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A Single-Tube Multiple-Locus Variable-Number Tandem-Repeat Analysis of *Mycoplasma pneumoniae* Clinical Specimens by Use of Multiplex PCR-Capillary Electrophoresis

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In this study, we developed a single-tube multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) assay to type *Mycoplasma pneumoniae* directly from respiratory samples collected from children with respiratory infections. The multiplex PCR included four fluorescently primed VNTRs (Mpn13, Mpn14, Mpn15, and Mpn16) and was carried out in a single tube. A total of 137 *M. pneumoniae*-positive specimens, collected in 2013 from Beijing, China, were divided among four types (M4-5-7-2, M4-5-6-2, M3-5-6-2, and M5-5-7-2) using the amended MLVA system. The most prevalent genotype was M4-5-7-2. No correlation was found between macrolide resistance in the *M. pneumoniae* samples and the MLVA types. To our knowledge, this is the first study to type and analyze *M. pneumoniae* clinical specimens using multiplex PCR-capillary electrophoresis in a single tube. This novel low-cost method can be used to rapidly type *M. pneumoniae* clinical specimens directly and shows great potential for monitoring outbreaks of *M. pneumoniae*.

Mycoplasma pneumoniae is one of the most common etiological agents of community-acquired pneumonia and other respiratory tract infections (1). Clinical symptoms include mild to severe pneumonia and even death. *M. pneumoniae* infections cause worldwide epidemics every 3 to 7 years, lasting up to 2 years at a time (2, 3). Rapid molecular typing of strains is critical to identify the source of an outbreak and monitor the progress of the epidemic. Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) is a common method for genotyping infectious agents as it has greater discriminatory power than other methods, such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) (4–8). Degrange et al. (9) successfully used MLVA to investigate *M. pneumoniae* isolates and grouped 265 strains into 26 MLVA types. Many studies use a multiplex approach with five loci (Mpn1, Mpn13, Mpn14, Mpn15, and Mpn16) and fluorescently labeled primers in two solutions to type *M. pneumoniae* isolates (9–15). Because cultivation of *M. pneumoniae* is difficult, other studies have used nested PCR in the MLVA approach to directly amplify the regions from the clinical specimens (16, 17). However, because of the instability of the VNTR locus Mpn1, we proposed an amended MLVA nomenclature system based on the remaining four VNTR loci in our preliminary study (17).

In this study, we typed 137 *M. pneumoniae*-positive clinical specimens collected from pediatric patients in Beijing, China, in 2013 using the amended MLVA system, which connects multiplex PCR (mPCR) with capillary electrophoresis (CE) in a single tube. We also analyzed macrolide resistance in all of the typed strains.

MATERIALS AND METHODS

Samples and reference strains. A total of 477 clinical specimens were collected from pediatric patients at the Affiliated Children's Hospital of the Capital Institute of Pediatrics in Beijing, China, in 2013. Each specimen was collected from a separate patient. The specimens included bronchoalveolar lavage fluid, sputum samples, throat swabs, and puncture fluid. The male-to-female ratio of the patients was 1.6:1 (293/184, male/female). The age range of the patients was 1 month to 12 years. The patients were divided into three age groups: 0 to 3 years (265/477), 4 to 6 years (112/477), and 7 to 12 years (100/477). The patients were diagnosed with pneumonia, bronchopneumonia, capillary bronchitis, or other respiratory tract infections. Two reference *M. pneumoniae* strains, FH (ATCC 15531) and M129 (ATCC 29342), were used as positive controls for the MLVA typing.

DNA extraction and real-time PCR detection. Bacterial DNA from the specimens was extracted using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *M. pneumoniae* was confirmed in the samples using real-time PCR as described previously (18).

Primer design for multiplex PCR. The primers flanking the four VNTR regions (Mpn13, Mpn14, Mpn15, and Mpn16) were designed based on the reference strain *M. pneumoniae* M129 (GenBank accession no. U00089) using Primer Premier software 5.0. The primer pairs for multiplex PCR were analyzed using Oligo Analyzer software to avoid primer loops and primer dimers and to determine primer properties like melting temperature (T_m) and GC content (Table 1). The specificities of the primers were checked by BLAST analysis (see http://blast.ncbi.nlm.nih.gov/Blast.cgi). We aimed to amplify four VNTRs in one multiplex reaction and to distinguish between them by the combination of product sizes and different fluorescent primer labels. Depending on the length of the locus to be amplified, forward primers Mpn13 (product length, 643 bp) and Mpn14 (product length, 243 bp) were fluorescently labeled at the 5′ end with 4,7,2′,4′,5′,7′-hexachloro-6-carboxy-fluorescein (HEX, green), while forward primers of Mpn15 (product length, 241 bp) and Mpn16 (product length, 465 bp) were fluorescently labeled at the 5′ end with 6-carboxyfluorescein (FAM, blue). The T_m of the primers was 62.1°C to 65.7°C. The GC contents were between 35.0% and 55.6%, and the

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TABLE 1 Oligonucleotide primers used for MLVA

VNTR	Repeat unit size (bp)	Sequence primer[a]	Sequence	Coordinates[b]	Label	T_m (°C)	GC content (%)	Secondary structure	Product size (bp)
Mpn13	16	Repeat	CTTATTAATAACTATT						
		F[b]	GTTTGCTAATTATCTCATGAATGGC	596328–596352	HEX[c]	63.34	36	None	643
		R	TGCGCAGTGACAATGCATA	596970–596952		65.25	47.37	Weak	
Mpn14	21	Repeat	ATGGAAGTAAAAATGGACAAA						
		F	AGATGAGCGATCGCTTGG	608636–608653	HEX	64.2	55.56	Weak	257
		R	TTTCAAAATCGC(T)[d]CAAAAAGC	608892–608873		63.69	35	None	
Mpn15	21	Repeat	TGTCCATTTTTACTTCCATCT						
		F	CAACAGCACCACATCTTTAGG	645491–645511	FAM	62.08	47.62	None	241
		R	GCTAATCTTGCAAACGCTGC	645731–645712		64.7	50	Weak	
Mpn16	47	Repeat	ATTTTTTAAAAGTTTTTATT ATACCGTTTTGACAACTGCTTTTTGTT						
		F	CCTTGATTGTGGTGTGCTGAG[e]	735940–735960	FAM	65.65	52.38	None	465
		R	GATTCCGCTTAAACTGCAGG[e]	736404–736385		63.48	50	None	

[a] F, forward primer; R, reverse primer. There was no primer-dimer formation.
[b] All the sequences refer to *M. pneumoniae* M129 (GenBank accession no. U00089).
[c] The color of HEX is green; the color of FAM is blue.
[d] T or C meets the need of mPCR.
[e] The primers Mpn16-F and Mpn16-R were used as described previously (16).

oligo(dG) values were determined to be <7 cal/mol by Oligo Analyzer software.

Multiplex PCR amplification for MLVA. Amplification of each of the four loci was multiplexed in one reaction. The reactions were performed on a Veriti 96-well thermal cycler (Applied Biosystems) in a final volume of 50 μl, containing 5 μl extracted template DNA, 0.4 μM of each primer, and 2× *Taq* PCR MasterMix (Tiangen, Hangzhou, China). The reaction consisted of one cycle at 95°C for 10 min followed by 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min.

The mPCR conditions were first optimized using DNA from control strains M129 and FH.

Accuracy determination (single PCR and sequencing). As this amended MLVA assay is a new method, we wanted to compare the results of CE with sequencing, which is considered the gold standard. Fifty *M. pneumoniae*-positive samples collected in a previous study, including reference strains M129 and FH, were chosen for use in the accuracy determination assay. Each sample was subjected to PCR using a single set of primers for each locus. The reaction mixtures contained 2 μl extracted DNA, 0.4 μM of each primer, and 2× *Taq* PCR MasterMix (Tiangen, Hangzhou, China), and reactions were carried out using the cycling conditions described above. The resulting amplicons were visualized on a 2% agarose gel and then sequenced (Invitrogen Life Technologies). The lengths of the products resulting from CE and sequencing were compared for each VNTR.

Detection of macrolide resistance genes. The 23S rRNA gene of each *M. pneumoniae* sample was amplified by a nested PCR-linked capillary electrophoresis and single-strand conformation polymorphism analysis (nPCR-CE-SSCP) as reported previously (19), and the amplified products of the specimens with mutations were sequenced. We then analyzed the common point mutations at positions 2063, 2064, 2611, and 2617 (*Escherichia coli* numbering) of the gene, including the relationships between the point mutations and the MLVA types.

Statistical analysis. The lengths of all products of the PCR were analyzed using the formula repeat number = (length of product-flanking size)/(length of each repeat). The labeled PCR products were separated by CE using an ABI 3130 genetic analyzer (Applied Biosystems), and the data were analyzed using GeneMapper software (version 4.0; Applied Biosystems). The 1200 LIZ size standard (Applied Biosystems) was used in capillary electrophoresis. The peak size analysis was performed using GeneMapper (Applied Biosystems). The size of each PCR product was then used to calculate the number of repeats at each locus. The loci were identified according to color and the length of the product. Table 2 shows the coding for loci Mpn13, Mpn14, Mpn15, and Mpn16. The single PCR sequencing results were also analyzed using the above formula. In this study, the MLVA type was designated by a prefix of M plus the number of repeats at each locus in the order Mpn13-Mpn14-Mpn15-Mpn16 (Mn-n-n-n), as described previously (17). The repeat number for partial sequences was rounded up to the next whole number (e.g., 3.2 was recorded as 4).

RESULTS

Detection of *M. pneumoniae* from clinical specimens. Of the 477 clinical samples, 146 specimens (30.6%; 95% confidence interval [CI], 26.7% to 34.5%) were positive for *M. pneumoniae* by real-time PCR, with the highest number of positive samples recovered in April (69.2%; $P < 0.005$). The positive rates for each month from January to December were 24.5%, 17.9%, 22.2%, 69.2%, 60.0%, 17.8%, 44.2%, 31.0%, 36.1%, 21.6%, 11.1%, and 23.5%, respectively.

The mean (± standard deviation [SD]) age of the patients was 4.07 years ± 3.95 years. The rates of *M. pneumoniae* infection in the samples for the three age groups were 25.7% (95% CI, 20.4% to 30.9%) in the 0- to 3-year age group, 42.9% (95% CI, 33.8% to 51.9%) in the 4- to 6-year age group, and 30.0% (95% CI, 20.8% to 39.2%) in the 7- to 12-year age group. No significant differences in the rates of *M. pneumoniae* infection were detected between the age groups ($P > 0.05$).

The male-to-female ratio of the *M. pneumoniae*-positive patients was 1.2:1 (77/69), with rates of 26.3% (95% CI, 21.4% to 31.2%) and 37.5% (95% CI, 35.3% to 39.7%) in males and females, respectively. This indicated that *M. pneumoniae* infection was more common in females ($P < 0.05$).

Typing of clinical specimens. Because of the low concentrations of target DNA in the samples, only 137 of the 146 samples were typed by MLVA (64 in the 0- to 3-year age group, 45 in the 4- to 6-year age group, and 28 in the 7- to 12-year age group). Four distinct MLVA types were identified, M4-5-7-2 (133/137, 97.1%; 95% CI, 94.34% to 99.82%), M4-5-6-2 (2/137, 1.45%; 95% CI, 0% to 3.41%), M3-5-6-2 (1/137, 0.72%; 95% CI, 0% to 2.09%), and M5-5-7-2 (1/137, 0.72%; 95% CI, 0% to 2.09%).

TABLE 2 Coding and correcting table of loci Mpn13, Mpn14, Mpn15, and Mpn16

Repeat no.	Data for locus (length of repeat):							
	Mpn13 (16 bp)		Mpn14 (21 bp)		Mpn15 (21 bp)		Mpn16 (47 bp)	
	Sequencing[a]	CE[b]	Sequencing	CE	Sequencing	CE	Sequencing	CE
0	592	NC[c]	152	NC	109	NC	371	NC
1	595	NC	173	NC	115	NC	418	NC
2	611	NC	194	NC	136	NC	465	460–469
3	627	626–628	215	213–216	157	NC	512	508–515
4	643	640–645	236	NC	178	NC	559	NC
5	659	657–660	257/258	245–253	199	NC	606	NC
6	675	674–680	278	NC	220	218–221	653	NC
7	691	NC	299	NC	241	238–245	700	NC
8	707	NC	320	NC	263	NC	747	NC
9	723	NC	341	NC	284	NC	794	NC
10	739	NC	362	NC	305	NC	841	NC

[a] Lengths of the repeats identified by sequencing and theoretical calculation.
[b] Lengths of the repeats identified by capillary electrophoresis were determined by the data of the specimens from 2003 to 2013 in our lab.
[c] NC, not classifiable in our study.

Type M4-5-7-2 appeared in every month in 2013, type M4-5-6-2 appeared in March and April, type M3-5-6-2 appeared in March, and type M5-5-7-2 appeared in July. Both of the patients typed M4-5-6-2 were female, while the patients typed M3-5-6-2 and M5-5-7-2 were male.

Of the four loci, locus Mpn13 showed three different repeat numbers ranging from three to five. Locus Mpn15 had two different-sized PCR products, with either six or seven repeats. Loci Mpn14 and Mpn16 were the most homogeneous, with all samples having five and two repeats, respectively.

In the 0- to 3-year age group (64/137), 95.3% (61/64) of isolates were M4-5-7-2, 3.12% (2/64) were M4-5-6-2, and 1.56% (1/64) were M5-5-7-2. In the 4- to 6-year age group (45/137), 97.8% (44/45) were M4-5-7-2, and 2.22% (1/45) were M3-5-6-2. All samples in the 7- to 12-year age group (28/137) were of the M4-5-7-2 genotype. Therefore, the M4-5-7-2 genotype was prevalent across all age groups. No significant relationships between age and genotype were detected ($P > 0.05$).

Accuracy determination. The types of the 50 *M. pneumoniae* samples as analyzed by CE were the same as the results produced by sequencing. Small differences in the product sizes of CE analysis were corrected by sequencing (Table 2). The fragment size and the actual repeat number at different loci were determined by sequencing and theoretical calculation. The CE results presented in Table 2 are standards used for comparison and were determined using specimens collected from 2003 to 2013 in our lab (details not described herein).

Detection of macrolide resistance gene. We used nPCR-CE-SSCP, as described previously, to detect macrolide resistance mutations in the 23S rRNA gene. The gene region was successfully amplified from 130 out of the 137 samples (94.9%; 95% CI, 91.4% to 98.44%), with 128 (98.5%; 95% CI, 96.34% to 100%) of the specimens containing an A2063G mutation. No mutations were detected at the other three sites associated with resistance (2064, 2611, and 2617). Of the 128 samples containing the macrolide resistance mutation, 124 (96.9%; 95% CI, 94.1% to 99.6%) were genotype M4-5-7-2, and the other four specimens were distributed across the remaining genotypes: M4-5-6-2 (2/4), M3-5-6-2 (1/4), and M5-5-7-2 (1/4). The two macrolide-sensitive samples were typed M4-5-7-2 and were detected in September and November.

DISCUSSION

In recent years, *M. pneumoniae* epidemics have been reported in many countries, including Denmark, England, and China (9–17). In this study, the rates of *M. pneumoniae* infection in the samples were highest in April and May 2013 and may be a continuance of the epidemic outbreak in 2012 in Beijing (17). We detected no significant differences in the rates of infection between the different age groups, but *M. pneumoniae* infection was more common in females ($P < 0.05$).

MLVA is a commonly used method for molecular typing of microorganisms that uses variation in the number of tandem-repeat sequences found at different loci in the genome. Degrange et al. (9) first used this method to investigate *M. pneumoniae* isolates. Because of the difficulties surrounding cultivation of *M. pneumoniae*, Dumke and Jacobs (16) developed a culture-independent method (nested PCR [nPCR]) for MLVA typing of *M. pneumoniae* clinical specimens.

Until now, all studies using MLVA to type *M. pneumoniae* isolates have targeted five select loci containing tandem repeats (Mpn1, Mpn13, Mpn14, Mpn15, and Mpn16). To date, >45 MLVA genotypes have been described, and multiple prevalent genotypes can exist, complicating the monitoring of *M. pneumoniae* outbreaks. The instability of Mpn1 in previous *in vitro* studies suggests that further evaluation of whether this locus is a reliable marker for MLVA typing is needed (9, 13, 17). Therefore, in this study, we focused on the remaining four VNTR loci (Mpn13, Mpn14, Mpn15, and Mpn16) to type *M. pneumoniae* in only one tube.

Previous studies all used the method described by Degrange et al. and multiplexed the five loci with fluorescently labeled primers in two solutions (Mpn1, Mpn14, and Mpn16 in solution 1; Mpn13 and Mpn15 in solution 2) to type the *M. pneumoniae* isolates (9–15). Because *M. pneumoniae* was difficult to culture, Dumke and Jacobs (16) and Sun et al. (17) used the nest PCR in the MLVA approach directly from the clinical specimens (16, 17). All of the methods developed to date for typing *M. pneumoniae*

isolates are time-consuming and costly and include a risk of contamination. Therefore, we developed this system for typing and analyzing *M. pneumoniae* isolates using mPCR-CE in only one tube. The MLVA system allows for the typing of *M. pneumoniae* directly from clinical specimens, meaning that it is simple, fast, and easy to apply to a wide variety of research. In this study, the prevalent genotype across all age groups was M4-5-7-2, and we found no associations between macrolide resistance and MLVA types. However, Qu et al. (20) found a possible association between M3-5-6-2 and macrolide susceptibility. Because of the low number of M3-5-6-2 type samples in our study, further investigation is needed to verify this association.

In summary, the highest rate of *M. pneumoniae* infections was found in April in Beijing, China, in 2013, and these infections may have been a continuance of the epidemic outbreak in 2012. M4-5-7-2 was the predominant genotype. This is the first study using multiplex PCR-capillary electrophoresis in only one tube to type and analyze *M. pneumoniae* clinical specimens directly. The method is rapid, simple, and inexpensive and shows great potential for detecting and investigating *M. pneumoniae* outbreaks in the future.

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