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Detection of *Mycoplasma pneumoniae* P1 subtype variations by denaturing gradient gel electrophoresis

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ABSTRACT

There were several methods to detect *p1* gene variations in *Mycoplasma pneumoniae*. In this study polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) assay was performed to establish a rapid and precise detection method for identifying *M. pneumoniae p1* gene variations. We detected *p1* gene variations in 109 *M. pneumoniae* clinical isolates from Shanghai, China, which were collected from 2009 to 2011 by DGGE, and compared this method with the PCR-based restriction fragment length polymorphism assay and sequencing. By PCR-DGGE method, among the 109 *M. pneumoniae* isolates, 101 (92.7%) isolates were classified into type I, and 8 (7.3%) were classified into type II. Seven (6.9%) type I variations and 8 (100%) type II variations were identified. The match rate of *p1* gene variation detected by DGGE reached 100% when compared to DNA sequencing and was more sensitive than restriction fragment length polymorphism. One new type II variant, designated as V2d, was found in this study. The sequence of the new variant was characterized. Our results indicated that PCR-DGGE is a rapid and reliable bio-technique for direct detection of *p1* gene variations.

1. Introduction

Mycoplasma pneumoniae is the most common pathogen of community-acquired respiratory tract infections (Waites and Talkington, 2004). Epidemiological studies have shown that *M. pneumoniae* is responsible for 20–50% of community-acquired pneumoniae (Atkinson et al., 2008). Strains typing or subtyping by molecular methods is a powerful tool for surveillance and outbreak investigation. Several molecular typing methods have been developed. Historically, typing schemes of *M. pneumoniae* were based upon restriction fragment length polymorphism (RFLP) analysis (Cousin-Allery et al., 2000), multiple-locus variable-number tandem-repeat analysis assay (Dégrange et al., 2009; Dumke and Jacobs, 2011; Liu et al., 2012), pyrosequencing (Spuesens et al., 2010; Spuesens et al., 2012) and sequencing (Zhao et al., 2011). Among them, restriction fragment length polymorphism (RFLP) analysis of *P1* gene (the gene encoding for the major adhesin of *M. pneumoniae*), which contains copies of repetitive elements (repMp4 and repMp2/3), is the most common genotyping method for *M. pneumoniae* molecular typing and identification of variants of each subtype (Cousin-Allery et al., 2000; Kenri et al., 1999). However, clinical isolates are poorly differentiated by PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis as *M. pneumoniae* is a genetically homogeneous species (Dégrange et al., 2009).

Denaturing gradient gel electrophoresis (DGGE) was used to detect single base mutations in DNA products (Su et al., 2012). This method is based on the different level of migration of DNA fragments following strand separation caused by chemical denaturants. DGGE was now applied to investigate the fingerprint of samples (Matussek et al., 2011; McAuliffe et al., 2003; Muyzer, 1999; Oates et al., 2012) and adopted for the investigation of the microbial diversity of food-contact surfaces (Koo et al., 2013).

The aim of this study was to determine the P1 variants of *M. pneumoniae* in DNA samples from 109 isolates commonly associated with respiratory tract infections (RTI) collected during 2009–2011 in Shanghai, China, using a novel and rapid PCR-based denaturing gradient gel electrophoresis (DGGE) method.

2. Materials and methods

2.1. M. pneumoniae clinical isolates and DNA preparation

One hundred nine unique *M. pneumoniae* clinical isolates, which had never been described before, were obtained from bronchial aspirations with low respiratory infections (each specimen collected from one patient) from January 2009 to march 2011 in Shanghai. Culture and identification of *M. pneumoniae* was carried out as

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Table 1
Primers used in for PCR-RFLP.

Primer	Sequence (5′-3′)	Length of amplified product (bp)
RepMp2/3 F	CCGTCCAATCCAAAATGTGG	1887
RepMp2/3R	CCAAATACCGGTGTCGTTGT	
RepMp4F	TAGTTCAAGATTGCCAAAAGCA	1639
RepMp4R	GTTGCATTATAGCGCACCCT	

described previously (Liu et al., 2009). The Genomic DNAs of each isolate were extracted using TIANamp Bacteria DNA kit. Two reference strains of *M. pneumoniae* M129 (ATCC 29342, type 1 of the *p1* gene) and FH (ATCC15531, type 2) were also included as the positive control for the *p1* gene analysis.

2.2. PCR-RFLP analysis

Two pairs of primers designed according to the *p1* gene polymorphism were summarized in Table 1. Primers of PCR-RFLP method were designed according to ADH1/ADH2 to amplify the P1 gene DNA containing RepMP4 sequences and ADH3/ADH4 were used to amplify the RepMP2/3 region. PCR reactions were prepared in a 50 μL volume with 5 μL 10× LA PCR buffer II (Mg^{2+} plus), 8 μL dNTP Mixture, 1 μL each primer set, 0.5 μL TaKaRa LA Taq polymerase (TaKaRa Biotechnology, Dalian, China), 5 μL template DNA, and 30.5 μL filtered sterile water. PCR conditions were as follows: initial denaturized at 94 °C for 5 min and then 30 cycles of 94 °C for 1 min, 62 °C for 2 min (65 °C for repMp 4), and 72 °C for 2 min, followed by a final polymerase extension step at 72 °C for 10 min.

The amplified products were subjected to an overnight restriction endonuclease digestion with 0.5 μL Rsa I/Taq I. Digested samples were run through nondenaturing 8% polyacrylamid–Bisacrylamid gel. Electrophoresis was performed on a vertical electrophoresis system with 0.05× Tris-Borate-EDTA solution buffer at 120 V for 90 min, then the gel was stained by silver staining method according to the procedure of molecular cloning (Sambrook and Russell, 2001).

2.3. PCR-DGGE analysis

The 6 pairs of primers used for the first PCR amplification are summarized in Table 2. Primers of DGGE method were also based on these regions. As the particularity of DGGE method-having a better capacity for analyzing 200–700 bp DNA pairs, the PCR products were limited <500 bp. In our study, the P1 gene of type1 or type2 was divided into 5 regions for analysis. The primers were designed according to these different regions respectively. To generate the 200–500 bp ideal PCR size for PCR-DGGE, the RepMp2/3 polymorphism region of *p1* gene was divided into 3 regions (Atkinson et al., 2008; Cousin-Allery et al., 2000; Dégrange et al., 2009; Dorigo-Zetsma et al., 2001; Dumke and Jacobs, 2011; Dumke et al., 2006; Kenri et al., 1999; Koo et al., 2013; Laguerre et al., 1994; Liu et al., 2009, 2010; Liu et al., 2012; Matussek et al., 2011; McAuliffe et al., 2003; Muyzer, 1999; Oates et al., 2012; Pereyre et al., 2007; Spuesens et al., 2010, 2012; Su et al., 2012; Waites and Talkington, 2004; Zhao et al., 2011) for amplification and the RepMp4 polymorphic region was divided into two region (Atkinson et al., 2008; Waites and Talkington, 2004).

GC-clamp was attached in the 5′ of forward primer for detection of single base substitutions in DNA fragments. PCR reactions were prepared in a 50 μL volume with 5 μL 10× LA PCR buffer II (Mg^{2+} plus), 8 μL dNTP Mixture, 1 μL each primer set, 0.5 μL TaKaRa LA Taq polymerase (TaKaRa Biotechnology, Dalian, China), 5 μL template DNA and 30.5 μL filtered sterile water. PCR conditions were as follows: initial denaturation at 94 °C for 10 min, and then 30 cycles of 94 °C for 50 s, 63 °C for 50 s (61 °C for region 4–1 and 4–2 type II) and 72 °C for 2 min, followed by a final polymerase extension step at 72 °C for 5 min.

Electrophoresis on parallel denaturing gradient gels was performed using DGGE-1001 system (BD Company). Parallel gradient gels for *p1* gene fragments were 10% acrylamide with 40–60% denaturant range and were run for 7 h at 60°C and 130 V (100% denaturant = 7 M urea +40% formamide; acrylamide: bisacrylamide = 37.5:1).

2.4. Sequencing

The full lengths of the *p1* genes of all 109 isolates were amplified using described primers (Zhao et al., 2011) and sequenced by Jieli Company (Shanghai, China) and sequences analysis was performed using DNAStar (DNASTAR, Madison, WI, USA).

3. Results

3.1. Results of p1 gene subtyping by PCR-RFLP

The *p1* gene PCR generated fragments of approximately 1686 bp (primers RepMp2/3 F and RepMp2/3R) and 1877 bp (primers RepMp4F and RepMp4R) from the 109 clinical isolates (data not shown). After digestion of these fragments with Rsa I/Taq I, 101 (92.7%) clinical isolates were classified into type I, and 8 clinical isolates (7.3%) were classified into type II (Fig. 1). Among these strains, 4 (4.0%) type I variants and 7 (87.5%) type II variants were identified.

3.2. Results of p1 gene variation by PCR-DGGE

Results of parallel gel electrophoresis were showed in Fig. 2. By PCR-DGGE method, among the 109 *M. pneumoniae* isolates, 101 (92.7%) were classified into classified into type I, 8 (7.3%) were

Table 2
Primers used for amplification of RepMp4 and RepMp2/3 of the *p1* gene of *M. pneumoniae*.

Nucleotide position[a]	Primer name	Sequence (5′-3′)	Size(bp) of product	Partition
repMp4 (180858–182404)	4/1-F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAATTTAGCTACACCCGCCCT	507	For type I/II(4-1)
	4/2-R	GCAACTGCTCATAGTACACC		
	4/3-F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGGTGTACTATGAGCAGTTGC	494	For type I(4-2)
	4/4-R	GGATCATTTGGGGAGGTCCT		
	4/3-F[b]	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGGTGTACTATGAGCAGTTGC	502	For type II(4-2)
	4/5-R	GAATTTGGCGGGGTCGTTGT		
repMp2/3 (182904–184704)	3/1-F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGTTGGATTATGTGCCCTGGA	482	For type I/II(3-1)
	3/2-R	AGTTGGTGGCCTCTTGTTGA		
	3/3-F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGTCAACAAGAGGCCACCAACT	458	For type I/II(3-2)
	3/4-R	TGCCACTGACAACGAGGTTA		
	3/5-F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGTAACCTCGTTGTCAGTGGCA	484	For type I/II(3-3)
	3/6-R	GTTGGGGTCCAACAGGTTTT		

[a] From the start of the *p1* gene.
[b] The same forward primer was used here for different subtype.