

Original Article

Multiple-Locus Variable-Number Tandem-Repeat Analysis of *Mycoplasma pneumoniae* Isolates between 2004 and 2014 in Yamagata, Japan: Change in Molecular Characteristics during an 11-year Period

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SUMMARY: Multiple-locus variable-number tandem-repeat analysis (MLVA) typing was performed for *Mycoplasma pneumoniae* strains isolated between 2004 and 2014 in Yamagata, Japan. The results were examined by considering the combination of the P1 type and prevalence of macrolide resistance-associated mutations. Four-locus (Mpn13–16) MLVA classified 347 strains into 9 MLVA types, including 3 major types: 3-5-6-2, 4-5-7-2, and 4-5-7-3. All type 3-5-6-2 strains (77 strains) were P1 type 2 variants (2a or 2c), while types 4-5-7-2 (181 strains) and 4-5-7-3 (75 strains) were P1 type 1. MLVA type 4-5-7-2 strains circulated and were dominant until 2010, accounting for 88.4% of the 121 strains isolated between 2004 and 2010. The prevalence of types 4-5-7-3 and 3-5-6-2 strains increased rapidly in 2011 and 2012, respectively, resulting in cocirculation of 3 MLVA types, including type 4-5-7-2, between 2011 and 2013. The prevalence of macrolide resistance-associated mutations in MLVA types 4-5-7-2, 4-5-7-3, and 3-5-6-2 strains was 59.7% (108/181), 25.3% (19/75), and 0% (0/77), respectively. Because the prevalence of macrolide resistance-associated mutations differed by current MLVA types in Yamagata, continued surveillance combined with molecular typing and identification of macrolide resistance-associated mutations is necessary.

INTRODUCTION

Mycoplasma pneumoniae is an important cause of upper and lower respiratory tract infections, mainly in children and young adults (1,2). This pathogen is responsible for up to 20% of all cases (3) and 30% of pediatric cases of community-acquired pneumonia (4,5).

Molecular typing of clinical isolates is important for understanding the epidemiology of *M. pneumoniae* infection. Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) was developed by Dégrange et al. (6) as a molecular typing method to compensate for P1 gene typing, the most common typing method for *M. pneumoniae* based on polymorphisms in the P1 gene encoding *M. pneumoniae* P1 adhesion protein (7). Although 5-locus MLVA (Mpn1, Mpn13–16) was reported by Dégrange et al. (6), exclusion of the Mpn 1 locus in future analysis is recommended because of its instability (8). Four-locus (Mpn13–16) MLVA can be used to classify *M. pneumoniae* into more than 20 MLVA types (9–13), whereas P1 gene typing classifies *M. pneumoniae* into 2 major types (types 1 and 2) and 3 P1 type 2 variants (types 2a, 2b, and 2c) (14–17).

M. pneumoniae infections can be treated with macrolides as first-line antibiotics (18,19). However, the prevalence of macrolide-resistant *M. pneumoniae* infections has increased since the year 2000, particularly in eastern Asian countries such as Japan and China (7). Since then, macrolide-resistant *M. pneumoniae* infections have spread worldwide, contributing to increasing global public health concerns (7,18).

Molecular epidemiological studies of *M. pneumoniae* examining MLVA type, P1 type, and macrolide susceptibility have been conducted in several countries and regions. The proportions of MLVA and P1 types were reported to vary among geographic regions, as has the prevalence of macrolide resistance-associated mutations (9–11,20,21). We also reported P1 types and the prevalence of macrolide resistance-associated mutations of *M. pneumoniae* isolates in Yamagata, Japan between 2004 and 2013 (22). However, few reports have described the MLVA types of *M. pneumoniae* and examined the association between MLVA type and macrolide susceptibility in Japan (23,24). In this study, we performed MLVA typing for *M. pneumoniae* isolated between 2004 and 2014 in Yamagata, Japan, and evaluated the molecular epidemiological features of *M. pneumoniae* including P1 type and prevalence of macrolide resistance-associated mutations during an 11-year period.

MATERIALS AND METHODS

Study design: A total of 347 *M. pneumoniae* strains isolated between January 2004 and December 2014 in Yamagata, Japan were used for MLVA typing. P1

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type and macrolide susceptibility profiles for 342 *M. pneumoniae* strains isolated between 2004 and 2013 have been reported previously (22). Profiles for the remaining 5 strains isolated in 2014 were determined in this study. All strains were isolated from clinical specimens collected at the Yamanobe Pediatric Clinic and Katsushima Pediatric Clinic in Yamagata, Japan (Fig. 1) in collaboration with the Yamagata Prefecture health authorities as part of the National Epidemiological Surveillance of Infectious Diseases, Japan.

Isolation of *M. pneumoniae* and DNA extraction: Isolation of *M. pneumoniae* from clinical specimens obtained in 2014 was performed as described previously (22,25). *M. pneumoniae* DNA was extracted from 200 µL of bacteria-enriched culture solution with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and eluted in 100 µL of DNase-free buffer.

MLVA typing: Four VNTR loci (Mpn13–16) were amplified by polymerase chain reaction (PCR) using extracted DNAs and primers as reported previously by Dégrange et al. (6). PCR products derived from 129 of 347 isolated strains were sequenced and the VNTR number of each locus was determined according to a previous study (8). The remaining 218 isolated strains were determined by their PCR product sizes using a microchip electrophoresis system (MCE-202; Shimadzu Corp., Kyoto, Japan), and the VNTR number of each locus was calculated according to the PCR product size. The MLVA type was represented by the combination of VNTR numbers at 4 loci as Mpn13–Mpn14–Mpn15–Mpn16.

P1 gene typing: P1 gene typing for 5 *M. pneumoniae* strains isolated in 2014 was performed using DNA extracted from *M. pneumoniae* strains isolated in accordance with the method based on PCR-restriction fragment length polymorphism as described previously (14,22,26). The P1 gene of *M. pneumoniae* was amplified by PCR using 2 primer pairs (ADH1 and ADH2, ADH3 and ADH4), and both amplicons were digested by the restriction enzyme *Hae*III (14,26). P1 type was identified by a combination of electrophoresis patterns of 2 digested amplicons as described previously (22).

Identification of macrolide resistance-associated mutations: Macrolide resistance-associated mutations in 5 *M. pneumoniae* strains isolated in 2014 were identified by sequencing as described previously (22,25). Macrolide resistance-associated mutations were defined as point mutations at nucleotides 2063, 2064, and 2617 (*M. pneumoniae* numbering) in domain V of the 23S rRNA gene (27,28).

Statistical analysis: Fisher's exact test was performed to determine the differences in the proportions of MLVA types between 2 periods, until 2010 and after 2011, and Fisher's exact test with the Holm method was performed to determine the differences in the prevalence of macrolide resistance-associated mutations by MLVA type of *M. pneumoniae* isolated between 2011 and 2013. These analyses were conducted using R version 3.3.2 (The R Foundation, Vienna, Austria). A *P* value < 0.05 was regarded as statistically significant.

RESULTS

A total of 347 *M. pneumoniae* strains were classified into 9 MLVA types including 3 major types: type 4-5-7-

Table 1. Molecular typing results of multiple-locus variable-number tandem-repeat analysis (MLVA) and P1 gene for 347 *Mycoplasma pneumoniae* strains isolated between 2004 and 2014 in Yamagata, Japan

MLVA type (Mpn13-14-15-16)	No. of isolates: P1 type ¹⁾				Total no. of isolates (%)
	1	2a	2b	2c	
3-5-6-2		9		68	77 (22.2)
3-6-6-2			5		5 (1.4)
4-4-7-3	1				1 (0.3)
4-5-6-2	3				3 (0.9)
4-5-7-1	3				3 (0.9)
4-5-7-2	181				181 (52.2)
4-5-7-3	75				75 (21.6)
4-6-7-2	1				1 (0.3)
5-5-7-3	1				1 (0.3)
Total	265	9	5	68	347 (100)

¹⁾ The profile of P1 type of 342 strains isolated between 2004 and 2013 were referred from our previous report (22).

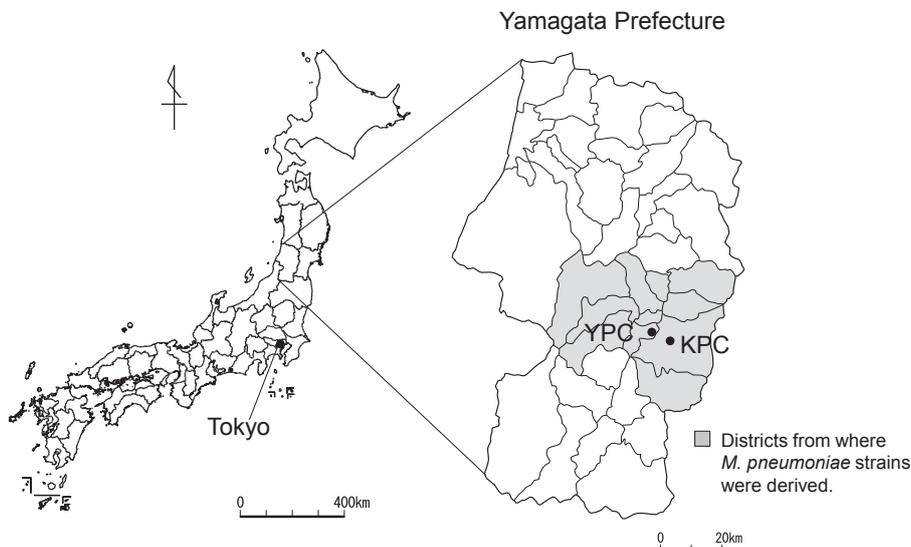


Fig. 1. Map of study area in Japan. Gray area in Yamagata Prefecture indicates districts from where *Mycoplasma pneumoniae* strains were derived. Abbreviations: YPC, Yamanobe Pediatric Clinic; KPC, Katsushima Pediatric Clinic.

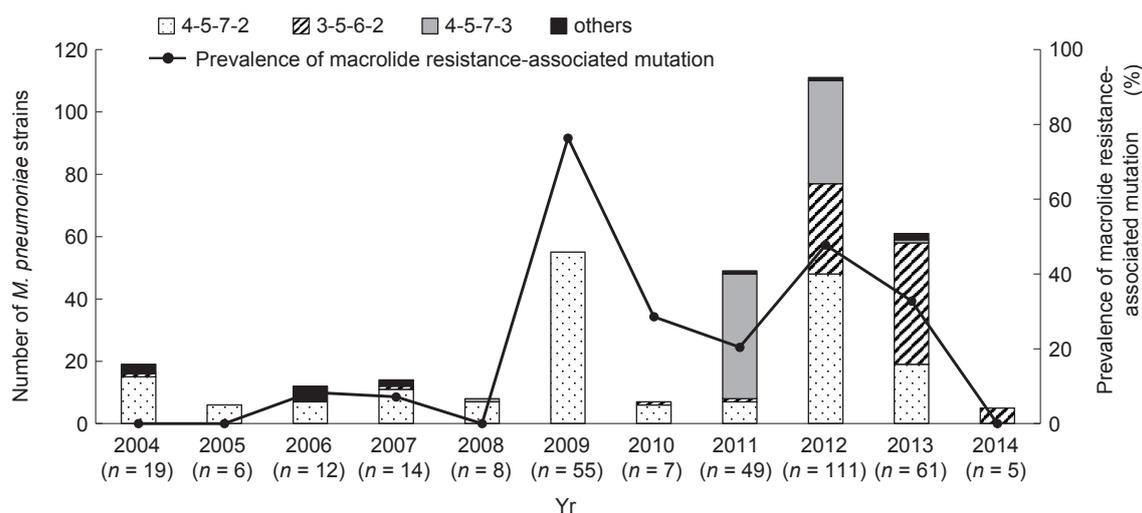


Fig. 2. Annual distribution of multiple-locus variable-number tandem-repeat analysis (MLVA) types and the prevalence of macrolide resistance-associated mutation of *Mycoplasma pneumoniae* strains isolated between 2004 and 2014 in Yamagata, Japan. The bar with dots shows MLVA type 4-5-7-2, the bar with an oblique line shows MLVA type 3-5-6-2, the gray bar shows MLVA type 4-5-7-3, and the black bar shows all other MLVA types (3-6-6-2, 4-5-6-2, 4-5-7-1, 4-4-7-3, 4-6-7-2, and 5-5-7-3). The number of *M. pneumoniae* strains per year is shown within parentheses. The line shows the prevalence of macrolide resistance-associated mutation of *M. pneumoniae* strains and the data between 2004 and 2013 based on a previous study (22).

2, 181 (52.2%); type 3-5-6-2, 77 (22.2%); and type 4-5-7-3, 75 (21.6%; Table 1). The P1 gene type of 5 strains isolated in 2014 was P1 type 2c. When combined with the P1 gene typing results determined in our previous study (22), all strains with a repeat number of 4 or 5 for Mpn13 were P1 type 1, whereas all strains with a repeat number of 3 for Mpn13 were P1 type 2 variants; type 3-5-6-2 were P1 type 2a or 2c, and type 3-6-6-2 were P1 type 2b.

The annual distribution of MLVA types is shown in Fig. 2. Type 4-5-7-2 was predominant between 2004 and 2010, with annual rates ranging from 58.3% (7/12 in 2006) to 100% (6/6 and 55/55 in 2005 and 2009, respectively). The numbers of types 4-5-7-3 and 3-5-6-2 increased rapidly in 2011 and 2012, respectively. The annual rate of increase for type 4-5-7-3 reached 81.6% (40/49) in 2011, while that for type 3-5-6-2 reached 26.1% (29/111) in 2012, 63.9% (39/61) in 2013, and 100% (5/5) in 2014.

The prevalence of the 3 major MLVA types (types 4-5-

7-2, 3-5-6-2, and 4-5-7-3) was statistically analyzed for 2 time periods, until 2010 and after 2011. Between 2004 and 2010, these 3 types accounted for 88.4% (107/121), 2.5% (3/121), and 0.8% (1/121) of strains, respectively. Between 2011 and 2014, the prevalence of types 4-5-7-2, 3-5-6-2, and 4-5-7-3 was 32.7% (74/226) for each type. Statistical analysis revealed that the prevalence of MLVA type differed significantly between the 2 time periods ($P < 0.001$).

Sequencing of domain V of the 23S rRNA gene revealed that 5 strains isolated in 2014 harbored no macrolide resistance-associated mutations. To reveal the association between MLVA type and macrolide resistance, the results for MLVA type were combined with the profile of macrolide resistance-associated mutations determined in this study and in our previous study (22) (Table 2). Among the 3 major MLVA types, none of type 3-5-6-2, 59.7% (108/181) of type 4-5-7-2, and 25.3% (19/75) of type 4-5-7-3 strains harbored macrolide resistance-associated mutations. The most

Table 2. Multiple-locus variable-number tandem-repeat analysis (MLVA) type and macrolide susceptibility of 347 *Mycoplasma pneumoniae* strains isolated between 2004 and 2014 in Yamagata, Japan

MLVA type (Mpn13-14-15-16)	Macrolide resistance-associated mutation ¹⁾		Rate of macrolide resistance (%)
	Negative	Positive (mutation of 23S rRNA, no.)	
3-5-6-2	77	0	0
3-6-6-2	5	0	0
4-4-7-3	0	1 (A2063G, 1)	100
4-5-6-2	3	0	0
4-5-7-1	3	0	0
4-5-7-2	73	108 (A2063G, 63; A2063T, 43; A2064C, 1; C2617G, 1)	59.7
4-5-7-3	56	19 (A2063G, 14; A2063C, 1; C2617A, 1; C2617G, 3)	25.3
4-6-7-2	1	0	0
5-5-7-3	0	1 (A2063G, 1)	100
Total	218	129	37.2

¹⁾ The profile of macrolide resistance-associated mutation of 342 *M. pneumoniae* strains isolated between 2004 and 2013 were referred from our previous report (22).

common mutation, A2063G ($n = 79$) (18), was detected in 34.8% (63/181) of type 4-5-7-2 and 18.7% (14/75) of type 4-5-7-3 strains. As shown in Fig. 2, the prevalence of macrolide resistance-associated mutations increased between 2009 and 2013. We previously reported that all strains harboring the A2063T mutation ($n = 43$) were isolated between July 2009 and January 2010 (22). The present study revealed that all strains isolated in 2009 ($n = 55$) were identified in type 4-5-7-2, with 76.4% (42/55) harboring A2063T mutations. When limited to the period between 2011 and 2013, during which the 3 major MLVA types coexisted, the prevalence of macrolide resistance-associated mutations in the MLVA type 4-5-7-2 strains (83.8% [62/74]) was significantly higher than that in type 4-5-7-3 (25.7% [19/74]) ($P < 0.001$) and type 3-5-6-2 (0% [0/69]) ($P < 0.001$) strains.

DISCUSSION

Epidemics of *M. pneumoniae* infections have been observed in 3- to 7-year intervals in many areas worldwide (7). In Japan, a large epidemic of *M. pneumoniae* infections was observed between 2011 and 2013 (29). In this study, the number of *M. pneumoniae* strains isolated increased with 2 peaks in 2009 and from 2011 to 2013 in Yamagata, Japan (Fig. 2). The increased incidence of *M. pneumoniae* infections in 2009 in Yamagata was due to a community outbreak that occurred mainly in a junior high school and primary school located in the same town as reported previously (25). The macrolide resistance-associated mutation A2063T was detected in 96.0% and 86.7% of strains isolated from students who attended the 2 schools, respectively (25). The present study revealed that the community outbreak in 2009 was due to a single MLVA type, type 4-5-7-2, which has circulated in Yamagata since 2004. Therefore, increased *M. pneumoniae* infections in 2009 potentially depended on acquisition of the A2063T mutation for MLVA type 4-5-7-2. In contrast, the increase in type 4-5-7-3 from 2011 to 2012 and type 3-5-6-2 from 2012 to 2013 resulted in cocirculation with 3 different MLVA types between 2011 and 2013 in Yamagata. MLVA types 3-5-6-2 and 4-5-7-3 were rarely isolated in Yamagata until 2010 (Fig. 2). Therefore, this result suggests that newly introduced MLVA types caused a large epidemic of *M. pneumoniae* infections between 2011 and 2013 in Yamagata. However, although Kubota et al. (23) reported that 100% (14/14) of *M. pneumoniae* strains isolated in Tokyo, Japan, between 2011 and 2013 were type 4-5-7-2, details of proportions of MLVA type in the other areas of Japan remain unclear because of insufficient study numbers. Thus, further studies of the molecular epidemiology of *M. pneumoniae* in other areas of Japan are needed.

The present study revealed that the prevalence of MLVA types changed in 2011. MLVA types 4-5-7-2 and 3-5-6-2 were the predominant types in studies from various geographic regions (9–11,20,21,23). A greater than 60% prevalence of type 4-5-7-2 was reported between 2004 and 2010 in Yamagata, Japan (this study); between 2004 and 2015 in Beijing, China (9); and between 2006 and 2009 in the United States (20). Additionally, type 4-5-7-2 showed a $\geq 30\%$ prevalence

in various regions worldwide during a respective study period, including Tokyo, Japan (23); Hong Kong (10); Germany (21); and Australia (11). The prevalence of MLVA type 3-5-6-2 was low: $< 30\%$, in Yamagata, Japan (this study); China (9); the United States (20); and Australia (11) until 2010. However, after 2011, the prevalence of MLVA type 3-5-6-2 was $> 30\%$ in those countries (the present study, 11,20), Hong Kong (10), and Germany (21) except for China (9). These observations suggest that changes in the proportion of MLVA types are due to a surge of type 3-5-6-2, which may have occurred worldwide except for in China. This and several previous studies demonstrated that most strains of MLVA type 3-5-6-2 were P1 type 2 or its variant, whereas MLVA type 4-5-7-2 was P1 type 1 (9,11–13,21,30). Therefore, the increase in MLVA type 3-5-6-2 after 2011 may be a type-shift phenomenon of *M. pneumoniae* P1 types (7).

MLVA type 4-5-7-3 was rarely detected worldwide. In China, only 5 (1.0%) strains were type 4-5-7-3 among the 480 *M. pneumoniae*-positive specimens collected between 2003 and 2015 (9). Moreover, 0%, 1.1%, and 3.3% prevalence rates of type 4-5-7-3 were reported between 2006 and 2013 in the United States (20), between 2011 and 2012 in Germany (21), and between 2008 and 2012 in Australia (11). Because approximately 10% or higher prevalence of type 4-5-7-3 was observed only in Yamagata, Japan (32.7% between 2011 and 2014), Hong Kong (14.1% between 2011 and 2014) (10), and Nakhon Phanom, Thailand (9.7% between 2009 and 2012) (31), the upsurge of MLVA type 4-5-7-3 in Yamagata after 2011 may have been associated with epidemics in those countries. Diaz et al. (32) recently reported that strains with 3 tandem repeats in the Mpn 16 region formed a distinct subgroup named as IN within the P1 type 1 branch by phylogenetic analysis using the *M. pneumoniae* core genome. Therefore, the increase in MLVA type 4-5-7-3 may represent the emergence of a new subtype of *M. pneumoniae* P1 type 1 (32).

In this study, during the period when 3 major MLVA types (types 4-5-7-2, 3-5-6-2, and 4-5-7-3) coexisted, the prevalence of macrolide resistance-associated mutations in MLVA type 4-5-7-2 (83.8%) strains was significantly greater than that in types 3-5-6-2 (0%) and 4-5-7-3 (25.7%) in Yamagata, Japan. In China, the rates of macrolide resistance-associated mutations were reported to increase with increases in MLVA type 4-5-7-2 strains, suggesting an association between MLVA type and macrolide resistance (9). However, several previous reports found that no correlation exists between MLVA or P1 type and macrolide resistance (6,20). In Yamagata, during the study period, the prevalence of the A2063G mutation in MLVA type 4-5-7-2 strains was only 0.9% (1/107) between 2004 and 2010, but was 83.8% (62/74) between 2011 and 2014. Taken together, these observations indicate that the frequency of macrolide resistance is related to the selection or development of resistant strains by macrolide use rather than by MLVA type. Although the prevalence of macrolide resistance-associated mutations in MLVA types 3-5-6-2 and 4-5-7-3 strains is currently low in Yamagata, Japan, careful monitoring is necessary to detect the emergence of macrolide resistance.

Several studies have indicated that the discrimination power of four-locus MLVA is low because the unstable Mpn 1 locus is excluded (12,21,24). Therefore, to improve the discrimination power of MLVA for *M. pneumoniae*, a new MLVA scheme should be developed that includes additional VNTR markers such as those identified in a recent study (33). In conclusion, MLVA combined with P1 gene typing is a useful tool to assess the dissemination of *M. pneumoniae* strains. Because the prevalence of macrolide resistance-associated mutations differed by MLVA/P1 type, continued surveillance combined with molecular typing and the identification of macrolide resistance mutations are necessary.

Conflict of interest None to declare.

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