Identification of *Mycoplasma pneumoniae* type 2b variant strains in Japan

*Mycoplasma pneumoniae* is an important causative pathogen of community-acquired pneumonia, particularly among children and young adults. Two major groups of this bacterium, subtypes 1 and 2, are known and the major differences between these groups are variations in the *p1* gene, encoding P1 cytadhesin, an essential factor for the pathogenicity of this bacterium (Nakane et al., 2011; Razin & Jacobs, 1992). The variations are observed in two parts of the *p1* gene, designated RepMP4 and RepMP2/3. These regions serve as targets for PCR-restriction fragment length polymorphism (RFLP) analysis (Cousin-Allery et al., 2000) in genotyping studies (Fig. 1a).

In the present study, we genotyped the *p1* gene of 48 *M. pneumoniae* strains deposited at the Kanagawa Prefectural Institute of Public Health. These strains were isolated from pneumonia patients in Japan from 2004 to 2005. Thirty-nine of these strains were genotyped as subtype 1, and six strains were identified as variant 2a; however, the remaining three strains (designated T-103, Y-135 and Y-169) exhibited a slightly different PCR-RFLP pattern compared to the known strains. The genotyping pattern of strain Y-169 is shown in Fig. 1b next to the subtype 1, 2 and variant 2a strains M129, FH and 309, respectively. In the typing of the RepMP4 region (Fig. 1b, lanes 1–4), the pattern of strain Y-169 was similar to that of strains FH and 309, suggesting a relation with the subtype 2 group. However, the RepMP2/3 pattern was slightly different from the known pattern for this gene region (Fig. 1b, lanes 5–8). We sequenced the *p1* gene of strains T-103, Y-135 and Y-169 and confirmed that strains Y-135 and Y-169 harboured a *p1* gene with an identical sequence. The *p1* gene of strain T-103 was also identical to the other two strains except for one additional AGT trinucleotide at the variable-number tandem-repeat (VNTR) site (Zhao et al., 2011; Fig. 1a). We compared the *p1* gene sequences published thus far with those derived in this study and found that a partial *p1* sequence reported by Dumke et al. (2006) (GenBank accession number DQ383277) was identical to the *p1* gene sequences determined here. The partial *p1* gene sequence of Dumke et al. (2006), classified as variant 2b, covered the 5′ half of the *p1* variation site determined in the

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Fig. 1. PCR–RFLP typing of the *p1* gene. (a) Schematic illustration of the *p1* gene. Filled triangles indicate the binding sites of the PCR primers designed to amplify target sequences for RFLP typing (Cousin-Allery et al., 2000; Kenri et al., 1999). Shaded boxes in the *p1* gene indicate variation sites between subtypes. Areas of repetitive elements (RepMP4 and RepMP2/3 regions) are indicated by square brackets. Sequences similar to these RepMP regions are present in the *Mycoplasma pneumoniae* genome in multiple copies (Schwartz et al., 2009; Wenzel & Herrmann, 1988). (AGT)* indicates a trinucleotide VNTR site (Zhao et al., 2011). The number of these repeats (n) for selected strains is shown in the upper right corner. (b) PCR–RFLP typing patterns of selected strains. Typing patterns were obtained by *Hae*III digestion of PCR products obtained using ADH1 and 2 (lanes 1–4) and ADH3 and 4 (lanes 5–6) primer sets. *Hae*III-digested fragments were visualized by 2% agarose gel electrophoresis and ethidium bromide staining. Strain names analysed are labelled above the lane numbers.

The DDBJ/EMBL/GenBank accession numbers for the variant 2b *p1* gene sequences of strains T-103, Y-135 and Y-169 are AB691539, AB691540 and AB678699, respectively.
present study. Recently, another partial p1 gene sequence similar to that reported by Dumke et al. (2006) was reported in the USA (Schwartz et al., 2009; Spuesens et al., 2010a). This sequence (GenBank GQ861494), obtained from a strain named isolate 3, matched the entire variation site of the p1 gene sequence determined here, except for a single base-pair mismatch (C→T at nucleotide position 4164 of the p1 gene sequence of strain Y-169). From these results, we concluded that the p1 gene sequences of strains T-103, Y-135 and Y-169 corresponded to that of variant 2b.

By using standard PCR-RFLP genotyping we were able to detect p1 gene sequences corresponding to variant 2b (Fig. 1b, lane 8). Except for the variant 2b-specific sequence in the RepMP2/3 region and the number of AGT trinucleotides at the VNTR site, the full-length p1 gene sequences of variant 2b were highly similar to subtype 2 and variant 2a (strains FH and 309; respectively; GenBank CP002077 and AP012303; Kenri et al., 2012; Krishnakumar et al., 2010) with only two single-nucleotide polymorphisms compared with strain FH and seven single-nucleotide polymorphisms compared with strain 309.

Sequence variations in the p1 gene are thought to be generated by DNA recombination events between the p1 locus and repetitive sequences similar to the RepMP4 and RepMP2/3 regions (RepMP elements) in the M. pneumoniae genome (Kenri et al., 1999; Spuesens et al., 2009; Wenzel & Herrmann, 1988). The region of the p1 gene containing sequence variations in variant 2b is about 640 bp in length. This 640 bp sequence is identical to a RepMP element outside the p1 locus of the strain FH genome (position 128399–129039), suggesting variant 2b variation was derived from this sequence. To date, four variants of the p1 gene in subtype 2 are known, including variants 2a, 2b and 2c, as well as the p1 gene of strain Mp3896. Variant 2c was recently reported in the Netherlands and in China (Spuesens et al., 2010b; Zhao et al., 2011). Variant 2c exhibits variations at the RepMP4 region, although the RepMP2/3 region of this variant is almost identical to that of variant 2a. Two slightly different variant 2c sequences were reported in China (GenBank JN048894 and JN048895; Zhao et al., 2011). Strain Mp3896 was isolated in France (Pereyre et al., 2007) and could not be classified by PCR-RFLP. The p1 gene of this strain has unique variations in both the RepMP4 and RepMP2/3 regions (GenBank EF656612); however, it is now believed that this sequence was created by DNA recombination in the genetic background of the subtype 2 group (Spuesens et al., 2009). Only a single strain of this variant has been reported so far. In contrast to subtype 2, subtype 1 variants are rarely detected; only one variant (strain Mp4817, GenBank AF290000) has been reported to date (Dorigo-Zetsma et al., 2001). The scarcity of subtype 1 variants may be linked to a reduced recombination activity in subtype 1 strains compared with subtype 2 (Krishnakumar et al., 2010).

In the present study, we identified three M. pneumoniae strains harbouring variant 2b p1 genes for what is believed to be the first time in Japan. These strains were collected from two distant areas (Y-135 and Y-169), and Y-169 was isolated at Yamagata prefecture in 2004 and T-103 was isolated from Kanagawa prefecture in 2005) and variant 2b strains are rarely detected in Japan at present. This situation is similar to other areas of the world. Variant 2b sequences were originally identified in central Europe by molecular methods (Dumke et al., 2006). In Germany, a relatively higher detection rate of variant 2b DNA was reported (5–15 % in specimens collected between 2003 and 2006); however, the bacterial strain has not been isolated (Dumke et al., 2010). A single variant 2b isolate was reported in the United States (Schwartz et al., 2009). Presently, variant 2b strains might constitute a minor proportion of clinically relevant strains; however, it is possible that they will become a major variant of clinical strains in the future, considering that the variant 2a strains that were rarely detected in 1990s are now commonly found worldwide (Dumke et al., 2010; Kenri et al., 2008; Zhao et al., 2011). Therefore, monitoring of variant 2b strains is needed to attain a better understanding of the epidemiology and evolutionary aspects of M. pneumoniae infection.

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