics and improving surveillance for MP and macrolide resistance is necessary to inform case and outbreak management and to understand the burden of disease.

ACKNOWLEDGEMENTS:

This work was supported by grants from the Health and Medical Research Fund of the Food and Health Bureau of the Government of the Hong Kong Special Administrative Region.

FIG 1. *M. pneumoniae* positive rate in respiratory specimens by date of request for a healthcare region in Hong Kong, January 2011-December 2014. Histogram shows the monthly number of specimens tested. Dotted line shows the percentage of specimens positive for MP in each month. Horizontal line showed the average positive percentage for the entire period. Only one specimen per patient was included.

FIG 2. MLVA type in *M. pneumoniae* specimens in a healthcare region in Hong Kong, 2011-2014. The percentage of each MLVA type for each year is shown. The number of patients in each year is shown within parentheses.

FIG 3. Macrolide resistance in *M. pneumoniae* specimens in a healthcare region in Hong Kong, 2011-2014. (A) MLVA type according to macrolide resistance genotype. The proportions of MLVA types for MRMP (n = 67) and MSMP (n = 138) groups are shown in the outer and inner doughnuts, respectively. Others in the MRMP group included types 4-6-7-3 and 5-5-7-2. Others in the MSMP group included types 3-6-6-2 and 4-4-7-3. (B) Changes in macrolide resistance rate of MLVA type 4-5-7-2 during 2011-2014. The number of patients in each year is shown within parentheses.

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