Identification of nine species of the *Chlamydiaceae* using PCR-RFLP

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The family *Chlamydiaceae* contains two genera and nine species. Rapid and easy identification of these species is essential for taxonomic, epidemiological and clinical determinations. Currently, DNA sequence analysis is the only accepted method that decisively distinguishes all nine species. In this study, a simple and rapid PCR-RFLP procedure was developed by which laboratory-cultured chlamydial specimens could be identified. To accomplish this, conserved oligonucleotide primers and restriction sites were deduced from 16S and 23S rRNA sequence data from >50 chlamydial strains representing all nine species. DNA from 25 previously characterized chlamydial strains were tested with these primers and restriction enzymes. All nine chlamydial species were reliably distinguished in the tests. The procedure was optimized by adjusting the annealing temperature using both a standard and a heat-activated DNA polymerase to reduce mismatch PCR amplification of mycoplasmas and other bacteria. The result was that a PCR method for species identification of chlamydial isolates and for distinguishing mycoplasmas and chlamydiae was created. This method can be used to rapidly identify known species of the family *Chlamydiaceae*.

Keywords: *Chlamydia*, PCR-RFLP, rRNA, human pathogens, animal pathogens

INTRODUCTION

The members of the order *Chlamydiales* are obligately intracellular bacteria that are proven or suspected pathogens of vertebrates. They cause reproductive, respiratory, cardiovascular, gastrointestinal or systemic disease, as well as conjunctivitis, arthritis and encephalitis (Birtles *et al.*, 1997; Everett & Andersen, 1997; Kahane *et al.*, 1996, 1998; Lieberman *et al.*, 1997). Recent reclassification of *Chlamydiales* identified four families: *Parachlamydiaceae*, *Simkaniaceae*, *Waddliaceae* and *Chlamydiaceae* (Everett *et al.*, 1999; Rurangirwa *et al.*, 1999). In the reclassification by Everett *et al.* (1999), more than 60 *Chlamydiaceae* strains belonging to *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae* and *Chlamydia pecorum* were regrouped into two genera and nine species based on differences in phenotype, 16S rRNA and 23S rRNA. All species of *Chlamydiaceae* are recognized by monoclonal antibodies (mAbs) that

detect the LPS trisaccharide epitope α Kdo-(2 $\rightarrow$ 8)- α Kdo-(2 $\rightarrow$ 4)- α Kdo (Löbau *et al.*, 1995), and under natural conditions each of the species has a relatively limited host range and a relatively limited disease range. Because many animals are host to more than one chlamydial species, a simple technique that distinguishes each *Chlamydiaceae* species is needed for taxonomic, epidemiological and pathological studies and for improved diagnostic capability.

Efforts to develop routine species identification of chlamydiae have been ongoing since the early days of chlamydial research. Early methods relied on presumed tissue or host affinities, presumed unique epidemiology, or on specific serology, all characteristics of host response to chlamydiae. In the mid-1960s, phenotypic traits of specific chlamydial strains were identified: glycogen accumulation and sulfadiazine sensitivity (Gordon & Quan, 1965; Lin & Moulder, 1966). These characteristics and inclusion morphology became the basis for the description and differentiation of *C. trachomatis* and *C. psittaci* species (Page, 1968). Later, DNA–DNA reassociation, serology and/or morphology of the infectious form were used to identify the new species *C. pneumoniae* and *C. pecorum*

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(Cox *et al.*, 1988; Fukushi & Hirai, 1989, 1992; Grayston *et al.*, 1989). Recent availability of 16S and 23S rRNA sequence data has made it possible not only to classify all currently known *Chlamydiaceae* strains into nine species, but also to use 'signature sequences' for rapid identification of species using PCR and DNA sequencing (Everett *et al.*, 1999). Methods other than sequencing, however, are needed for identification of species because DNA sequencing is limited to specialized laboratories.

Detection of many strains, groups of strains and species has been possible using mAbs, PCR, PCR-RFLP or DNA hybridization procedures (e.g. Andersen, 1991; Black, 1997; Kaltenböck *et al.*, 1997; Morre *et al.*, 1998; Sayada *et al.*, 1995; Vretou *et al.*, 1996; Wang *et al.*, 1985). However, these assays were not designed for speciation *per se*, so when a test fails to identify an isolate, additional effort must be invested. *C. trachomatis* and *C. pneumoniae* can be identified with commercially available mAbs. Species identification of *C. trachomatis* may also use mAbs that recognize a linear, variable-segment-IV epitope in the major outer-membrane protein (MOMP) (Batteiger *et al.*, 1996). However, the epitope recognized by these mAbs is also present in the MOMP of *Chlamydia muridarum* and *Chlamydia suis* (Everett *et al.*, 1999). With the change in classification, these mAbs are no longer species-specific but are, instead, genus-specific. Other mAbs previously characterized as being genus-specific must now be described as family-specific (Everett *et al.*, 1999). These mAbs recognize an LPS epitope in all of the species mentioned above and also in species belonging to the new genus *Chlamydophila*, which includes *C. pecorum*, *C. pneumoniae*, *C. psittaci*, *Chlamydophila abortus*, *Chlamydophila caviae* and *Chlamydophila felis*.

DNA-based tests for chlamydiae generally target *ompA*, plasmid or rDNA. Nested PCR and PCR-RFLP methods have been developed to identify two to four chlamydial species (Holland *et al.*, 1990; Kaltenböck *et al.*, 1997; Meijer *et al.*, 1997; Messmer *et al.*, 1997; Tong & Sillis, 1993). Examination of > 50 complete *ompA* sequences, however, indicates that nested PCR procedures cannot satisfactorily be expanded to distinguish all nine species because of the excess diversity of this gene. PCR-RFLP targeting of the rRNA operon (Meijer *et al.*, 1997) has differentiated four chlamydial species with sub-groups in both *C. trachomatis* and *C. psittaci*. The primers that are used in that test generate a ≥ 800 bp PCR product spanning the 16S–23S ribosomal intergenic spacer. This 800 bp segment has too many restriction sites for identification of nine species. Meijer's 23S primer also has a 2–4 base mismatch with chlamydial 23S rRNA genes, which reduces both sensitivity and specificity.

The rRNA operon, particularly the 16S–23S intergenic spacer, is an appropriate target for PCR-RFLP identification of species of *Chlamydiaceae* because it is a phylogenetic marker for the nine chlamydial species and contains conserved sequence

interspersed with species-specific segments (Everett & Andersen, 1997; Everett *et al.*, 1999). In the present report, rDNA sequences from over 50 *Chlamydiaceae* strains representing all nine species were used as a basis to design PCR primers and to select enzymes for a PCR-RFLP method of identification that would distinguish the nine chlamydial species. Sizes of the PCR-RFLP fragments were consistent with reported DNA sequences. Comparative amplification using an alternative DNA polymerase made it possible to also detect mycoplasmas which may contaminate host-cell cultures or chlamydial preparations. This PCR-RFLP identification method has taxonomic, epidemiological and clinical value.

METHODS

Bacterial strains. Twenty-five strains of *Chlamydiaceae* that had been previously characterized using microimmunofluorescence, serology, *ompA* sequence analysis, RFLP, DNA hybridization and/or *in vitro* growth were used in this study (Table 1). The NADC strains were grown in Vero or McCoy cell cultures or in embryonating chicken eggs (Andersen, 1991) (Table 1). Ten mycoplasmas and DNA from other chlamydial isolates, *Legionella pneumophila* and *Simkania negevensis* were provided by laboratories where they had been characterized or isolated (Table 1). The first preparation of *Simkania* DNA proved to contain template from *Chlamydia trachomatis* L2/434/BU that was being grown in the same laboratory. Upon discovery of the contamination in the course of this study, *Simkania* from an earlier passage was obtained, confirmed to be free of contamination, and used in subsequent analyses. In addition, a plasmid clone was used that contained an rRNA segment from *Mycoplasma orale* (Everett & Andersen, 1997).

DNA template preparation. Chlamydial template DNA was prepared by alkaline lysis. The first step in alkaline lysis was to pellet chlamydiae and/or chlamydial-infected cells by centrifugation (10000 g). The pellet was resuspended in 30 mM Tris pH 9.0, 10 mM EDTA, pH 9.0, 50 mM DTT and incubated for 1 h at 37 °C. An equal volume of 1% Nonidet P-40 was added to each sample, and 5 µl DNase-free RNase (2 µg) (Boehringer Mannheim) was added to each 200 µl mixture. This mixture was incubated 1 h at 37 °C, extracted with phenol/chloroform, and extracted with chloroform (Sambrook *et al.*, 1989).

PCR conditions. Primers 16SF2 (5' CCGCCGTCACATCATGG 3') and 23R (5' TACTAAGATGTTTCAGTTC 3') were selected based on their conservation among all known *Chlamydiaceae* strains and on taxon-specific sequence variation within the predicted PCR amplicon (Everett & Andersen, 1997). Primer 16SF2 was located at approximately nucleotide position 1400 in the chlamydial 16S rRNA gene; primer 23R was located at approximately nucleotide position 200 in the chlamydial 23S rRNA gene (Everett & Andersen, 1997). Primer 23R had a 3 base mismatch with the *Simkania* sequence. These primers were used to produce PCR amplicons using 0.25 µg of each DNA template. Templates were amplified in a GeneAmp PCR System 9600 thermocycler using PCR Core Reagents, AmpliTaq DNA polymerase and/or AmpliTaq Gold DNA polymerase (Perkin-Elmer). Reaction mixes were prepared on ice, and each 50 µl mixture consisted of 1 × GeneAmp

Table 1. Strain references and sources

Abbreviations: ^TType strain for this species; NADC = USDA, Agriculture Research Service, National Animal Disease Center, Avian and Swine Respiratory Disease Research Unit, Ames, IA, USA; NVSL = USDA, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories, Diagnostic Virology Laboratory, Ames, IA, USA.

Species	Strain	Source	Reference
<i>Chlamydia muridarum</i>	MoPn ^T (VR 123)	H. D. Caldwell	Nigg (1942)
	SFPD	J. G. Fox	Stills <i>et al.</i> (1991)
<i>Chlamydia suis</i>	H5	NADC	Everett & Andersen (1997)
	R22	NADC	Everett & Andersen (1997)
	S45 ^T (VR 1474)	B. Kaltenböck	Kaltenboeck <i>et al.</i> (1993)
<i>Chlamydia trachomatis</i>	A/Har-13 ^T (VR 5718)	H. D. Caldwell	Wang & Grayston (1962)
	D/UW-3/CX (VR 885)	H. D. Caldwell	Wang <i>et al.</i> (1985)
	F/IC/CAL3	H. D. Caldwell	Hanna (1962)
<i>Chlamydophila abortus</i>	A22	A. J. Herring	McClenaghan <i>et al.</i> (1984); Stamp <i>et al.</i> (1950)
	B577 ^T (VR 656)	NADC	Storz (1966)
	EBA	NADC	Perez-Martinez & Storz (1985)
<i>Chlamydophila caviae</i>	GPIC ^T (VR 813)	NADC	Murray (1964)
<i>Chlamydophila felis</i>	FP Baker ^T (VR 120)	NADC	Baker (1942)
	FP Cello	NVSL	Cello (1967)
	FP Vaccine	Solvay Animal Health Inc., MN	
<i>Chlamydophila pecorum</i>	1710S	B. Kaltenböck	Kaltenboeck <i>et al.</i> (1993); Kölbl <i>et al.</i> (1970)
	BP-1	NADC	Fraser & Berman (1965)
	E58 ^T (VR 628 ^T)	NADC	McNutt & Waller (1940)
<i>Chlamydophila pneumoniae</i>	CM-1	C. M. Black	Black <i>et al.</i> (1992)
	N16	C. Storey	Wills <i>et al.</i> (1990)
	TW-183 ^T (VR 2282)	L. A. Campbell	Kuo <i>et al.</i> (1995)
<i>Chlamydophila psittaci</i>	6BC ^T (VR 125)	NADC	Golub & Wagner (1947)
	CP3 (VR 574)	NADC	Page & Bankowski (1960)
	Daruma	H. Fukushi	Pudjiatmoko <i>et al.</i> (1997)
	GD	NADC	Illner (1960)
<i>Simkania negevensis</i>	Z ^T (VR 1471)	S. Kahane	Kahane <i>et al.</i> (1995)
<i>Legionella pneumophila</i>	SVir	P. S. Hoffman	
<i>Acholeplasma laidlawii</i>	Field strain	J. M. Saupe	
<i>Mycoplasma arginini</i>	96-159	J. M. Saupe	
<i>Mycoplasma bovis genitalium</i>	96-192	J. M. Saupe	
<i>Mycoplasma bovirhinis</i>	97-005	J. M. Saupe	
<i>Mycoplasma bovis</i>	97-002	J. M. Saupe	
<i>Mycoplasma californicum</i>	Field strain	J. M. Saupe	
<i>Mycoplasma gallinaceum</i>	97-044	J. M. Saupe	
<i>Mycoplasma mycoides</i>	97-087	J. M. Saupe	
<i>Mycoplasma ovipneumoniae</i>	Field strain	J. M. Saupe	
<i>Mycoplasma pullorum</i>	96-188	J. M. Saupe	

PCR Buffer II, 2.5 mM MgCl₂, 0.15 µM each primer, 200 µM each dNTP and 0.25 µl (1.25 U) DNA polymerase. PCR amplicons for restriction analysis were produced using AmpliTaq DNA polymerase and 35 cycles of amplification: 25 s at 94 °C, 15 s at 50 °C, and 30 s at 72 °C, followed by incubation for 5 min at 72 °C. The specificity of the chlamydial primers was tested using both AmpliTaq DNA polymerase and AmpliTaq Gold DNA polymerase. Optimal annealing specificity was determined using seven duplicate sets of template and annealing temperatures of 50, 52, 54, 56, 60, 64 or 66 °C. Several sets were also tested with higher annealing temperatures. PCR cycling using both enzymes

was done simultaneously at each temperature, except that AmpliTaq Gold cycling was directly preceded by a 5 min incubation at 94 °C. A modified three-stage PCR cycling procedure was also developed that reduced the amplification of non-chlamydial PCR product but not the amount of chlamydial product. This modified procedure took advantage of improved techniques for using AmpliTaq Gold (Perkin-Elmer technical bulletin 771601-001, 1997). The three-stage procedure consisted of a pre-cycling 10 min enzyme activation stage at 95 °C, a 10-cycle amplification stage using 54 °C annealing, and a 30-cycle amplification stage using 52 °C annealing.

Sequence comparison to identify restriction enzymes. Cleavage sites for restriction enzymes were identified in 41 aligned chlamydial sequences that spanned the ribosomal 16S–23S intergenic spacer (Everett & Andersen, 1997). The sequence analysis programs MAP, PILEUP and PRETTY were used (Genetics Computer Group, 1994). Restriction enzymes were selected based on cleavage site conservation within the nine chlamydial species and predicted sizes of fragments (Table 2).

Restriction analysis and gel electrophoresis. Restriction enzymes *AccI*, *BclI*, *BfaI*, *HpaI*, *SfcI* (New England Biolabs) *DdeI* and *RsaI* (Boehringer Mannheim) were used at a ratio of 1 U enzyme to 3 µl PCR product in each 10 µl reaction mix, according to the manufacturers' instructions. Restriction fragments and DNA Molecular Weight Marker VI (Boehringer Mannheim) were separated by electrophoresis in VisiGel separation matrix (Stratagene). DNA bands in the VisiGel matrix were most effectively detected by including a small amount of ethidium bromide in the gel mix. Two other typical staining techniques were unsatisfactory with VisiGel: ethidium bromide in the running buffer only partially diffused into the gel and smeared the DNA bands; gels stained by immersion resisted destaining. Running conditions for these minigels in TAE were 100 mA for 15 min.

Optimization of PCR amplification conditions for comparison of chlamydial and mycoplasma amplicon production was carried out in 1.5% agarose gels and with 1 kb DNA Ladder (Gibco Life Technology). DNA bands were visualized using ethidium bromide.

RESULTS

PCR and restriction analysis of 25 *Chlamydiaceae* strains

Sequence analysis predicted that primers 16SF2 and 23R would produce a PCR amplicon with restriction endonuclease sites that would distinguish chlamydial species by PCR-RFLP (Table 2). Using these primers and an annealing temperature of 50 °C, *AmpliTaq* DNA polymerase produced a single amplicon from all of the *Chlamydiaceae*. The size of the PCR product was 600 bp, using template from two strains of *Chlamydia muridarum* and three strains each of *Chlamydia suis* and *Chlamydia trachomatis*. A 585 bp PCR product was produced from *Chlamydomydia caviae*, three strains each of *Chlamydomydia abortus*, *Chlamydomydia felis*, *Chlamydomydia pecorum* and *Chlamydomydia pneumoniae*, and four strains of *Chlamydomydia psittaci*. These conditions were also used to amplify PCR product from three control-DNA templates: *Legionella pneumophila*, *Simkania* and a cloned rRNA segment of *Mycoplasma orale*. Although the *Simkania* products were predicted to be 65 bases longer than the *Chlamydia* products, all of the products examined by electrophoresis appeared to be approximately the same size, except that multiple bands were produced from the plasmid clone of *M. orale* and from *L. pneumophila* (not shown).

Restriction analysis of 24 of the 25 *Chlamydiaceae* PCR products using *AccI*, *BclI*, *BfaI*, *HpaI*, *SfcI*, *DdeI*

and *RsaI* resulted in fragment sizes that were fully consistent with fragment sizes predicted by sequence analysis (Fig. 1). Chlamydial restriction fragments could be readily distinguished from *M. orale* and *L. pneumophila* fragments by size. Extra fragments in *Simkania* and *C. pneumoniae* N16 were not fully consistent with sequence predictions (Fig. 1, Table 2). Optimization was required before the N16 bands could be resolved (see below). Examination of Table 2 suggested that extra *Simkania* fragments might be products from *C. trachomatis* L2/434/BU. To test this, the *Simkania* preparation and a different preparation of *Simkania* DNA were PCR amplified using *AmpliTaq* and also using *AmpliTaq* Gold. When *AmpliTaq* was used, both templates produced PCR products, but the products differed in size: the *Simkania* preparation used in Fig. 1 generated a product that was identical in size to chlamydial products; the new *Simkania* preparation generated a distinctly larger product (not shown). Sequence analysis of the *AmpliTaq* products confirmed that the apparent *Simkania* PCR product in Fig. 1 actually derived from contaminating *C. trachomatis* L2/434/BU template. This template was preferentially amplified over the *Simkania* template because the primers were considerably more specific for L2/434/BU. Sequence analysis of the L2/434/BU product was unambiguous. Sequence analysis of *AmpliTaq* mismatch-amplified PCR product from the uncontaminated *Simkania* preparation was also unambiguous, matching sequence data previously obtained for *Simkania*. When *AmpliTaq* Gold was used, the new *Simkania* preparation produced no PCR product at all, indicating that this enzyme improved the specificity of the amplification (Fig. 2).

Increasing specificity to reduce amplification of mycoplasmas

DNA from the *C. pneumoniae* N16 specimen produced a mixture of PCR-RFLP fragments, some predicted and some not predicted by rDNA sequence analysis (Fig. 1, Table 2). N16 fragment sizes were predicted to be nearly identical to those for other strains of *C. pneumoniae* (Table 2). Because the strain was prepared in cell culture, contamination of the sample with mycoplasmas was suspected. To determine if mycoplasmas could be a problem for chlamydial PCR-RFLP analysis, 10 *Mollicutes* template DNAs were obtained for PCR specificity studies: nine field isolates from the family *Mycoplasmatales* and one from *Acholeplasmatales*. A positive control was prepared from a pellet of *C. psittaci*-NJ1-infected Vero cells and included. DNA from *L. pneumophila* and the new preparation of *Simkania* were also used (Fig. 2). To enhance chlamydial template specificity, two method changes were used: first a hot-start amplification technique (*AmpliTaq* Gold) vs the standard amplification technique (*AmpliTaq*), and then also changes in annealing temperature (Fig. 2).

Table 2. Predicted restriction endonuclease fragments of chlamydial 16S/23R amplicons, based on rDNA sequence analyses

Products that do not cut are shown in bold. Sizes are rounded to nearest 5 or 10 bases.

Taxon and strain	Restriction enzymes and fragment sizes (bp):						
	<i>Bfa</i> I	<i>Sfi</i> I	<i>Hpa</i> I	<i>Bcl</i> I	<i>Dde</i> I	<i>Aci</i> I	<i>Rsa</i> I
<i>C. psittaci</i> 6BC ⁺ , CP3, Ct1, GD, M56, MN, NJ1, Par1, WC	400, 180	440, 145	350, 235	585	405, 170, 10	585	585
<i>C. abortus</i> B577 ⁺ , A22, EBA, OSP	400, 180	440, 145	585	585	405, 170, 10	585	585
<i>C. felis</i> FP Baker ⁺ , FP Cello, FP Vaccine	400, 180	440, 145	350, 235	585	585	400, 185	585
<i>C. pecorum</i> ES8 ⁺ , BP1, IPA, L71, Z, 1710S	400, 180	585	585	320, 255	585	585	585
<i>C. caviae</i> GPIC ⁺	400, 180	440, 145	350, 235	585	405, 170, 10	400, 185	585
<i>C. pneumoniae</i> TW-183 ⁺ , CWL-1011, CWL-029, CM1, FML-12, FML-16	400, 180	440, 145	350, 235	320, 255	585	210, 190, 160, 25	585
N16	400, 180	440, 145	350, 235	320, 255	585	210, 190, 185	585
<i>C. trachomatis</i> A/Har-13 ⁺ , B/TW-5/OT, D/UW-3/CX, F/IC/CAL3	420, 90, 90	380, 220	600	330, 270	420, 170, 10	350, 200, 50	600
L2/434/BU	420, 90, 90	380, 220	600	330, 270	420, 170, 10	550, 50	600
<i>C. suis</i> H5, H7, R19, R22, R24, R27	420, 90, 90	600	600	330, 270	600	550, 50	355, 245
S45 ⁺	420, 90, 90	600	600	330, 270	430, 170	350, 160, 50, 40	355, 245
<i>C. muridarum</i> MoPn ⁺ , SFPD	420, 180	600	600	330, 270	420, 170, 10	550, 50	355, 245
<i>S. negevensis</i> Z ⁺	350, 180, 120	665	665	665	665	520, 80, 50	225, 160, 150, 115

With *AmpliTaq*, *Acholeplasma laidlawii* produced little or no PCR product at any annealing temperature. At the highest possible annealing temperature (72 °C, because PCR extension took place at 72 °C), neither chlamydial nor mycoplasma template generated PCR products. Annealing temperatures of 64 °C and 62 °C produced non-specific amplicons of many sizes from most templates (not shown). With annealing of 60 °C (not shown) and 56 °C (Fig. 2), an approximately 600 bp product was amplified from all chlamydial and non-chlamydial templates except *A. laidlawii* and two *Mycoplasma* templates; low molecular mass PCR artifacts were apparent. The low molecular mass artifacts were not present with 54 °C annealing. With 52 °C and 50 °C annealing, all chlamydiae and *Mycoplasma* yielded single-band PCR products with *AmpliTaq*, although the amount of *Mycoplasma mycoides* and *Mycoplasma arginini* products were somewhat reduced at 52 °C.

In contrast, with *AmpliTaq* Gold and annealing at 52 °C, only 2 mycoplasmas were amplified (Fig. 2, Table 3). Chlamydial DNA was amplified using 50–56 °C annealing temperatures and PCR artifacts were not generated. With ≥ 54 °C annealing, mycoplasma PCR products were barely detectable; however, chlamydial and *L. pneumophila* PCR products were still readily apparent (Fig. 2, Table 3). The *L. pneumophila* amplification suggested that the *L. pneumophila* sequence had significant homology with both

of the chlamydial primers. This homology was confirmed by sequence analysis (Table 3).

Because specificity was improved with use of *AmpliTaq* Gold and 52 °C and 54 °C annealing (Fig. 2), a modified three-stage PCR cycling procedure was used to more specifically amplify *C. pneumoniae* N16 template so that it could be re-examined using PCR-RFLP. This procedure was designed to improve specificity for N16 without a quantitative reduction in product. The amount of improvement in N16 amplification, relative to apparent N16 fragments in Fig. 1, could be readily assessed by electrophoresis of *Aci*I fragments. *Aci*I was predicted to cut N16 PCR product into three fragments, each approximately 200 bp long (Table 2). The 200 bp chlamydial *Aci*I fragments stained intensely with ethidium bromide when the three-stage N16 amplicon was used, but an uncleaved 650 bp band was still evident (Fig. 3). In Fig. 1, 200 bp *Aci*I products had been almost undetectable. In Fig. 1, four N16 bands had been distinctly inconsistent with N16 sequence data (Table 2): the 650 bp *Aci*I band, 500 bp *Dde*I band, 600 bp *Hpa*I band and 500 bp *Rsa*I band. With the three-stage N16 product, the intensities of these bands were greatly reduced (Fig. 3). In addition, a 430 bp *Bfa*I band showed itself to be a doublet of N16 and a non-chlamydial fragment, indicating that a *Bfa*I site was conserved in both the chlamydial and the non-chlamydial products.

Direct DNA sequence analysis of the uncleaved 650 bp

Fig. 1. For legend see facing page.

Fig. 2. Control of mismatch PCR amplification. A pure culture of *Simkania* was used for this analysis. Identical template sets were PCR amplified, as shown, at various annealing temperatures using either AmpliTaq Gold (immediately preceded by a 5 min activation of AmpliTaq Gold at 94 °C) or AmpliTaq. Lanes – S, *Simkania*; L, *L. pneumophila*; Chlamydiae: 1, NJ1 whole infected-cell nucleic acid preparation with RNase added to the PCR amplification mix; 2, NJ1 whole infected-cell RNA and DNA; 3, NJ1 whole infected-cell DNA. Mollicutes: 1, *Mycoplasma pullorum*; 2, *Mycoplasma mycoides*; 3, *Mycoplasma bovirhinis*; 4, *Mycoplasma bovis*; 5, *Mycoplasma gallinaceum*; 6, *Mycoplasma arginini*; 7, *Mycoplasma bovis*; 8, *Mycoplasma californicum*; 9, *Mycoplasma ovipneumoniae*; and 10, *Acholeplasma laidlawii*.

Fig. 1. PCR-RFLP of 25 *Chlamydiaceae* strains distinguishes nine species, using AmpliTaq and 50 °C annealing. Three non-*Chlamydiaceae* templates were also used. Amplicons were generated with primers 16SF2 and 23R. Restriction enzymes were selected on the basis of predicted fragment sizes (Table 2). The *Simkania* preparation in this analysis contained a small amount of *C. trachomatis* L2/434/BU template.

Table 3. Reduced mismatch amplification using AmpliTaq Gold, with primers 16SF2 and 23R

PCR products resulted from all of these templates when AmpliTaq was used, but only from the templates indicated by '+' when AmpliTaq Gold was used. Mismatches were ≥ 5 bases away from the 3' end of the primer, except where indicated. Detectable PCR amplicon formation is shown as a function of annealing temperature. NA, Not available; —, no PCR product.

Template DNA	Strain (sequence accession no.)	No. mismatched bases in primer:		Strain, this study	Amplification at:	
		16SF2	23R		50 °C	56 °C
<i>Chlamydiaceae</i>	All strains	0	0	All	+	+
<i>M. bovis</i>	Donetta ^T (U02968)	2	NA	97-002	+	—
<i>M. californicum</i>	ST-6 (M24582)	3	NA	Field strain	—	—
<i>M. hyorhinis</i>	ATCC 17981 (M24658)	1	1	NA	+	—
<i>M. mycoides</i>	Eight strains	2*	NA	97-087	—	—
Other <i>Mycoplasma</i> tales	All strains	1	NA	All	+	—
<i>A. laidlawii</i>	JA1 and PG8 (M23932 and U14905)	3*	NA	Field strain	—	—
<i>S. negevensis</i>	Z ^T (U68460)	0	3†	Z ^T	—	—
<i>L. pneumophila</i>	Many strains	1	1†	SVir	+	+

* 3' end mismatch.

† Determined in this study.

Fig. 3. Optimized PCR of *C. pneumoniae* N16 to reduce mismatch amplification of *M. hyorhinis* template. AmpliTaq Gold and the three-stage PCR amplification procedure were used (10 min enzyme activation at 95 °C; 10-cycle amplification with 54 °C annealing; 30-cycle amplification with 52 °C annealing). Comparison of this analysis with predicted N16 fragment sizes in Table 2 and with the intensity of predicted N16 fragments in Fig. 1 indicated that the proportion of N16–*M. hyorhinis* fragments increased with this procedure.

AclI PCR product from N16 unambiguously showed that the non-chlamydial template in this specimen was *Mycoplasma hyorhinis*. Mapping of a 16SF2/23R contig formed from previously reported *M. hyorhinis* sequences (GenBank accession nos X68421 and X58555) showed that *M. hyorhinis* had a 1 base mismatch with each chlamydial primer and that the predicted *M. hyorhinis* RFLP fragments could account for observed non-N16 fragments in the N16 lanes of Figs 1 and 3.

DISCUSSION

This study provided a PCR-RFLP method that can be used as a rapid, specific and inexpensive procedure for speciating *Chlamydiaceae* isolates. The method uses primer 16SF2, primer 23R and restriction enzymes *AclI*, *BclI*, *BfaI*, *DdeI*, *HpaI*, *RsaI* and *SfiI*, selected on the basis of rDNA sequence analysis of > 50 chlamydial strains. The primers generated 600 bp amplicons from *Chlamydia* templates and 585 bp amplicons from *Chlamydophila* templates. Species-specific RFLP fragment sizes were correctly predicted by sequence analysis (Fig. 1, Table 2). Some diversity was evident in the fragment sizes produced by *C. suis* templates, consistent with the substantial sequence diversity in this species (Everett & Andersen, 1997).

This study also provided a methodology that discriminates between chlamydiae and mycoplasmas by the substitution of one type of *Taq* polymerase for another. AmpliTaq DNA polymerase produced amplicons from both mycoplasmas and chlamydiae, due to primer mismatch amplification. In contrast, AmpliTaq

Gold DNA polymerase, which is heat activated, preferentially amplified chlamydial template because the primers matched it exactly. Mycoplasmas from natural sources may be cultured along with chlamydiae when new isolations are made, so the discrimination provided by these enzymes enables chlamydiologists to determine whether mycoplasmas are present in new isolates while using a single test method and primer set.

Primers and restriction enzymes in this study were selected after a comprehensive comparison of sequence data (Everett & Andersen, 1997). Primers were chosen that produced a relatively short PCR amplicon, improving the probability that amplification would be successful from even old or damaged template. A search of the GenBank database using BLAST in January 1998 showed that the 16SF2 primer matched 16S rRNA sequences from 59 chlamydial strains, several strains in the order *Verrucomicrobiales*, and a planctomycete; primer 23R complemented 23S rRNA sequences from >50 known chlamydial strains and from a number of chloroplasts. Thus, the selection of these primers should reduce or eliminate mismatch amplification. Mg^{2+} concentration and K^+ concentration in the reaction were kept moderate, to prevent EDTA and other compounds that might be added with the template from limiting amplification. When AmpliTaq DNA Gold DNA polymerase was used, amplification was quite specific for chlamydial template (Fig. 2). However, when AmpliTaq DNA polymerase was used, the chlamydial primers amplified most mycoplasma templates, even at elevated annealing temperatures (Fig. 2). The improvement of specificity with AmpliTaq Gold occurred because this enzyme is activated by a temperature-dependent increase in buffer pH as a first step in the amplification process (Perkin-Elmer technical bulletin 771601-001, 1997). Enhanced specificity provided by AmpliTaq Gold made it possible to identify a small amount of *C. trachomatis* L2/434/BU DNA in a sample of closely related *Simkania* template. Use of AmpliTaq Gold and a three-stage PCR amplification procedure enhanced the production of *C. pneumoniae* N16 PCR product against a background of mismatch-amplified *M. hyorhinis* product.

It was determined that the mycoplasmas amplified by the chlamydial primers in this study had one or more mismatches with the primers (Table 3). The apparent quantity of mismatch-produced amplicons was inversely related to the actual number of mismatches (especially 3' mismatches). Use of AmpliTaq Gold eliminated most of the mismatch amplification. It did not eliminate *L. pneumophila* amplification, however, because each primer had only a 1 base mismatch, according to 16S rRNA data in GenBank and partial sequence analysis of the *L. pneumophila* 23S rRNA gene. Whether a 2 base mismatch could prevent amplification when AmpliTaq Gold was used was not clear. However, the 3 base mismatch of the *Simkania* template with the 23R primer clearly prevented mismatch amplification by AmpliTaq Gold.

Mismatch amplification may prove useful for identifying new members of the order *Chlamydiales*. Primer 16SF2 matched the 16S rRNA from all four families in *Chlamydiales*; primer 23R perfectly complemented the 23S rRNA from *Chlamydiaceae* and *Parachlamydiaceae* spp., had a 2 base mismatch with the *Waddliaceae* type species, and a 3 base mismatch with the *Simkania* type species. Thus, when AmpliTaq DNA polymerase is used, these primers can be expected to generate an amplicon from the template of a culture of chlamydia-like bacteria. The relatedness of these bacteria to *Chlamydiaceae* can be determined by direct sequence analysis of these amplicons. Previously, analyses of chlamydia-like isolates were hindered because these bacteria were not recognized by mAbs specific for the *Chlamydiaceae*. This work indicates that primers 16SF2 and 23R can aid in the discovery and characterization of new chlamydial families.

This PCR-RFLP identification method readily distinguished all nine proposed species of *Chlamydiaceae* and can be performed in most laboratories. Identifications may be confirmed by testing isolates with specific mAbs. Use of this method with AmpliTaq DNA polymerase contrasted with AmpliTaq Gold DNA polymerase discriminates between chlamydial isolates and mycoplasma contaminants. This assay may be useful in recognizing mixed cultures of chlamydiae and will help improve our understanding of chlamydial pathogenesis, taxonomy and epidemiology.

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