Genotyping analysis of *Mycoplasma pneumoniae* clinical strains in Japan between 1995 and 2005: type shift phenomenon of *M. pneumoniae* clinical strains

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Mycoplasma pneumoniae clinical isolates obtained between 1995 and 2005 were examined to determine the prevalent genotype. One hundred and twenty-seven strains isolated from bronchitis and pneumonia patients were genotyped by a PCR-RFLP method based on nucleotide sequence polymorphisms of the *p1* gene, which encodes the major adhesin protein. The typing results established that 66 of the isolates were group I strains, 45 were group II strains and 16 were group II variants. Analysis of the annual occurrence of these isolates showed a predominance of group II strains between 1995 and 2001 ($n=37$). No group I strain was found during this period. However, group I strains appeared in the isolates from 2002 (2/5 isolates, 40 %) and increased in specimens taken after 2003, thereby constituting a large proportion of the isolates. In 2004 and 2005, no group II strains were found among the isolates ($n=49$), although there were nine group II variants. Throat swabs and sputum samples obtained from patients with respiratory infections between 1997 and 2005 were also analysed by PCR-RFLP or a new nested PCR to detect the *p1* gene DNA. Typing analysis of these *p1* gene DNAs also showed that the group I *p1* gene was not present in specimens taken before 2000, but was present and dominant in specimens taken after 2001. These results indicate that, in Japan, the prevalent type of *M. pneumoniae* changed from a group II strain to a group I strain around 2002.

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INTRODUCTION

Mycoplasma pneumoniae is an aetiological agent of bronchitis and primary atypical pneumonia in humans. Adhesion of *M. pneumoniae* to the human respiratory epithelium is a critical step in the colonization of this pathogen and onset of these diseases (Jacobs, 1991; Razin & Jacobs, 1992; Waites & Talkington, 2004). The adhesion step is mediated by a terminal tip-like structure, which is the attachment organelle of this bacterium (Krause, 1998; Razin *et al.*, 1998). At the surface of the attachment organelle is a 170 kDa adhesin protein, P1, which is densely

clustered and plays a major role in binding to the receptor molecule of host epithelial cells (Feldner *et al.*, 1982; Hu *et al.*, 1982; Krause & Balish, 2001). The complete gene for P1 (the *p1* gene or MPN141) is present as a single copy in the *M. pneumoniae* genome and is known to exhibit nucleotide sequence polymorphism across isolates (Su *et al.*, 1990). So far, two major subtypes of the *p1* gene (subtypes 1 and 2) are known, and there are some minor variants of these (1, 2a, 2b and Mac) (Kenri *et al.*, 1999; Dorigo-Zetsma *et al.*, 2001; Dumke *et al.*, 2006). The difference between *p1* gene subtypes exists in two regions of the sequence, RepMP4 and RepMP2/3, which are known as repetitive sequences in the *M. pneumoniae* genome (Ruland *et al.*, 1990; Himmelreich *et al.*, 1996). The polymorphism

Abbreviation: HA, haemadsorption.

of the *p1* gene may have been generated by DNA recombination between the *p1* gene and RepMP repetitive sequences in the *M. pneumoniae* genome (Su *et al.*, 1993; Kenri *et al.*, 1999). This polymorphism of the *p1* gene allows *M. pneumoniae* clinical strains to be classified into two distinct groups (groups I and II, also called subtype 1 and 2 strains) and their variant strains based on its minor variations. In a previous study, we genotyped 215 clinical strains isolated in Japan between 1976 and 1994 and found that the isolation rate of the two groups was not constant but varied from year to year. Group I strains dominated during the 1970s and the latter half of the 1980s, whilst group II strains dominated at the beginning of the 1980s and after 1993 (Sasaki *et al.*, 1996). A similar trend in epidemiological status was also reported from studies in Germany, France and Denmark (Jacobs *et al.*, 1996; Cousin-Allery *et al.*, 2000; Dumke *et al.*, 2003), suggesting that a type shift of the clinical strain is a general epidemiological phenomenon of this bacterium. To confirm and characterize this phenomenon in Japan, we analysed *M. pneumoniae* clinical strains isolated between 1995 and 2005. We examined 127 clinical isolates from pneumonia patients and also genotyped *p1* gene DNA detected in throat swabs and sputum samples. We also designed a new nested PCR-based method that enabled easy detection and genotyping of *p1* gene DNA from clinical specimens.

METHODS

Bacterial strains and clinical specimens. *M. pneumoniae* was isolated independently from throat swabs of 127 pneumonia or bronchitis patients between 1995 and 2005 from three separate areas in Japan. One hundred and seven strains were isolated in Kanagawa prefecture, 16 in Hokkaido prefecture and four in Kochi prefecture. *M. pneumoniae* type strains M129 (Lipman *et al.*, 1969) and FH (Barile *et al.*, 1988) were used as representatives of groups I and II, respectively. These *M. pneumoniae* strains were cultured in PPLO medium [2.1% PPLO broth (Becton Dickinson), 0.25% glucose, 0.002% phenol red, 2.5% fresh yeast extract, 10% horse serum (Gibco-BRL) and 50 µg ampicillin ml⁻¹] at 37 °C and filter cloned (Tully, 1983) prior to the genotyping analysis. Throat swabs were collected from 89 primary atypical pneumonia or bronchitis patients who exhibited clinical signs of *M. pneumoniae* infection in Nagasaki and Hokkaido prefectures between 1997 and 2000. Between 2000 and 2005, 1568 sputum samples were collected from patients suspected of having a respiratory infection at Saitama Medical School Hospital, Japan. The throat swabs and sputum samples were treated with a QIAamp DNA extraction kit (Qiagen) and the extracted DNAs were used for detection and genotyping of the *M. pneumoniae p1* gene.

Genotyping analysis of *M. pneumoniae*. The synthetic oligonucleotide DNA primers used for detecting and typing *M. pneumoniae* are listed in Table 1. Typing of *M. pneumoniae* clinical isolates was performed as described previously using a PCR-RFLP method (Sasaki *et al.*, 1996). *M. pneumoniae* cells were collected from 1 ml PPLO culture by centrifugation (15 000 g for 2 min), suspended in 100 µl 1% Triton X-100 and heated at 95 °C for 2 min to release genomic DNA from the cells. One microlitre of this crude solution was subjected to PCR-RFLP analysis. Primers ADH1, ADH2, ADH3 and ADH4 and the restriction enzyme *Hae*III were used in this analysis. DNA extracted from throat swabs was also analysed by the PCR-RFLP method. DNA from the sputum samples was screened by *p1*

Table 1. PCR primers used for the detection and typing of *M. pneumoniae*

Primer	Sequence (5'→3')
ADH1	CTGCCTTGTCCTCAAGTCCACT
ADH2	AACCTTGTCGGAAGAGCTG
ADH3	CGAGTTTGCTGCTAACGAGT
ADH4	CTTGACTGATACCTGTGCGG
ADH2F	GGCAGTGGCAGTCAACAAACCACGTAT
ADH2R	GAACCTAGCGCCAGCAACTGCCAT
ADH3F	GAACCGAAGCGGCTTTGACCGCAT
ADH3R	GTTGACCATGCCTGAGAACAGTAA
ADH4F	GACCGCATCAACCACCTTTGCGTTACG
N1	CCCGGTGGTGGAAGTATTTT
2N2C	TGCCTTGGTCACCGGAGTTG
MP2/3-R1	AGATTGACCTGAGCCTGAAG
MP2/3-F2	CACAAGTGGTTCGCGTTTCCT
MP2/3-R2	GGCTGGGTGGAATGGTCTGT
MP2/3-F3	TCGACCAAGCCAACCTCCAG
R3-1	TTGGAATCGGACCCACTTCG
R3-2	CGACGTGTGTTGTGCCAC
R3-2V	CGGTATAGCTAATTTGGTAC

gene-specific nested PCR. For detection of the RepMP4 region of the *p1* gene, the ADH2F and ADH2R primer pair was used for the first PCR, and the ADH3F and ADH3R primer pair for the second PCR (Fig. 1a). For detection of the RepMP2/3 region, the ADH3 and MP2/3-R1 primer pair was used for the first PCR, and the MP2/3-F2 and MP2/3-R2 primer pair was used for the second PCR (Fig. 1a). The *p1* gene fragments amplified by these nested PCRs were diluted 1:10 000 and subjected to multiplex PCR to determine the type of the *p1* gene. The multiplex PCR primer set consisting of ADH4F, N1 and 2N2C was used for typing the RepMP4 region. This multiplex PCR gives a 343 or 560 bp amplification product from subtype 1 or 2 (group I or II) *p1* gene DNA, respectively (Fig. 1b). The multiplex PCR primer set consisting of MP2/3-F3, R3-1, R3-2 and R3-2V was used for typing of the RepMP2/3 region. This multiplex PCR gives a 394 bp product from the *p1* gene DNA of group I strains, a 617 bp product from that of group II strains, and 617 and 1008 bp products from that of group II variant strains (variant 2a) (Fig. 1c). The reaction conditions used in the nested and multiplex typing PCR were 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Premix Ex Taq (Takara) was used for the reaction, in accordance with the manufacturer's instructions.

RESULTS AND DISCUSSION

Genotyping of *M. pneumoniae* clinical isolates

M. pneumoniae strains collected from three areas in Japan (Kanagawa, Kochi and Hokkaido prefectures) were genotyped. Both polymorphic regions of the *p1* gene (RepMP4 and RepMP2/3) were examined using a PCR-RFLP method developed originally by Cousin-Allery *et al.* (2000). The results are shown in Table 2. The typing result revealed that, between 1995 and 2001, there were no group I strains among the isolates. During this period, 35 isolates were group II strains whilst the other two strains were group II variants. In contrast, group I strains were found in the specimens taken after 2002. In 2002, two out of five isolates

Fig. 1. Strategy used for PCR detection and genotyping of the *M. pneumoniae* *p1* gene. (a) Schematic illustration of the *p1* gene and location of the primers used in the PCR. The two shaded boxes indicate the RepMP4 and RepMP2/3 repetitive regions of the *p1* gene (Ruland *et al.*, 1990). The lightly shaded boxes within the RepMP regions represent a polymorphic region between the group I and group II (subtype 1 and 2) *p1* genes. The filled box in the RepMP2/3 region indicates the polymorphic region of the group II variant (variant 2a) (Kenri *et al.*, 1999; Dumke *et al.*, 2006). Arrows indicate the positions of the primer sequences listed in Table 1. The primer sites of ADH1, ADH2R, ADH2, ADH3, MP2/3-R1 and ADH4 are located outside the RepMP4 and RepMP2/3 regions. Primers N1 and R3-1 are specific to the group I *p1* gene. Primers 2N2C and R3-2 are specific to group II and group II variant. Primer R3-2V is specific to group II variant (variant 2a). (Primer R3-2V is also specific to group II variant 2b (Dumke *et al.*, 2006) and is thought to give an identical typing result to that of variant 2a. However, we have not confirmed this experimentally, as we do not have a variant 2b strain.) (b) Example of genotyping of the RepMP4 region by multiplex PCR. The agarose gel (2%) electrophoresis pattern of the final PCR products from *p1* genes of group I and II strains is shown. The genomic DNAs of *M. pneumoniae* group I standard strain M129 (lane 1) and group II standard strain FH (lane 2) were used as templates in this example. Lane M, size marker. The RepMP4 region of group II variant *p1* is identical to that of group II. Genotyping of the RepMP4 region of groups II and II variant *p1* gave identical results (data not shown). (c) Example of PCR genotyping of the RepMP2/3 region by multiplex PCR. The agarose gel (2%) electrophoresis pattern of the final PCR products from *p1* genes of groups I, II and II variant (II') strains are shown (lanes 1–3). The genomic DNAs of *M. pneumoniae* M129, FH and group II variant strain 309, respectively, were used as templates in this example. Lane M, size marker.

were group I strains (40%). Apparently, after 2003, the number of group I strains increased. In 2003, 24 out of 36 isolates (67%) were group I strains, and five were group II variants. Between 2004 and 2005, no group II strains were found among the 49 isolates, but nine group II variants were present. Although isolates could not be obtained in 1999 because there were few pneumonia patients in that year, our typing results strongly suggest that the type of prevalent clinical *M. pneumoniae* strain shifted from group II to group I around 2002.

Typing of *M. pneumoniae* *p1* genes detected in clinical specimens

As the growth of *M. pneumoniae* is fastidious, a great deal of effort is required to obtain a sufficient number of

isolates. This is a major difficulty in epidemiological studies on *M. pneumoniae* infection. To increase the number of samples and to confirm the type shift phenomenon of *M. pneumoniae*, we tried to detect and genotype the *p1* gene DNA present in clinical specimens without isolating *M. pneumoniae* itself; therefore, we applied a PCR-RFLP method to throat swab specimens. We analysed 89 throat swabs collected from bronchitis and pneumonia patients suspected of having *M. pneumoniae* infection between 1995 and 2000 from two prefectures in Japan (Hokkaido and Nagasaki). In this analysis, *p1* genes were detected in 24 specimens (27%) and genotyped. The result established that all *p1* gene DNAs detected in throat swabs were from group II strains (Table 2), indicating the apparent dominance of group II strains between 1995 and 2000. We then attempted to analyse sputum samples from

Table 2. Genotyping results of clinical isolates and clinical specimens

–, Samples not collected; NT, not tested (group II and group II variants were not distinguished by the analysis of RepMP4 in this period).

Isolate/specimen	Group	Year											Total
		1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	
Clinical isolates	Group I	0	0	0	0	0	0	0	2	24	22	18	66
	Group II	7	13	2	5	0	2	6	3	7	0	0	45
	Group II variant	0	0	0	1	0	0	1	0	5	7	2	16
Swabs	Group I	–	–	0	0	0	0	–	–	–	–	–	0
	Group II	–	–	6	4	1	13	–	–	–	–	–	24
Sputum	Group I	–	–	–	–	–	0	14	24	2	6	4	50
	Group II	–	–	–	–	–	9	13	19	0	0	0	41
	Group II variant	–	–	–	–	–	NT	NT	NT	NT	2*	0	2

*Group II variant 2a or 2b.

patients with respiratory infections. Before this analysis, we considered that a more sensitive *p1* detection method would be required, because the sputum samples were derived from patients with respiratory infections that were not specified as *M. pneumoniae* infections. In our evaluation experiments, the PCR-RFLP method required about 300 copies of purified genomic DNA of *M. pneumoniae* M129 for detection of the *p1* gene (data not shown). As an analysis method for clinical specimens, the availability of the present PCR-RFLP method is limited. Thus we designed a new nested PCR method to detect and genotype *p1* genes effectively from clinical samples (Fig. 1). This nested PCR could detect *p1* genes from three copies of purified *M. pneumoniae* M129 genomic DNA (data not shown). In this new method, the RepMP4 or RepMP2/3 polymorphic region of the *p1* gene was specifically amplified. The amplified *p1* gene fragments were then subjected to multiplex PCR to determine the genotype (Fig. 1b and c). The multiplex PCR for the RepMP4 region was designed for genotyping of group I and II *p1* genes (at the time of method design, no *p1* gene variants in RepMP4 were known). In contrast, the multiplex PCR for the RepMP2/3 region was designed to distinguish groups I, II and II variant. We employed the RepMP4 typing method for the sputum analysis between 2000 and 2003, and both the RepMP4 and RepMP2/3 methods for analysis after 2004. These analyses gave 93 typing results from 1568 sputum specimens (5.9%). The results (shown in Table 2) established that 50 *p1* gene DNAs were from group I strains, 41 were from group II strains and two were from group II variants (2a or 2b). Similar to the results of the throat swab analysis, no group I *p1* DNA was found in sputum samples collected in 2000. However, group I *p1* DNA appeared in samples collected after 2001, and group II DNA disappeared from sputum samples collected after 2003. These results from the genotyping of *p1* gene DNA from throat swabs and sputum samples were consistent with those of *M. pneumoniae* isolates and strongly supported the presence of a type shift phenomenon from group II to group I in Japan around 2002.

Type shift phenomenon in Japan

The genotyping data of this study (1995–2005) were integrated with those of a previous study (1976–1994) (Sasaki *et al.*, 1996). As a result of this integration (Fig. 2a and b), it became even more evident that a type shift phenomenon occurs in Japan every 8–10 years. The type shift from one group to another requires 2–3 years (i.e. 1983–1984, 1992–1993 and 2001–2003). Once a group becomes dominant, the trend continues for approximately 7 years. A great topic of interest about the type shift phenomenon is to explore whether the type shift is involved in *M. pneumoniae* outbreaks. It was reported in the 1970s that outbreaks of mycoplasma pneumonia occur every 4 years in Japan, coinciding with the year of the Olympic Games (Niitu, 1984). Consistent with this report, large outbreaks were observed in 1984 and 1988 in the national surveillance of primary atypical pneumonia and mycoplasma pneumonia in Japan (Fig. 2c). These outbreaks were in the years of type transition of *M. pneumoniae* clinical strains (from group II to group I, 1984) and in the middle of a group I-dominated period (1988). These data suggest possible correlations between the type shift phenomenon and pneumonia outbreaks (i.e. the outbreaks may be caused by an antigenic change in the pathogen or by propagation of the dominant pathogen in the human population). However, periodic outbreaks were not apparent in the 1990s, whilst the type shift from group I to group II occurred in the early 1990s. There were only minor increases in patient numbers in 1992 and 1996. After 2000, the number of mycoplasma pneumonia patients increased gradually and a large outbreak was observed in 2006. There is a possibility that this outbreak was linked to the type shift phenomenon. However, we think at this point that more information, including focusing on local area surveillance, is needed before we conclude that there is a connection between the type shift phenomenon reported in this study and the pneumonia epidemic.

Although the mechanism underlying this type shift phenomenon is unclear, it may be caused by interactions

Fig. 2. Integrated data from *M. pneumoniae* typing in Japan between 1976 and 2005. (a) Typing results of *M. pneumoniae* clinical strains (1976–2005) and the genotyping results of *p1* genes from throat swabs (1997–2000) and sputum samples (2000–2005) are integrated (see text). The group II variant strain isolated in 1998 was designated strain 309 in the previous report (Kenri *et al.*, 1999). (b) Annual detection rate of each group. (c) National surveillance of primary atypical pneumonia and mycoplasma pneumonia cases in Japan. Primary atypical pneumonia cases were surveyed weekly from April 1981 to March 1999. Mycoplasma pneumonia cases were surveyed weekly after April 1999 under the new law for infectious diseases. The data were collected from nationwide sentinel clinics and integrated by the Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Japan (<http://idsc.nih.go.jp/index.html>).

between *M. pneumoniae* and the immunological status of the human population, as explained below. It is well known that patient sera from *M. pneumoniae* infections often exhibit an activity (called haemadsorption or HA inhibitory activity) that inhibits the adherence of *M. pneumoniae* to red blood cells (Razin & Jacobs, 1992; Waites & Talkington, 2004). This HA inhibitory activity in a patient's serum is thought to serve as a type of immunity that reduces the possibility of re-infection with *M. pneumoniae*. Jacobs *et al.* (1996) previously reported that, in the serum of some patients, the HA inhibitory activity against a particular *M. pneumoniae* group was stronger than that against another. Furthermore, this group-specific HA inhibitory activity depended on the time of serum sampling (possibly this activity occurred during the period in which a particular group predominated), suggesting the induction of group-specific antibodies in patient serum during *M. pneumoniae* infection. It was also reported that pre-infection of guinea pigs with one subtype of *M. pneumoniae* reduced the recovery rate of the same *M. pneumoniae* subtype in subsequent infection experiments (Dumke *et al.*, 2004). This also suggests that there is subtype-specific protection against *M. pneumoniae* as a result of immunity. If this kind of subtype-specific immunity is induced among the human population during the period in which a particular group predominates, it could possibly drive the type shift phenomenon. Although the real target of group-specific HA inhibitory activity or protective immunity is unclear at present, the most likely candidate is the P1 protein. This is because P1 is the most important adhesin molecule and is responsible for successful infection; its polymorphism is also a major difference between group I and II *M. pneumoniae* strains. Further studies on the correlation between anti-P1 antibodies present in the human population and their protective effect against *M. pneumoniae* infection may be crucial in understanding the mechanisms of the type shift phenomenon.

In this study, a considerable number of group II variants were detected. All group II variants examined by PCR-RFLP in our study (*Hae*III digestion) showed an identical typing pattern, suggesting that these strains were almost homogeneous (variant 2a type). The variant 2b strain reported recently by Dumke *et al.* (2006) and the variant 1 strain (Dorigo-Zetsma *et al.*, 2001) were not detected in our study. Group II variants were rare in earlier studies; however, in this study, they appear to be increasing, despite the decrease in the number of group II strains after 2003. At present, it is difficult to predict whether group II variants will become the dominant type in the future. It will be interesting to monitor the status of group II variants over the next decade. The nested multiplex PCR method designed in this study (Fig. 1) is useful for monitoring group II variants (including variant 2b). In addition, our method is applicable to monitoring of other variants by combining additional multiplex primers. However, this method is not applicable to new *p1* genes of unknown

sequence. As the first *p1* gene with variation in the RepMP4 region was reported recently (Pereyre *et al.*, 2007), there is a possibility that more variants may emerge by recombination between the *p1* gene locus and RepMP repetitive sequences scattered in the *M. pneumoniae* genome. To survey and characterize new variant *p1* gene strains, it is important to continue to isolate clinical strains of this bacterium.

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