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1 **Title:** *PI* gene of *Mycoplasma pneumoniae* isolated from 2016 to 2019 and relationship between
2 genotyping and macrolide resistance in Hokkaido, Japan

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38 **Key words:** Mycoplasma pneumoniae, macrolides, antibiotic resistance, Japan

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40 **Running Title:** Macrolide resistance of mycoplasma pneumoniae in Japan

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42 **GenBank accession number:** The complete nucleotide sequence of the isolate Mp3896 P1 protein
43 gene (4,908 bp) has been assigned GenBank accession no. EF656612.

44

45 **Abstract**

46 We characterized 515 Mycoplasma pneumoniae specimens in Hokkaido. In 2013 and 2014, *pl*
47 gene type 1 strain, mostly macrolide-resistant, was dominant and the prevalence of macrolide
48 resistance was over 50%. After 2017, *pl* gene type 2 lineage, mostly macrolide-sensitive, increased
49 and the prevalence of macrolide resistance became 31.0% in 2017, 5.3% in 2018 and 16.3% in 2019.
50 (57 words)

51

52 **Text**

53 *Mycoplasma pneumoniae* (*M. pneumoniae*) is a common bacterial cause of pneumonia and
54 bronchitis, particularly in children and young adults [1]. Since about the year 2000, macrolide-
55 resistant *M. pneumoniae* (MRMP) has been appearing in Asia, Europe, Canada, and the USA [1, 2]
56 and has been gradually increasing in other areas of the world as well [1, 2]. Periodical increases in
57 the number of patients with *M. pneumoniae* pneumonia in 3-7-year cycles have been reported from

various parts of the world [3, 4]. In Japan, large epidemics of *M. pneumoniae* pneumonia occurred in 2011, 2012, 2015, and 2016 according to reports by the National Epidemiological Surveillance of Infectious Diseases (Figure 1A). In Hokkaido, the northernmost island of Japan, large epidemics of *M. pneumoniae* pneumonia occurred in 2018 and 2019 (Figure 1B). In the epidemics in 2011 and 2012, a high prevalence of MRMP strains among clinical isolates from wide areas of Japan was reported [5, 6]. It is unclear at present whether the emergence and spread of MRMP strains affect epidemiological patterns of *M. pneumoniae* pneumonia. To obtain a better understanding of these aspects of *M. pneumoniae* infections, it is important to examine the genetic properties of *M. pneumoniae* in patients with *M. pneumoniae* pneumonia. In this study, we performed genotyping analysis of 515 *Mycoplasma pneumoniae* specimens from patients with *M. pneumoniae* pneumonia in Hokkaido, which is the northernmost island of Japan and comprises about 22% of the total land area of the country. The aim of this study was to clarify the longitudinal changes in genotype and macrolide resistance (MR) of *M. pneumoniae* in one area of Japan.

Nasopharyngeal swab samples were collected from pediatric patients who were suspected of having respiratory tract infections associated with *M. pneumoniae* from January 2013 to December 2019 at 16 pediatric clinics and in the department of pediatrics in 17 hospitals in Hokkaido, Japan. DNA of *M. pneumoniae* was detected by real-time PCR using Mp181-F and Mp181-R primer pairs and an Mp181-P probe as described elsewhere [7, 8]. Mutations associated with resistance to macrolides at sites 2063, 2064, and 2617 in the *M. pneumoniae* 23S rRNA domain V gene region

77 were detected by a sequencing method described elsewhere [9]. *M. pneumoniae* showing a point
78 mutation in domain V of the 23S rRNA gene was defined as MRMP. The *p1* gene, encoding P1
79 cytidhesin, an essential pathogenic factor of *M. pneumoniae*, was subtyped by a PCR-based method
80 [10]. All statistical analyses were performed using JMP software version 13.2.0 (SAS Institute,
81 Cary, NC, USA). Written informed consent was obtained from all patients or guardians.

82 In this study, we collected 829 nasopharyngeal swab samples, and DNA of *M. pneumoniae* was
83 detected from 515 specimens by real-time PCR. MR-associated mutations were found in 157
84 (30.5%) of the 515 specimens (Table 1). The prevalence of MR was higher than 50% in 2013 and
85 2014 (51.1% - 52.0%) but decreased significantly to 31.0% in 2017, 5.3% in 2018 and 16.3% in
86 2019 ($P < 0.01$, Fisher's exact test) (Figure 2A, Table 1). These results are consistent with the
87 results of a previous study conducted in Osaka Prefecture, which is the southwestern part of Japan,
88 showing a high prevalence of MR between 2011 and 2014 and a decrease in the prevalence of MR
89 from 2015 [11]. The decrease of MR in *M. pneumoniae* strains after 2015 can probably be
90 explained as follows. The *p1* subtyping analysis in the present study revealed that the type 1 strain
91 was dominant in specimens in 2013 and 2014 (56.0% - 64.8%) (Figure 2A, Table 1). Most of the
92 type 1 strains were macrolide-resistant (Figure 2C, Table 1). Afterwards, the type 2 lineage (types
93 2, 2b, 2c and 2d) increased and accounted for more than half of all specimens between 2015 and
94 2019 (62.3%-92.9%) (Figure 2A, Table 1). *M. pneumoniae* strains belonging to type 2 lineage were
95 mostly macrolide-sensitive (Figure 2C, Table 1). Such a genotype shift from type 1 lineage to type

2 lineage may be the major reason for the decrease in the prevalence of MR of *M. pneumoniae* after 2015. A decrease in the sales volume of antibiotics including macrolides in Japan (<http://amrcrc.ncgm.go.jp/surveillance/020/20181128172618.html>) may be another reason. The decrease in the sales volume of antibiotics may have contributed to the decrease of MR in type 1 strain (92.9% in 2014 to 63.6% in 2019) and continuous low MR rate of type 2 lineage (19.4% in 2013 and 10.8% in 2019) (Table 1 and Figure 2B, 2C). However, the major factor for the current decrease of MR prevalence in *M. pneumoniae* in Japan is thought to be the genotype shift from type 1 lineage to type 2 lineage of *M. pneumoniae*, which was probably caused by interactions between the pathogen and herd immunity of the human population [6, 12, 13].

The real reason for the correlation between MR and *p1* gene type revealed in this study is unknown. The macrolide-resistant type 1 strain was probably selected and emerged by extensive clinical use of macrolides during the 2000s, when the type 1 strain was dominant [5, 6, 12-14]. Although type 2 lineage strains were also present in the 2000s, they were not major causes of *M. pneumoniae* pneumonia. Therefore, the type 2 strains were not exposed to macrolide therapy in 2000s and they sustained macrolide sensitivity.

Information on macrolide administration before sampling was available for 301 of the 515 patients. In the type 1 strain group, the prevalence of MRMP in patients with macrolide pre-administration was 92.3% (24 of 26 patients) and that in patients without macrolide pre-administration was 83.7% (41 of 49 patients) ($P = 0.48$, Fisher's exact test). In the type 2 lineage

115 group, the prevalence of MRMP in patients with macrolide pre-administration was 16.7% (6 of 36
116 patients) and that in patients without macrolide pre-administration was 4.7% (9 of 190 patients), and
117 the difference was statistically significant ($P=0.01$, Fisher's exact test). These results suggest that
118 the prevalence of MRMP could be increased in the type 2 lineage *M. pneumoniae* as in the type 1
119 strain if the type 2 lineage is exposed frequently to macrolides. Although most strains of the type 2
120 lineage isolated from patients are still macrolide-sensitive in Japan at present, surveillances of
121 macrolide sensitivities of *M. pneumoniae*, genotypes of *M. pneumoniae* and sales volume of
122 antibiotics should be continued.

123

124 **Conflicts of interest**

125 The authors declare that there are no conflicts of interest.

126

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130

131 **Ethical approval**

132 All of the necessary ethics approval for this study was obtained from the Institutional Review Board
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134 Written informed consent was obtained from all patients or guardians.

135

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141

142 **References**

- 143 1. **Waites KB, Xiao L, Liu Y, Balish MF, Atkinson TP.** Mycoplasma pneumoniae from the
144 Respiratory Tract and Beyond. Clinical microbiology reviews 2017;30(3):747-809.
- 145 2. **Pereyre S, Goret J, Bebear C.** Mycoplasma pneumoniae: Current Knowledge on
146 Macrolide Resistance and Treatment. Front Microbiol 2016;7:974.
- 147 3. **Eun BW, Kim NH, Choi EH, Lee HJ.** Mycoplasma pneumoniae in Korean children: the
148 epidemiology of pneumonia over an 18-year period. J Infect 2008;56(5):326-331.
- 149 4. **Brown RJ, Nguipdop-Djomo P, Zhao H, Stanford E, Spiller OB et al.** Mycoplasma
150 pneumoniae Epidemiology in England and Wales: A National Perspective. Front Microbiol
151 2016;7:157.
- 152 5. **Kubota H, Okuno R, Hatakeyama K, Uchitani Y, Sadamasu K et al.** Molecular typing

- 153 of *Mycoplasma pneumoniae* isolated from pediatric patients in Tokyo, Japan. *Jpn J Infect Dis*
154 2015;68(1):76-78.
- 155 6. **Suzuki Y, Seto J, Itagaki T, Aoki T, Abiko C et al.** [Gene Mutations Associated with
156 Macrolide-resistance and *p1* Gene Typing of *Mycoplasma pneumoniae* Isolated in Yamagata, Japan,
157 between 2004 and 2013]. *Kansenshogaku Zasshi* 2015;89(1):16-22.
- 158 7. **Winchell JM, Thurman KA, Mitchell SL, Thacker WL, Fields BS.** Evaluation of three
159 real-time PCR assays for detection of *Mycoplasma pneumoniae* in an outbreak investigation. *J Clin*
160 *Microbiol, Evaluation Studies* 2008;46(9):3116-3118.
- 161 8. **Ishiguro N, Koseki N, Kaiho M, Ariga T, Kikuta H et al.** Therapeutic efficacy of
162 azithromycin, clarithromycin, minocycline and tosufloxacin against macrolide-resistant and
163 macrolide-sensitive *Mycoplasma pneumoniae* pneumonia in pediatric patients. *PLoS One*
164 2017;12(3):e0173635.
- 165 9. **Matsuoka M, Narita M, Okazaki N, Ohya H, Yamazaki T et al.** Characterization and
166 molecular analysis of macrolide-resistant *Mycoplasma pneumoniae* clinical isolates obtained in
167 Japan. *Antimicrob Agents Chemother, Research Support, Non-U.S. Gov't* 2004;48(12):4624-4630.
- 168 10. **Cousin-Allery A, Charron A, de Barbeyrac B, Fremy G, Skov Jensen J et al.** Molecular
169 typing of *Mycoplasma pneumoniae* strains by PCR-based methods and pulsed-field gel
170 electrophoresis. Application to French and Danish isolates. *Epidemiology and infection*
171 2000;124(1):103-111.

- 172 11. **Katsukawa C, Kenri T, Shibayama K, Takahashi K.** Genetic characterization of
173 *Mycoplasma pneumoniae* isolated in Osaka between 2011 and 2017: Decreased detection rate of
174 macrolide-resistance and increase of *pl* gene type 2 lineage strains. PLoS One 2019;14(1):e0209938.
- 175 12. **Yamazaki T, Kenri T.** Epidemiology of *Mycoplasma pneumoniae* Infections in Japan and
176 Therapeutic Strategies for Macrolide-Resistant *M. pneumoniae*. Front Microbiol 2016;7:693.
- 177 13. **Kenri T, Okazaki N, Yamazaki T, Narita M, Izumikawa K et al.** Genotyping analysis of
178 *Mycoplasma pneumoniae* clinical strains in Japan between 1995 and 2005: type shift phenomenon of
179 *M. pneumoniae* clinical strains. J Med Microbiol, Research Support, Non-U.S. Gov't 2008;57(Pt
180 4):469-475.
- 181 14. **Ando M, Morozumi M, Adachi Y, Ubukata K, Iwata S.** Multilocus Sequence Typing of
182 *Mycoplasma pneumoniae*, Japan, 2002-2016. Emerg Infect Dis 2018;24(10):1895-1901.

183

184 **Figure legends**

185 Figure 1. Surveillance of pneumonia cases associated with *M. pneumoniae* infection in Japan: (A)
186 national average and (B) Hokkaido. The latest data are available from the website of the National
187 Institute of Infectious Diseases (<https://www.niid.go.jp/niid/ja/idwr.html>).

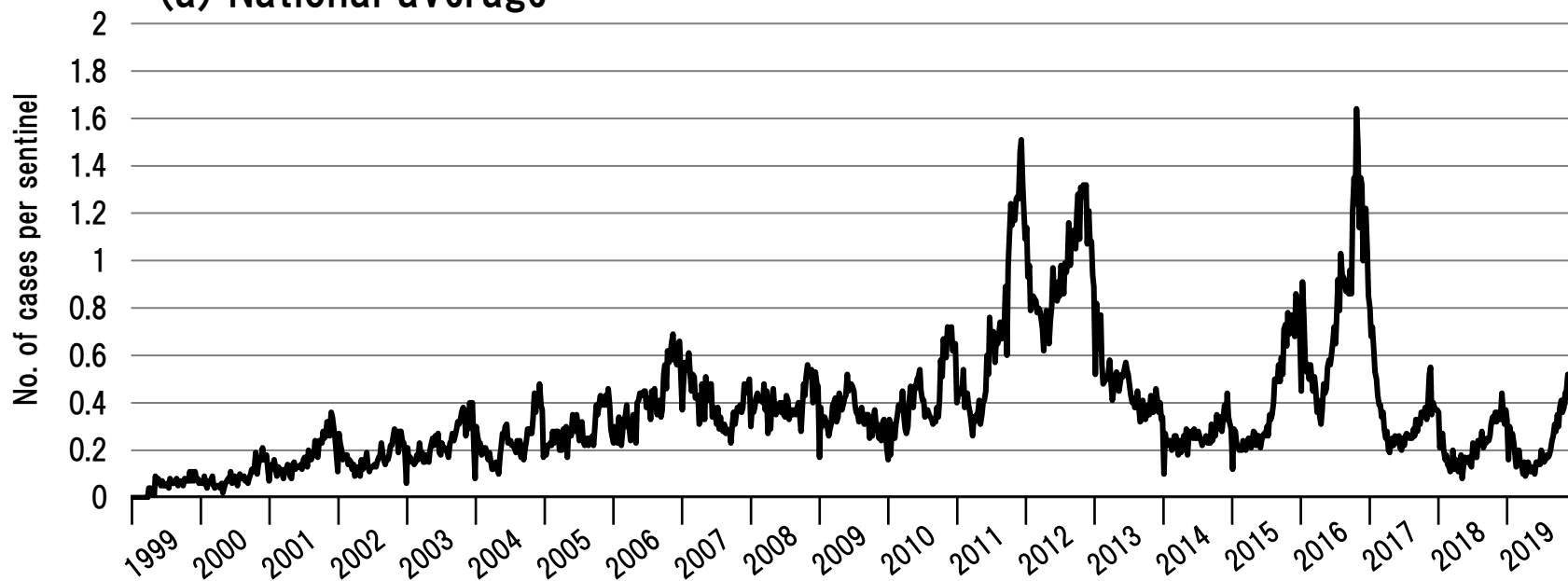
188

189 Figure 2. Annual rates of macrolide resistance and *pl* gene types of *Mycoplasma pneumoniae* in
190 Hokkaido in 2013-2019. (A) Macrolide resistance and *pl* gene types. (B) Macrolide resistance and

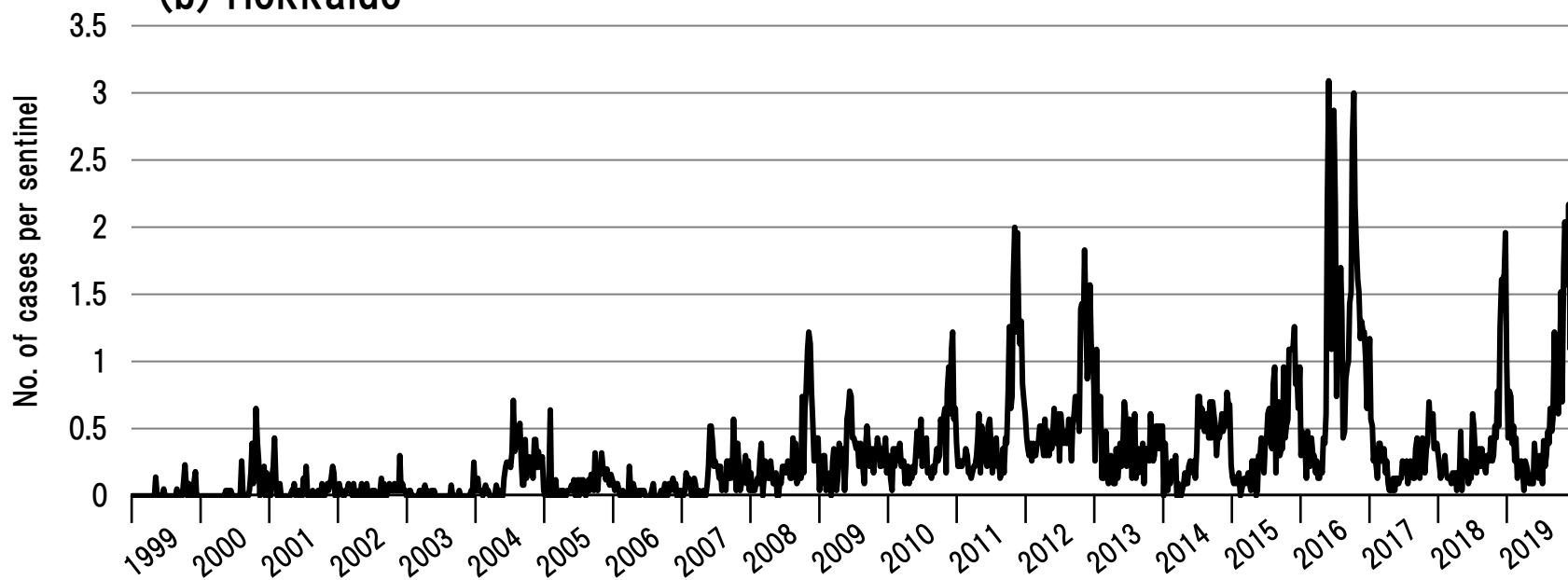
191 *pl* gene type 1 strain. (C) Macrolide resistance and *pl* gene type 2 lineage strains. Also see Table

192 1.

(a) National average



(b) Hokkaido



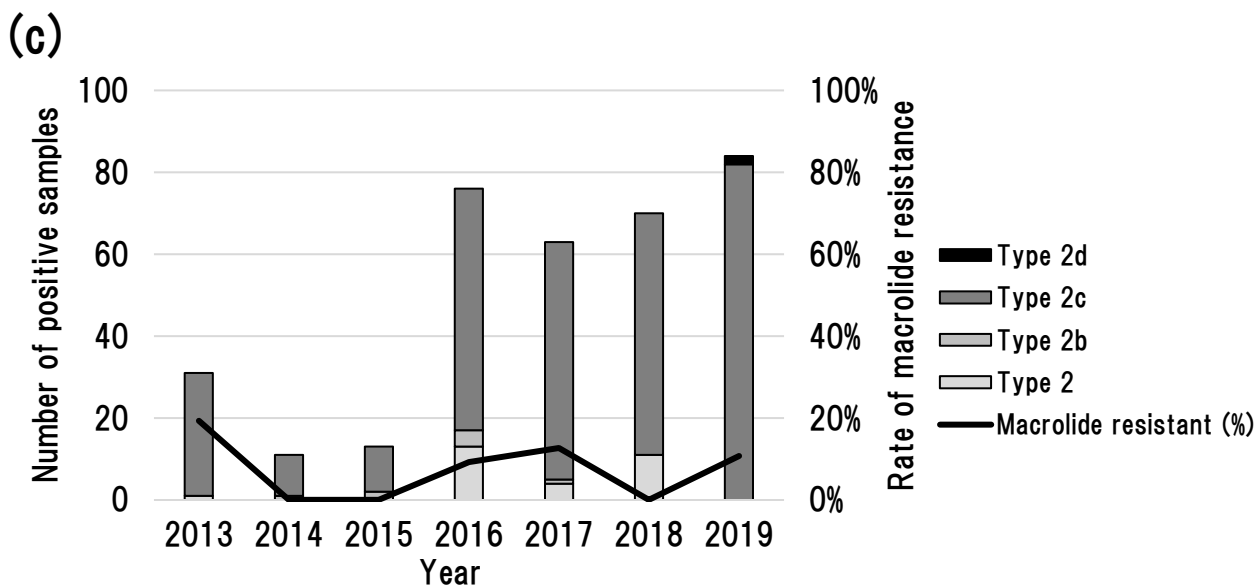
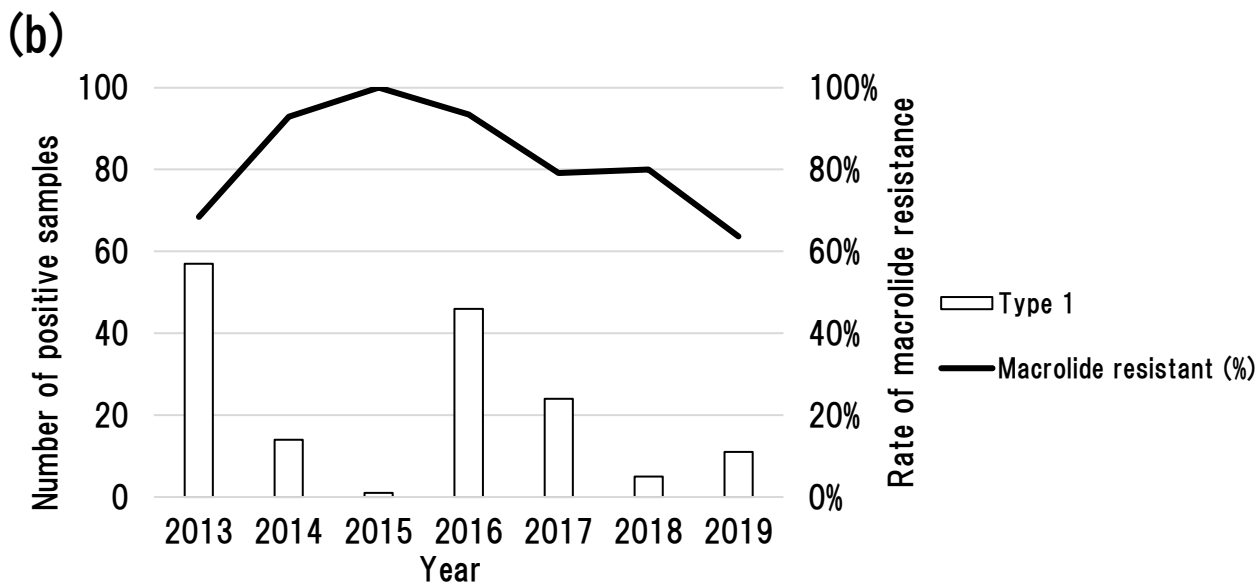
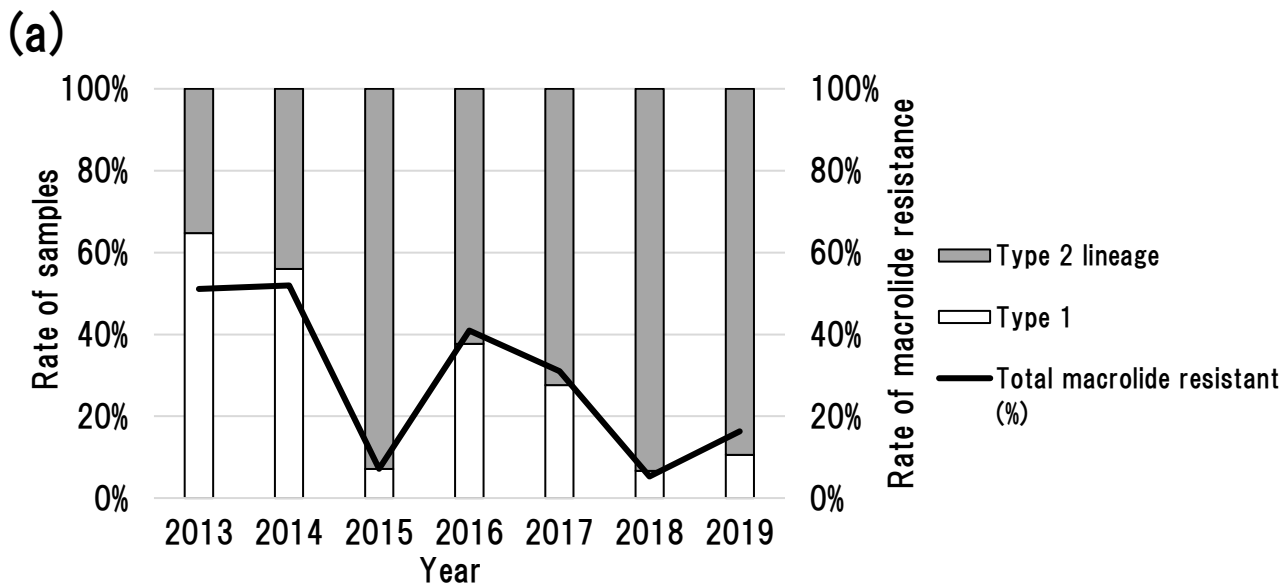


Table 1. Annual number of samples of *Mycoplasma pneumoniae* categorized by macrolide resistance and *p1* gene types*

p1 typing		Macrolide susceptibility	mutation in the 23S rRNA gene	2013	2014	2015	2016	2017	2018	2019
Type 1		Susceptible		18 (31.6%)	1 (7.1%)	0 (0.0%)	3 (6.5%)	5 (20.8%)	1 (20.0%)	4 (36.4%)
		Resistant	A2063G	39 (68.4%)	13 (92.9%)	1 (100%)	43 (93.5%)	19 (79.2%)	4 (80.0%)	7 (63.6%)
		(subtotal)		57 (64.8%)	14 (56.0%)	1 (7.1%)	46 (37.7%)	24 (27.6%)	5 (6.7%)	11 (10.6%)
Type 2	2	Susceptible		1	0	0	12	1	11	9
		Resistant	A2063G	0	0	0	1	2	0	0
			C2617G	0	0	0	0	1	0	0
	2b	Susceptible		0	1	2	4	1	0	0
	2c	Susceptible		24	10	11	53	53	59	72
		Resistant	A2063G	6	0	0	4	4	0	10
			A2064G	0	0	0	1	1	0	0
			C2617G	0	0	0	1	0	0	0
	2d	Susceptible		0	0	0	0	0	0	2
	(subtotal)			31 (35.2%)	11 (44.0%)	13 (92.9%)	76 (62.3%)	63 (72.4%)	70 (93.3%)	93 (89.4%)
		Susceptible		25 (80.6%)	11 (100%)	13 (100%)	69 (90.8%)	55 (87.3%)	70 (100%)	83 (89.2%)
		Resistant		6 (19.4%)	0 (0.0%)	0 (0.0%)	7 (9.2%)	8 (12.7%)	0 (0.0%)	10 (10.8%)
	Total			88 (100%)	25 (100%)	14 (100%)	122 (100%)	87 (100%)	75 (100%)	104 (100%)
		Susceptible		43 (48.9%)	12 (48.0%)	13 (92.9%)	72 (59.0%)	60 (69.0%)	71 (94.7%)	87 (83.7%)
		Resistant		45 (51.1%)	13 (52.0%)	1 (7.1%)	50 (41.0%)	27 (31.0%)	4 (5.3%)	17 (16.3%)

* DNA was extracted with a DNA extraction kit (Smitest EX-R&D, Medical & Biological Laboratories Co., Nagoya, Japan) from the sample buffer in various assays described below. These assays were used to find *M. pneumoniae*-positive samples. FUJI DRI-CHEM IMMUNO AG cartridge Myco (FUJIFILM Co., Tokyo, Japan), loop-mediated isothermal amplification (LAMP) assay kit (Eiken Chemical Co., Tokyo, Japan), Ribotest Mycoplasma (Asahi Kasei Pharma Co., Tokyo, Japan), ImunoAce Mycoplasma (Tauns Laboratories Inc., Shizuoka, Japan), QuickNavi-Mycoplasma (Otsuka Pharmaceutical Co., Tokyo, Japan), Prolast Myco (LSI medience Co., Tokyo, Japan), Quick Chaser Auto Myco (Mizuho Medy Co., Saga, Japan), Genecube Mycoplasma Pneumoniae (Toyobo Co., Tokyo, Japan), Prime Check Mycoplasma (Alfresa Pharma Co., Osaka, Japan) and TRCReady MP (Tosoh Co., Tokyo, Japan).