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Title: *P1* gene of Mycoplasma pneumoniae isolated from 2016 to 2019 and relationship between genotyping and macrolide resistance in Hokkaido, Japan

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

Running Title: Macrolide resistance of mycoplasma pneumoniae in Japan

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

Abstract

We characterized 515 Mycoplasma pneumoniae specimens in Hokkaido. In 2013 and 2014, *p1* gene type 1 strain, mostly macrolide-resistant, was dominant and the prevalence of macrolide resistance was over 50%. After 2017, *p1* gene type 2 lineage, mostly macrolide-sensitive, increased and the prevalence of macrolide resistance became 31.0% in 2017, 5.3% in 2018 and 16.3% in 2019. (57 words)

Text

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

58 various parts of the world [3, 4]. In Japan, large epidemics of *M. pneumoniae* pneumonia occurred
59 in 2011, 2012, 2015, and 2016 according to reports by the National Epidemiological Surveillance of
60 Infectious Diseases (Figure 1A). In Hokkaido, the northernmost island of Japan, large epidemics of
61 *M. pneumoniae* pneumonia occurred in 2018 and 2019 (Figure 1B). In the epidemics in 2011 and
62 2012, a high prevalence of MRMP strains among clinical isolates from wide areas of Japan was
63 reported [5, 6]. It is unclear at present whether the emergence and spread of MRMP strains affect
64 epidemiological patterns of *M. pneumoniae* pneumonia. To obtain a better understanding of these
65 aspects of *M. pneumoniae* infections, it is important to examine the genetic properties of *M.*
66 *pneumoniae* in patients with *M. pneumoniae* pneumonia. In this study, we performed genotyping
67 analysis of 515 Mycoplasma pneumoniae specimens from patients with *M. pneumoniae* pneumonia
68 in Hokkaido, which is the northernmost island of Japan and comprises about 22% of the total land
69 area of the country. The aim of this study was to clarify the longitudinal changes in genotype and
70 macrolide resistance (MR) of *M. pneumoniae* in one area of Japan.
71 Nasopharyngeal swab samples were collected from pediatric patients who were suspected of
72 having respiratory tract infections associated with *M. pneumoniae* from January 2013 to December
73 2019 at 16 pediatric clinics and in the department of pediatrics in 17 hospitals in Hokkaido, Japan.
74 DNA of *M. pneumoniae* was detected by real-time PCR using Mp181-F and Mp181-R primer pairs
75 and an Mp181-P probe as described elsewhere [7, 8]. Mutations associated with resistance to
76 macrolides at sites 2063, 2064, and 2617 in the *M. pneumoniae* 23S rRNA domain V gene region

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

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

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

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Ethical approval

All of the necessary ethics approval for this study was obtained from the Institutional Review Board of Hokkaido University Hospital for Clinical Research (014-0269, 016-0097, 017-0443, 018-0051).

Written informed consent was obtained from all patients or guardians.

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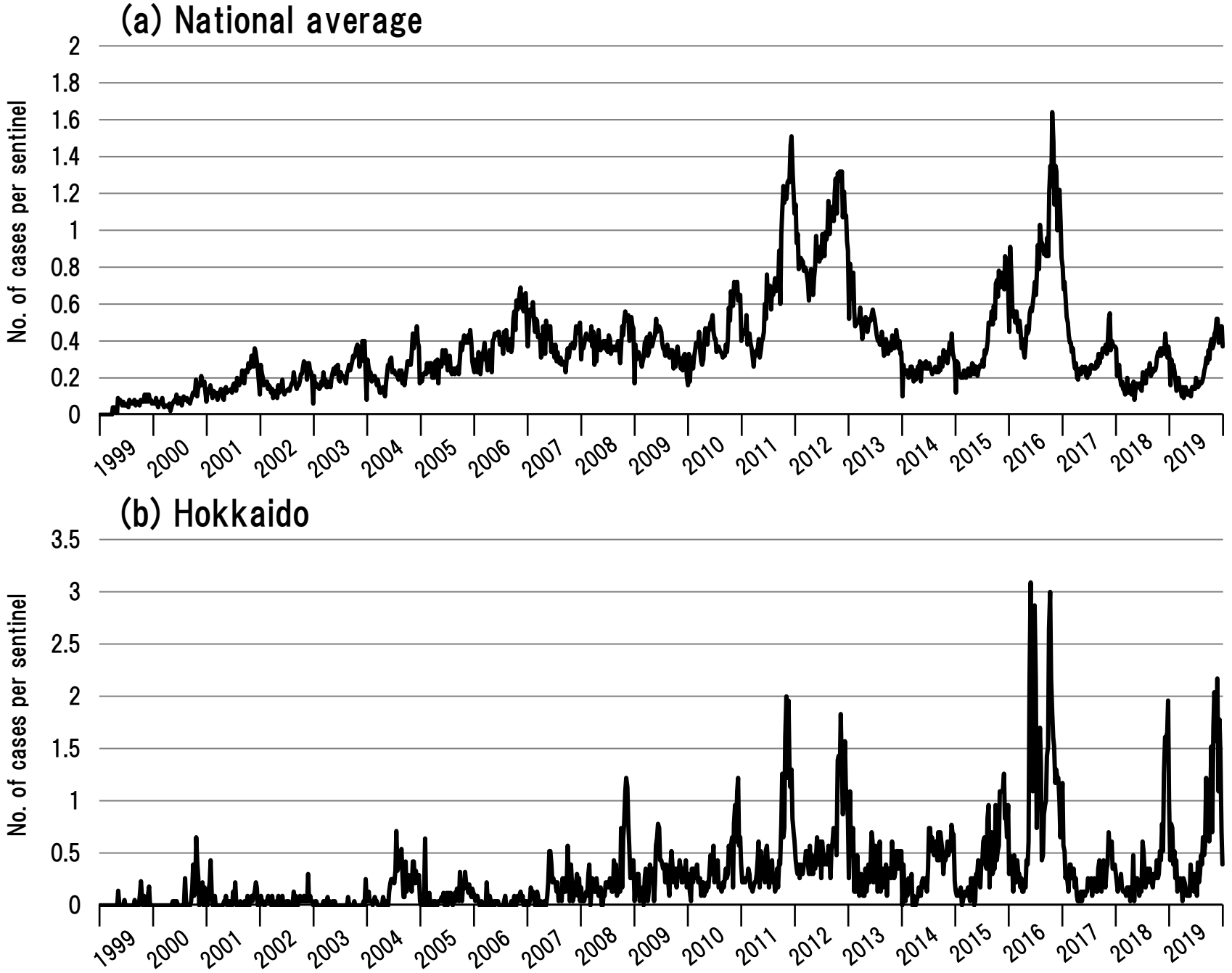
Figure legends

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

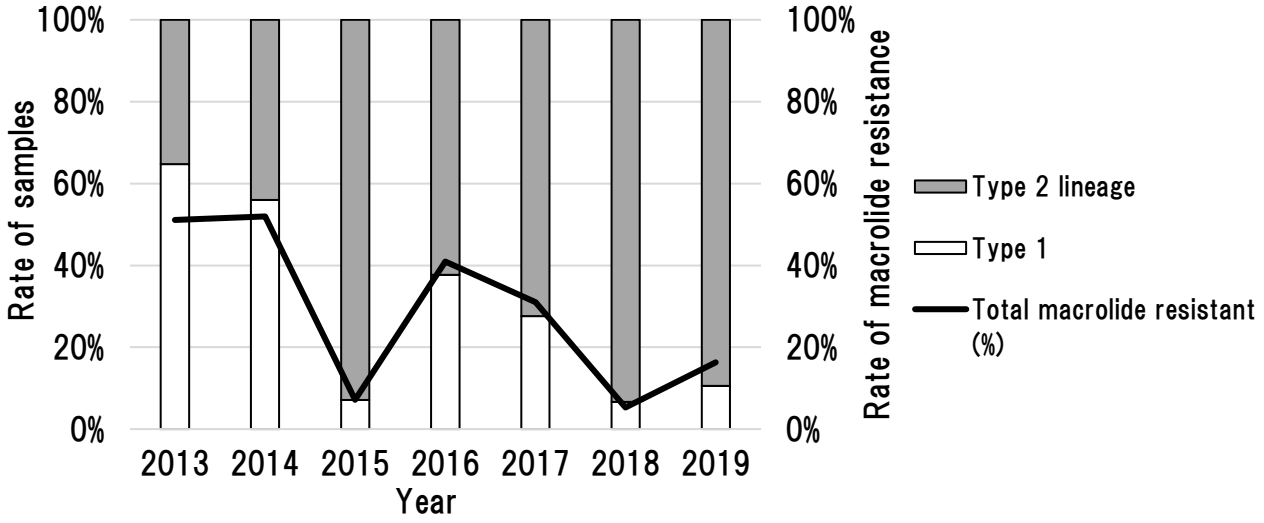
Figure 2. Annual rates of macrolide resistance and *p1* gene types of Mycoplasma pneumoniae in Hokkaido in 2013-2019. (A) Macrolide resistance and *p1* gene types. (B) Macrolide resistance and

191 *p1* gene type 1 strain. (C) Macrolide resistance and *p1* gene type 2 lineage strains. Also see Table

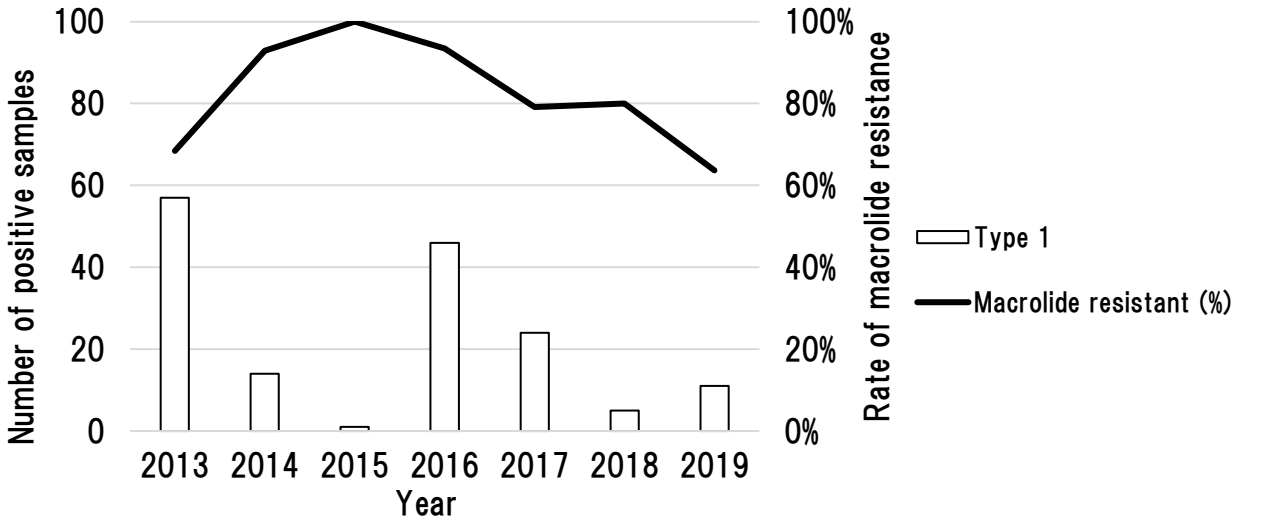
192 1.


(a) National average
No. of cases per sentinel
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
(b) Hokkaido
No. of cases per sentinel
3.5
3
2.5
2
1.5
1
0.5
0
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019

(a)


100%
80%
60%
40%
20%
0%
Rate of samples
Rate of macrolide resistance
2013
2014
2015
2016
2017
2018
2019
Year
Type 2 lineage
Type 1
Total macrolide resistant (%)

(b)


100
80
60
40
20
0
100%
80%
60%
40%
20%
0%
Number of positive samples
Rate of macrolide resistance
2013
2014
2015
2016
2017
2018
2019
Year
Type 1
Macrolide resistant (%)

(c)

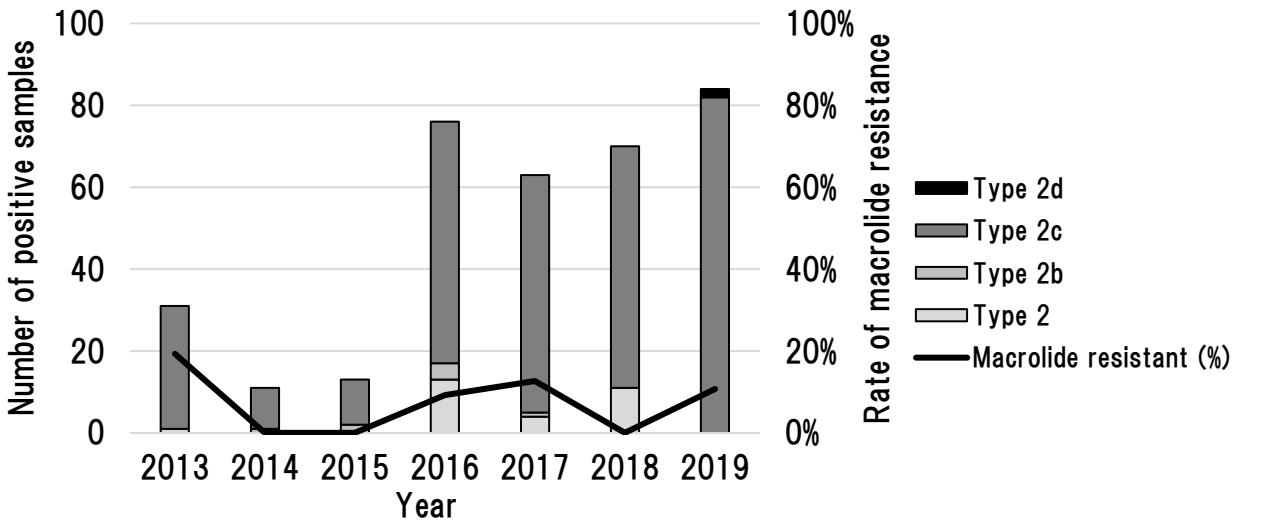

100
80
60
40
20
0
100%
80%
60%
40%
20%
0%
Number of positive samples
Rate of macrolide resistance
2013
2014
2015
2016
2017
2018
2019
Year
Type 2d
Type 2c
Type 2b
Type 2
Macrolide resistant (%)

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).