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

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24 **ABSTRACT**

25 Macrolide-resistant *Mycoplasma pneumoniae* (MRMP) is rapidly emerging in Asia but
26 information on the temporal relationship between the increase in macrolide resistance and
27 changes in strain types is scarce. Between 2011 and 2014, *M. pneumoniae* infection was
28 diagnosed by PCR as part of routine care in a healthcare region in Hong Kong. Testing was
29 initiated by clinicians, mainly in patients with suspected *M. pneumoniae* pneumonia.
30 Specimens positive for *M. pneumoniae* were retrospectively investigated by macrolide
31 resistance genotyping and a four loci (Mpn13-16) multilocus variable-number tandem-repeat
32 analysis (MLVA) scheme. The overall percentage of *M. pneumoniae*-positive specimens was
33 17.9% with annual rates ranging from 9.8%-27.2%. Prevalence of MRMP had rapidly
34 increased from 13.6% in 2011, 30.7% in 2012, 36.6% in 2013 to 47.1% in 2014 ($P = 0.038$).
35 Two major MLVA types 4-5-7-2 and 3-5-6-2 accounted for 75%-85% of the infections each
36 year. MLVA types 4-5-7-2 and 3-5-6-2 predominated among macrolide-resistant and
37 macrolide-sensitive groups, respectively. Increase in MRMP was mainly caused by
38 increasing macrolide resistance in the prevalent MLVA type 4-5-7-2, changing from 25.0%
39 in 2011, 59.1% in 2012, 89.7% in 2013 to 100% in 2014 ($P < 0.001$). In conclusion,
40 increasing MRMP in Hong Kong was linked to a single MLVA type which was both
41 prevalent and increasingly resistant to macrolides.

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43

44 **INTRODUCITON**

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

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69 MATERIALS AND METHODS

70 **Study design.** This retrospective study was conducted in a healthcare region in Hong Kong
71 comprising one university-affiliated hospital with 1600 beds, three extended-care hospitals
72 with a total of 1600 beds, and one paediatric hospital with 160 beds. Diagnostic PCR assay
73 for MP was provided as a routine service for inpatients by a clinical microbiology laboratory
74 (5,6). Testing was initiated by clinicians, mainly in patients with features suspicious of MP
75 pneumonia (2,15). Nasopharyngeal aspirate was collected in viral transport medium (7).
76 Sputum and other respiratory specimens were collected using standard techniques (5).
77 Patients were included if their respiratory specimens were obtained for MP testing by PCR
78 between January 2011 and December 2014. During the study period, a total of 1657
79 respiratory specimens from 1433 patients were investigated by real-time PCR test for the
80 presence of MP. Overall, 257 (17.9%) patients, including 274 (16.5%) specimens were
81 positive for MP. The 274 MP-positive specimens comprised 264 nasopharyngeal
82 aspirates/swabs, five pleural specimens and five other respiratory specimens (sputum,
83 bronchial aspirate). The data was analysed by five age groups: 0-1 year (infants, $n = 11$), 2-
84 11 years (children, $n = 195$), 12-17 years (teenagers, $n = 33$), 18-64 years (adults, $n = 16$),
85 ≥ 65 years (seniors, $n = 2$). The patients were diagnosed with pneumonia ($n = 231$), upper
86 respiratory tract infection ($n = 7$), non-specific respiratory illness ($n = 9$) and acute
87 bronchiolitis ($n = 1$). In nine patients, no information on the syndromic diagnosis was
88 available. Clinical features and macrolide resistance genotyping results for 101 of the patients
89 have been reported previously (5,6). Nucleic acid extracts from the 257 patients with positive
90 MP results were retrospectively retrieved for further testing. Only one specimen from each
91 patient was included.

92

93 **Nucleic acid extraction.** Nucleic acid extraction was performed by using the NucliSENS
94 easyMAG extraction system (bioMérieux, France) and stored at -80 °C, as described
95 previously (5). All testing was performed on nucleic acid extracted from the clinical
96 specimens. Culture for MP was not performed.

97

98 **Real-time qPCR for the detection of *M. pneumoniae*.** Real-time quantitative (qPCR) was
99 conducted for the detection of MP using TaqMan universal PCR master mix (Applied
100 Biosystems) in a StepOnePlus instrument (Applied Biosystems, Foster City, CA), as
101 previously described (5). A series of 6 log₁₀ dilutions equivalent to ten to 1 × 10⁶ copies per
102 reaction mixture were prepared from a plasmid (pC-RII-TOPO vector; Invitrogen, CA)
103 containing the corresponding target bacterial sequence to generate calibration curves; these
104 were ran in parallel with the test specimens. The detection limit of the qPCR assay was
105 approximately ten copies per reaction mixture (5).

106

107 **MRMP genotype detection.** SimpleProbe real-time PCR coupled to melting curve analysis
108 (SimpleProbe PCR) was performed on the extracted nucleic acid from specimens to identify
109 MRMP. The MRMP assay was done by using the LightCycler FastStart DNA master
110 HybProbe kit (Roche Diagnostics, Germany) according to a published protocol (5). The
111 detection limit of SimpleProbe PCR for both wild-type and mutant was 10³ copies per
112 reaction. A randomly chosen subset of the specimens was subjected to Sanger sequencing for
113 confirmation.

114

115 **MLVA typing.** Previously published primers were used to amplify four variable-number
116 tandem-repeat (VNTR) loci (Mpn13-16) (24). Our initial testing showed that nonspecific
117 bands were commonly observed if all loci were amplified together in one multiplex reaction.

118 After optimization, good results were obtained through amplification of the loci in two
119 duplex reactions, one for Mpn13 and Mpn15, and one for Mpn14 and Mpn16. The PCR was
120 performed using 2 μ l nucleic acid, 15 μ l 2 \times QIAGEN multiplex master mixes (QIAGEN),
121 concentrations for each primers were 0.2 μ M for Mpn13 and Mpn15, 0.4 μ M for Mpn14 and
122 0.08 μ M for Mpn16. The total reaction mixture volume was made up to 30 μ l with nuclease-
123 free water. A Veriti 96-well thermal cycler (Applied Biosystems) was used for amplification.
124 Cycling conditions were as follows: denaturation step of 15 min at 95 $^{\circ}$ C, amplification step
125 of 40 cycles of 30 s at 95 $^{\circ}$ C, 30 s at 62 $^{\circ}$ C, and 45 s at 72 $^{\circ}$ C. The products were then pooled
126 into one tube for product size determination in one lane by capillary electrophoresis using an
127 ABI 3130 genetic analyser (Applied Biosystem), and the data was analysed using
128 GeneMapper software (version 4.0, Applied Biosystems). The primers were fluorescently
129 labelled at the 5' end with VIC (green, Mpn13 and Mpn15), NED (yellow, Mpn14) or 6-
130 FAM (blue, Mpn16) (Applied Biosystem). The fluorescent labels for the targets together with
131 the expected product sizes of each locus allowed sizing of the amplicons for all four loci in
132 one reaction mixture. The number of repeats for each locus was calculated according to the
133 PCR fragment size. The MLVA type was designated by the numeric combination of the
134 number of tandem repeats at four loci (Mpn13-16), as suggested previously (1,9). The
135 number of repeats was rounded up to an integer value (8,24). The number of repeats in each
136 locus (1 to 2 specimens for each product size) was confirmed by Sanger sequencing.

137

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

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142

143 **RESULTS**

144 **Prevalence of *M. pneumoniae*.** The percentage of test-positive patients per month from 2011
145 to 2014 is shown in Fig 1. Higher positive rates (more than 1 standard deviation above
146 average for the entire period) were observed in 2012 (April, June, July, September) and 2013
147 (May to July). The percentage of test-positive patients by year was 9.8% in 2011, 27.2% in
148 2012, 24.3% in 2013 and 11.4% in 2014 ($P < 0.001$).

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

153

154 **Prevalence of macrolide-resistant genotype.** The MP-positive specimens for 16 patients
155 were of an insufficient amount and not investigated further. Macrolide-resistant genotyping
156 could be successfully carried out on all specimens from the remaining 241 patients.
157 SimpleProbe real-time PCR coupled to melting curve analysis identified 34.9% (84/241) of
158 the unique patient specimens as MRMP genotype. The A2063G transition was the only
159 mutation identified. A subset of 88 specimens, including 61 with MSMP genotype and 27
160 with MRMP genotype was further analysed by Sanger sequencing. The results were 100%
161 concordant with melting curve analysis. The annual prevalence of MRMP among all MP-
162 positive patients had significantly increased from 13.6% (3/22) in 2011 to 30.7% (23/75) in
163 2012, 36.6% (34/93) in 2013 and 47.1% (24/51) in 2014 ($P = 0.038$). The prevalence of
164 MRMP genotype was higher among children (aged 0-1 years, 30.0%; aged 2-11 years, 36.1%;
165 aged 12-17 years, 39.7%) than in adults (aged 18-64 years, 20.0%, aged ≥ 65 years, 0%) but

166 the difference was not statistically significant ($P = 0.122$). MRMP prevalence among males
167 (33.7%) and females (35.7%) were similar ($P = 0.742$).

168

169 **Temporal changes in macrolide resistance rate and MLVA types.** Specimens from the
170 241 patients with sufficient DNA extracts were further investigated by MLVA typing and
171 successful results were obtained for 205 (85.1%) patients. The number of repeats in the four
172 loci were 3 to 5 for Mpn13, 4 to 6 for Mpn14, 6 to 7 for Mpn15 and 2 to 3 for Mpn16, giving
173 seven distinct MLVA types. The major types were 3-5-6-2 (44.4%), 4-5-7-2 (36.6%) and 4-5-
174 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
175 4-4-7-3 ($n = 1$) only accounted for 4.9% of the total.

176 During the four year period, types 4-5-7-2 and 3-5-6-2 were predominant (Fig. 2).
177 Together the two types comprised 75% to 85% of all positive specimens in each year. The
178 proportion of type 4-5-7-2 had an increasing trend from 29% in 2011, 34% in 2012, 36% in
179 2013 and 43% in 2014 but the difference was not statistically significant ($P = 0.686$). Of the
180 seven MLVA types, five and four MLVA types were found among specimens with
181 macrolide-sensitive *M. pneumoniae* (MSMP) and MRMP genotypes, respectively (Fig. 3A).
182 The two major MLVA types (3-5-6-2 and 4-5-7-2) occurred in the MRMP and MSMP groups
183 at different frequencies. The prevalence of MLVA type 4-5-7-2 was substantially higher in
184 the MRMP group than in the MSMP group (89.6% versus 10.9%, $P < 0.001$). In contrast,
185 MLVA type 3-5-6-2 was more prevalent in the MSMP group than in the MRMP group
186 (64.5% vs. 3.0%, $P < 0.001$). The other five MLVA types were found at low frequencies in
187 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-
188 6-6-2) only. Stratification by year revealed that macrolide resistance rate of MLVA type 4-5-
189 7-2 had significantly increased from 25.0% in 2011 to 100% in 2014 (Fig. 3B, $P < 0.001$).

190

191 **DISCUSSION**

192 In this study, an increase in the rate of MP infection was noted in 2012 and 2013, suggesting
193 that there was an epidemic outbreak during this period. During the entire period, changes in
194 the annual cycle of positive rates were irregular, with peaks in early summer (in 2012), mid-
195 summer (in 2013) or early autumn (in 2014). This is in line with reports describing more MP
196 infections with increased relative humidity and ambient temperature (17,22). In our
197 neighborhood areas, recent epidemic outbreaks of MP infections were also noted in South
198 Korea from 2010-2011, in Japan from 2011 to 2012 and in Beijing and Shanghai, China in
199 2012 (13,16,20). Notably, a substantial increase in the prevalence of MRMP was noted in the
200 areas during or shortly after those epidemics (12-14,16,20). Our data revealed that the MRMP
201 rate had increased by more than three folds from 13.6% in 2011 to 47.1% in 2014. Reported
202 rates of MRMP range from 62.9% in South Korea (12) and >80% to 90% in China and Japan
203 (14,26), compared to $\leq 10\%$ in Europe and the United States (9,19). The relationship between
204 MP epidemics and MRMP emergence is likely to be complex, involving selection pressure
205 from widespread administration of macrolides (12).

206 Two MLVA types (3-5-6-2, 44.4% and 4-5-7-2, 36.6%) accounted for 81.0% of the
207 infections during the study period. Both types occur worldwide and were among the
208 predominant types in many studies. Amongst international collections of MP isolates
209 collected over decades, 14.0%-20.8% and 50.6%-55.1% were of MLVA types 3-5-6-2 and 4-
210 5-7-2, respectively (1,8). During the periods with a high proportion of MP-positive specimens,
211 the major genotypes did not change (Figure 2A). This suggests that increased detection of
212 MP infections is likely a result of increased transmission of co-circulating MLVA types,
213 rather than introduction of new types to the community.

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

216 increased from 25% in 2011 to 100% in 2014. All the other MLVA types including the
217 prevalent 3-5-6-2 type remained largely macrolide-sensitive. Qu *et al* previously reported the
218 same significant correlation between macrolide resistance and susceptibility with type 4-5-7-
219 2 and type 3-5-6-2, respectively (20). In the two international MP collections described by
220 Degrange *et al* and Benitez *et al*, four (33.3%) of 12 and nine (90%) of ten MRMP isolates
221 were of MLVA type 4-5-7-2, respectively (1,8). In the United States, 13 (68.4%) of 19
222 MRMP isolates identified through CDC-assisted investigations across the country between
223 2006 and 2013 belonged to MLVA type 4-5-7-2 (9). In China, this MLVA type accounted for
224 >90% of the MRMP isolates identified in Beijing from 2010 to 2013 (20,24,25). Of the 21
225 distinct types that could be distinguished by the amended four loci MLVA scheme (1,3,4,8-
226 11,18,20,21,23-25) (Table S1, supplementary file), MRMP has been detected in 12 types.
227 Among the published reports, prevalence of the other 11 types among MRMP were low and
228 occurrence was sporadic (1,8,9,11,18,20,21,23-25).

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

240 In summary, we demonstrated a link between increasing macrolide resistance and the
241 expansion of MRMP strains of the MLVA type 4-5-7-2 in Hong Kong during 2011-2014. It
242 is worrying that type 4-5-7-2 may be associated with more severe disease (20). Increasing
243 public awareness, enhancing access to rapid diagnostics and improving surveillance for MP
244 and macrolide resistance is necessary to inform case and outbreak management and to
245 understand the burden of disease.

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251 and Health Bureau of the Government of the Hong Kong Special Administrative Region.

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

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261

262 **FIG 2.** MLVA type in *M. pneumoniae* specimens in a healthcare region in Hong Kong, 2011-
263 2014. The percentage of each MLVA type for each year is shown. The number of patients in
264 each year is shown within parentheses.

265

266

267 **FIG 3.** Macrolide resistance in *M. pneumoniae* specimens in a healthcare region in Hong
268 Kong, 2011-2014. (A) MLVA type according to macrolide resistance genotype. The
269 proportions of MLVA types for MRMP ($n = 67$) and MSMP ($n = 138$) groups are shown in
270 the outer and inner doughnuts, respectively. Others in the MRMP group included types 4-6-7-
271 3 and 5-5-7-2. Others in the MSMP group included types 3-6-6-2 and 4-4-7-3. (B) Changes
272 in macrolide resistance rate of MLVA type 4-5-7-2 during 2011-2014. The number of
273 patients in each year is shown within parentheses.

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