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Rapid-Cycle PCR for Detection and Typing of *Mycoplasma pneumoniae* in Clinical Specimens

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We designed several new primers and modified previously described species- and type-specific primers targeting the *Mycoplasma pneumoniae* P1 adhesin gene. Optimized thermal profiles allowed one-step or nested PCR to be completed in less than 1 h. In 10 patients with pneumonia, *M. pneumoniae* type 1 was identified in 3 and type 2 in 7.

Mycoplasma pneumoniae is a causative agent of tracheobronchitis and primary atypical pneumonia in children (5) and one of the commonest causes of community-acquired pneumonia in adults, ranging in severity from mild to life-threatening (13, 25). *M. pneumoniae* also causes extrapulmonary complications and infections, involving the heart (16), central nervous system (3), and genitourinary tract (18).

Rapid confirmation of the diagnosis is important for clinical and epidemiological reasons (14), but culture is slow, technically demanding, and relatively insensitive (4, 18). Currently, diagnosis of *M. pneumoniae* infection usually relies on serology, which also has major limitations (4, 19). Recently, PCR has been accepted as a valuable method for diagnosis of *M. pneumoniae* infections (1, 4).

M. pneumoniae can be separated into two types on the basis of divergence of P1 gene sequence (21, 22, 23). PCR is the simplest and the most practical typing method (10). The relationship between these types and virulence, epidemic activity, reinfection, cross-protection or clinical severity, and complications of *M. pneumoniae* infection requires further investigation (10, 20).

In this study, we aimed to develop a faster and more practical PCR for detection and typing of *M. pneumoniae*.

Reference strains used were *M. pneumoniae* M129 (ATCC 29342), *M. pneumoniae* FH (ATCC 15531), and *Mycoplasma genitalium* (ATCC 33530), which were purchased directly from the American Type Culture Collection. Nasopharyngeal aspirates were obtained from 176 children admitted to the hospital with pneumonia during a 12-month period (April 1998 to March 1999). Specimens were stored at −70°C. Before processing, they were thawed and separated into two portions, each about 200 to 500 μl. One portion (the "original" specimen) was used directly for DNA extraction and PCR examination, and the other was inoculated into 2 ml of SP4 broth ("broth-enhanced" specimen). After 24 h of incubation at 37°C, 500 μl of broth was used for DNA extraction. DNA was prepared as previously described (6, 11, 26).

The following reference sequences were used to improve and design new primers: P1 adhesin gene sequences of *M. pneumoniae* type 1 (M129 [ATCC 29342]) (GenBank accession numbers M18639, M21519, and M20916); P1 adhesin gene sequences of *M. pneumoniae* type 2 (TW 7-5 and FH [ATCC 15531]) sequences as previously described (21); and the MgPa adhesin gene sequence of *M. genitalium* (G-37 [ATCC 33530]) (GenBank accession number M31431). The oligonucleotide primers used are listed in Table 1. The primer pairs used for initial screening for *M. pneumoniae* in clinical specimens and for optimizing thermal profiles in single-step and nested PCRs for detection and typing of *M. pneumoniae*, as well as the conditions used for PCRs, are shown in Tables 2 and 3.

The 25-μl amplification reaction mixtures were used as previously described (11). For nested PCR, 1 to 5 μl of the first-step PCR product was used as the template in the second-step PCR reaction systems. All PCRs were performed in a Perkin-Elmer thermocycler 9600. Twelve microliters of PCR products was analyzed by electrophoresis on 2% agarose gels, which were stained with 0.5-μg/ml ethidium bromide. If all controls were satisfactory, a visible band of the appropriate size on UV translumination was accepted as evidence of the presence of *M. pneumoniae* DNA.

Unmodified primer pairs P4A-P4B (18) and MP-P11–MP-P12 (2) were used in parallel to screen all original and broth-enhanced specimens for *M. pneumoniae*. The thermal profiles used were as previously described (2, 18). *M. pneumoniae* DNA was detected in 10 of 176 (6%) specimens from patients with pneumonia by using this single-round PCR—both specimen types were positive in six cases, while original or broth-enhanced specimens alone were positive in two cases each. Results were the same with both sets of detection primers for all but one specimen, which was positive only with primers P4A-P4B.

Nested PCR, including the use of type-specific inner primers, showed that three specimens contained *M. pneumoniae* type 1, while seven specimens contained type 2 P1 gene fragments. Amplicons (P1-40/MPAW2 or P1-40/MPAW1)—containing highly heterologous regions (partial Rep MP4) of the P1 adhesin gene—from all *M. pneumoniae*-positive specimens were sequenced to confirm the PCR typing results and the specificity of new type-specific primer pairs. Sequences of all amplicons were identical with those of the corresponding *M. pneumoniae* P1 adhesin gene sequences in GenBank (M129, type 1) or published previously (TW 7-5 and FH, type 2) (21).

It has been suggested that the times commonly used in PCR cycles are often unnecessarily long, which may reduce the spec-

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TABLE 1. Oligonucleotide primers used in this study

Primer name	T_m (°C) (a/b)[a]	Sequence[b]	Reference
P1-40	76.1/78	**39**TTGG[c] ATT CTC ATC CTC ACC GCC ACC**63**	25
P1-178	77.0/78	**177** CAA TGC CAT CAA CCC GCG CTT AAC C**201**	25
P1-285	76.8/76	**285**GTT GTC GCG CAC TAA GGC CCA CG**263**	25
P1-331	78.3/80	**330**CGT GGT TTG TTG ACT GCC ACT GCC G**306**	25
MP-P11	77.0/78	**176**CCAA TGC CAT CAA CCC GCG CTT AAC**200**	2
MP-P12	73.6/82	**648**CCC CCT/(G)[d] T/(C)TG CAA CTG CTC ATA GTA CACC**621**	2
P4A	78.9/88	**3944/3968**CTTC AGG CTC AGG TCA ATC TGG CGT GGA**3971/3995**	18
P3A3BS	73.4/84	**4138/4162**GAT GTT GAT GGT ATT GTA CGCACCCCAC**4165/4189**	18
P3A3BA	73.5/84	**4162/4186**GGGTGCG TAC AAT ACC ATC AAC ATC GTC**4135/4159**	18
P4B	76.7/84	**4296/4320**TACC GGA TCA AAC AGA TCG GTG ACT GGG T**4268/4292**	18
MPSW1 (MP-1)	78.1/90	**459**CGAC CAA ACC GGG CAG ATC ACC TTT AACC**487**	2
MPSW2	53.0/66	**526**GTG AAA CGA GGT CAA AAA CAA GG**550**	This study
MPAW	55.0/64	**1143/1158**ATA AGG CGC ATC GTAC AGA A TC**1122/1137**	This study
MPAW1	79.3/88	**1149/1164**GCG CGC ATA AGG CGC ATC GTAC AGA A TC**1122/1137**	This study
MPAW2	83.4/90	**1175/1190**TCA ACG CGG TCA ATG GCG GTA CGG TTG C**1148/1163**	This study
PnG1S	76.2/88	**96**ACACTTCA CAA GTA CCA CGA CGC TCA A**125**	12
PnG1A	78.7/92	**984**TTGAGTTGG ACG GAC TGA CCC GAC TCC TC**955**	12
PnG2S	75.6/90	**667**GGGAGTT CGT CAG GCT CAG ACA GCA CTA A**695**	12
PnG2A	83.5/86	**931**CCACCTGT TCG GTG CCT TGG TCA CCG GAG**903**	12
MPI-A1	72.2/84	**700**CGG TGG TGG AAG TAT TTT GAC CAC TCT C**673**	This study
MPI-A2	75.1/86	**702**CCC GGT GGT GGA AGT ATT TTG ACC ACT C**675**	This study
MPII-A1	73.6/86	**701**GTT TGG TTA GTG CTG TCT GAG CCT GAC G**674**	This study
MPII-A2	73.5/86	**704**CCT GTT TGG TTA GTG CTG TCT GAG CCT G**677**	This study

[a] The two primer T_m values are those provided by the primer synthesizer (Sigma-Aldrich) (a) and those calculated by the formula $T_m = [4 \times \text{numbers of (G+C)}] + [2 \times \text{numbers of (A+T)}]$ (b).
[b] Boldface numbers represent the numbered base positions at which primer sequences start and finish (numbering from the start codon of the P1 adhesin gene).
[c] Underlined sequences show bases added to modify previously published primers and/or probes.
[d] Letters in parentheses indicate alternative nucleotides in type 2.

ificity and sensitivity of reactions (8, 27). Rapid-cycle PCR can improve product specificity significantly (9). Newer types of thermocyclers have been used successfully for rapid-cycle PCR, with total reaction times between 90 s and 20 min (7, 12, 17, 24). However, because conventional heat-block thermocyclers are still most commonly used, our aim was to develop a faster PCR cycle that could be used with this type of equipment (e.g., Perkin-Elmer thermocycler 9600). To allow shorter ramp and/or incubation times and increase the specificity, we modified the most commonly used primer pairs targeting the P1 adhesin gene to increase their melting temperatures (T_m) (≥72°C), so that high annealing temperatures (≥70°C) could be used.

After clinical specimens containing *M. pneumoniae* (in one or both portions) had been identified by PCR as described above, both portions of those 10 specimens were used to op-

TABLE 2. Primer pairs[a] and conditions used for one-step PCRs for the detection and typing of *M. pneumoniae*

Purpose or specificity	Primer pairs	Conditions[b]
Screening for *M. pneumoniae*	P4A-P4B (unmodified) MP-P11–MP-P12 (unmodified)	95°C, 60 s; 55°C, 60 s; 72°C, 60 s; 35 cycles
M. pneumoniae detection	P1-40–P1-331	96°C, 1 s; 70°C, 1 s; 74°C, 1 to 10 s (according to amplicon length); 40 cycles
	P1-178–P1-285	
	MP-P11–MP-P12	
	MP-P11–P1-331	
	MPSW1–MP-P12	
	P4A-P4B	
	P4A-P3A3BA	
	P3A3BS-P4B	
M. pneumoniae type 1	PnG1S-PnG1A	
	PnG1S–MPI-A1	
	PnG1S–MPI-A2	
	MPSW1-PNG1A	
M. pneumoniae type 2	PnG2S-PnG2A	
	MPSW1–MPII-A1	
	MPSW1–MPII-A2	

[a] Primers are as shown in Table 1 (i.e., with modification, if applicable), unless otherwise stated.
[b] Denaturation, annealing, and elongation temperatures and times, respectively.

TABLE 3. Primer pairs and conditions used for nested PCRs for the detection and typing of *M. pneumoniae*

Purpose or specificity	Outer primer pairs	Inner primer pairs	Conditions[a]
M. pneumoniae detection	P1-40–MPAW2 or P1-40–P1-331; P1-40–MPAW2 or MP-P11–MP-P12; P4A-P4B	P1-178–P1-331 or P1-178–P1-285; MP-P11–P1-331 or MPSW1–MP-P12; P4A-P3A3BS; P3A3BS-P4B	1st step: 96°C, 1 s; 70°C, 1 s; 74°C, 10 s; 2nd step: 96°C, 1 s; 70 to 72°C, 1 s; 74°C, 1 to 10 s (according to amplicon length); 25 to 30 cycles for each step or 1st step: 96°C, 1 s; 70°C, 10 s; 2nd step: 96°C, 1 s; 70 to 72°C, 1 to 10 s (according to amplicon length); 25 to 30 cycles for each step
M. pneumoniae type 1	P1-40–MPAW2 or PnG1S-PnG1A	PnG1S-PnG1A PnG1S-MPI-A1 PnG1S-MPI-A2 MPSW1-PnG1A	
M. pneumoniae type 2	P1-40–MPAW2 or MPSW1-PnG2A	MPSW1–MPII-A1 MPSW1–MPII-A2 PnG2S-PnG2A	
Sequencing	P1-40–MPAW2 or P1-40–MPAW1	P1-178–MPAW1 or P1-178–MPAW2	

[a] Denaturation and annealing-elongation temperatures and times, respectively.

timize thermal profiles for the modified and new primers. The methods were adjusted to achieve sensitivities at least as high as those obtained by using unmodified primer pairs P4A-P4B (18) and MP-P11–MP-P12 (2), i.e., to give positive results in all known *M. pneumoniae*-positive specimens. This was achieved using denaturation and annealing and elongation at times and temperatures of 1 s at 96°C, 1 s at 70°C, and 1 to 10 s (depending on the amplicon length) at 70 to 74°C, respectively, in a Perkin-Elmer thermocycler 9600 (Tables 2 and 3).

With use of modified and new primers with optimized thermal profiles, the one-step PCR could be finished within 40 min and the nested PCR within 1 h (15). Thus, the whole procedure—DNA preparation (<2 h), detection of *M. pneumoniae* in clinical specimens (1 h), typing of *M. pneumoniae* for positive specimens (1 h), and electrophoresis (<2 h for both detection and typing)—could be completed in less than one working day. We believe that this is the fastest *M. pneumoniae* detection and typing method reported so far. In future, more rapid DNA preparation and automated amplicon detection systems (instead of traditional electrophoresis) will allow PCR detection and typing time to be further reduced.

An algorithm was developed for clinical use of a rapid, nested PCR for detection and typing of *M. pneumoniae* (Fig. 1). We used a single outer primer pair (either P1-40–MPAW2 or P1-40–MPAW1) for the first round, followed by species- and type-specific primers for the second round, as required. The choice of primers was based on their sensitivity, band clarity, and suitable amplicon size. Our typing results showed that contrary to results in several other countries (20), type 2 was more commonly implicated than type 1 in a group of Australian children with pneumonia.

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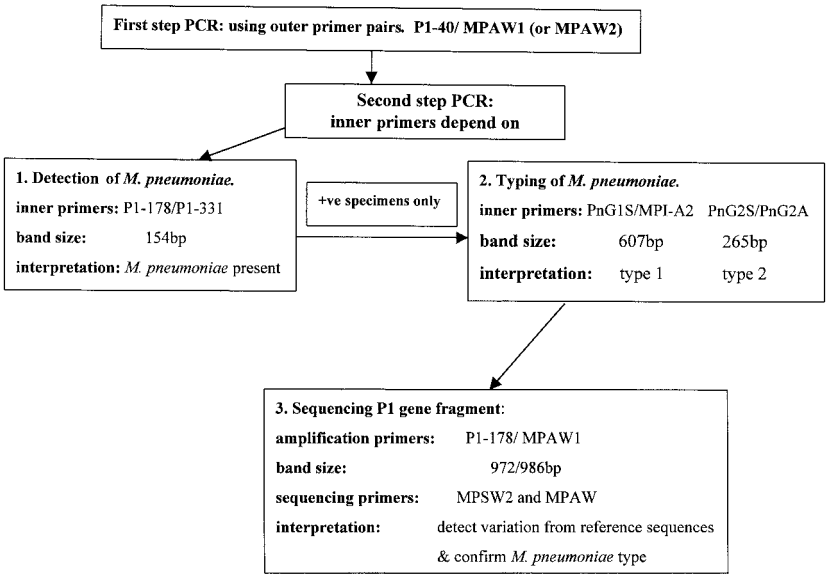

FIG. 1. Algorithm for detection, typing, and sequencing of *M. pneumoniae* using nested PCR.

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