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Species Identification and Subtyping of *Ureaplasma parvum* and *Ureaplasma urealyticum* Using PCR-Based Assays

FANRONG KONG, ZHENFANG MA, GREGORY JAMES, SUSANNA GORDON, AND GWENDOLYN L. GILBERT*

Centre for Infectious Diseases and Microbiology, Institute of Clinical Pathology and Medical Research, Westmead, New South Wales, Australia

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There is good evidence that the organism currently known as *Ureaplasma urealyticum* should be divided into two species—*U. parvum* (previously *U. urealyticum* biovar 1) and *U. urealyticum* (previously *U. urealyticum* biovar 2). In this study, we designed a series of primers, targeting the 16S rRNA gene and 16S rRNA-23S rRNA intergenic spacer regions, the urease gene subunits, and the 5′ ends of the multiple-banded antigen (MBA) genes, to identify and subtype these *Ureaplasma* species. All of the species-specific primer pairs could distinguish the two species, but only subtype-specific primer pairs targeting the MBA genes could distinguish subtypes within each species. *U. parvum* was separated into three subtypes, represented by serovars 1, 3/14, and 6. *U. urealyticum* was also separated into three subtypes by PCR and/or direct sequencing. Subtype 1 consisted of serovars 2, 5, 8, and 9; subtype 2 contained serovars 4, 10, 12, and 13; and subtype 3 contained serovars 7 and 11. A selection of primer pairs was used to identify and subtype 78 clinical ureaplasma isolates from vaginal swabs of pregnant women and to identify and subtype ureaplasmas directly in 185 vaginal swabs in which they had been previously detected. *U. parvum* was identified in 228 (87%) of 263 isolates or specimens, and *U. urealyticum* was identified in 50 (19%) (both were present in 6%). Serovars 3/14 (48%) and 1 (43%) were most common among *U. parvum* isolates, and subtypes 2 (62%) and 1 (34%) were most common among *U. urealyticum* isolates. This new PCR-based typing system will facilitate future studies of the relationship between individual *Ureaplasma* species or subtypes and human disease.

Evidence has been presented that the species currently known as *Ureaplasma urealyticum* should be separated into two new species, namely, *U. parvum* (previously *U. urealyticum* biovar 1) and *U. urealyticum* (previously *U. urealyticum* biovar 2) (9). Ureaplasmas are commensals in the genital tract, recognized causes of disease (15), and suspected contributors to a number of other pathological conditions (1). Because they are commonly found in healthy people, their pathogenic role can be difficult to prove (10, 18). The majority of human ureaplasma isolates belong to the proposed new species *U. parvum* (1, 3, 5, 7, 8), which includes serovars 1, 3, 6, and 14. *U. urealyticum* (biovar 2) is isolated less often but is not uncommon. Some ureaplasma serovars have been associated with disease syndromes more commonly than with normal flora (19), but data are limited because of difficulties with conventional serotyping methods. Rapid molecular methods for identification of species and subtypes would be of great value in studies of the epidemiology and pathogenesis of infections with *U. parvum* and *U. urealyticum* (8).

Recently, PCR-based methods have been used successfully to distinguish the two *Ureaplasma* species (biovars), but there is a need to improve their specificity and sensitivity (2, 8). Target sequences of the 16S rRNA gene and 16S rRNA-23S rRNA intergenic spacer regions (6, 12), the urease gene subunits (2, 11), and the 5′ ends of the multiple-banded antigen (MBA) genes (8, 16, 17) have all been used in PCR-based assays to differentiate *U. parvum* from *U. urealyticum*. Previously, we have sequenced portions of these genes from all 14 *Ureaplasma* serovars (9) and described PCR-based assays for the identification of *U. parvum* and *U. urealyticum* and the subtyping of *U. parvum* (8). In the present study, we evaluated the specificity of a large range of primers and used a small subset to develop an algorithm for the detection, species identification, and subtyping of *Ureaplasma* species.

* Corresponding author. Mailing address: Centre for Infectious Diseases and Microbiology, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Darcy Rd., Westmead, New South Wales, 2145 Australia. Phone: (612) 9845 6255. Fax: (612) 9893 8659. E-mail: lyng@icpmr.wsahs.nsw.gov.au.

MATERIALS AND METHODS

Bacterial strains. Two sets of reference strains of all 14 serovars of *U. urealyticum* (including biovars 1 and 2, referred to hereafter as *U. parvum* and *U. urealyticum*, respectively) were used as previously described (8, 9). These included one set obtained directly from the American Type Culture Collection (ATCC reference strains) and another kindly provided by H. L. Watson, Department of Microbiology, University of Alabama at Birmingham (UAB reference strains). Additional reference strains from the ATCC were used to test the specificity of primers: *Mycoplasma pneumoniae* strains M129 (ATCC 29342) and FH (ATCC 15531), *Mycoplasma genitalium* (ATCC 33530), *Mycoplasma fermentans* (ATCC 19989), *Mycoplasma hyorhinis* (ATCC 17981), and *Acholeplasma laidlawii* (ATCC 23206). *Mycoplasma hominis* isolates were grown from clinical specimens on A7 agar in our laboratory and identified by colonial morphology and partial sequencing of the 16S rRNA gene and the 16S rRNA-23S rRNA gene spacer regions.

Clinical isolates and specimens. A total of 78 *Ureaplasma* isolates obtained from vaginal swabs of pregnant women and recently cultured in our laboratory and 185 vaginal swabs obtained from pregnant women and women attending a sexually transmitted disease clinic and in which *Ureaplasma* species had been previously detected (8) were used in this study.

Oligonucleotide primers. The 29 individual primers used in this study to amplify portions of three genes of all 14 serovars are shown in Table 1. They include 4 primers that have been previously described (12, 16, 17) and 25 new primers designed by us. The nomenclature of our primers is based on specificity (e.g., UU and UP for *U. urealyticum* and *U. parvum*, respectively), gene target (e.g., UM for the MBA genes), the direction of the sequence (S, sense; A, antisense), and the numbered base position at which the primer sequence starts (9).

DNA preparation and PCRs. DNA preparation and PCR were performed as previously described (8, 9). In each reaction, positive and negative controls were processed in parallel with the tested samples to detect false-negative results or contamination. Melting temperature values are shown in Table 1. The annealing temperature was 58°C for all primer pairs except UPS2-UPA2, UUS2-UUA2,

TABLE 1. Primers targeting the sequences of three different genes or regions

Target	Primer	Specificity	T_m (°C)[a]	Sequence[b]	Reference(s)
16S rRNA gene and 16S rRNA-23S rRNA intergenic spacer regions	UPS1	*U. parvum*	68	175 ATG AGA AGA TGT AGA AAG TCG CTC 198	
	UPA	*U. parvum*	68	852 TTA GCT ACA ACA CCG ACC CAT TC 830	
	UPA1	*U. parvum*	68	854 CGT TAG CTA CAA CAC CGA CCC A 833	
	UPS	*U. parvum*	72	814 CAT CAT TAA ATG TCG GCC CGA ATG G 835	
	UPSA[c]	*U. parvum*	64	1626 TAG AAT CCG ACC ATA TGA ATT TTT A 1602	
	U8	*U. urealyticum*	70	188 GAA GAT GTA GAA AGT CGC GTT TGC 206	12
	UUA	*U. urealyticum*	68	848 CTA CAA CAC CGA CTC GTT CGA G 827	
	UUS	*U. urealyticum*	68	814 CAT TAA ATG TCG GCT CGA ACG AG 836	
	UUSA[c]	*U. urealyticum*	68	1625 AGA GTC CGA CCA TAT GAA CTT TTG 1602	
Urease gene subunits and adjoining spacer regions	UPS2[c]	*U. parvum*	60	501 CAG GAT CAT CAA GTC AAT TTA G 522	
	UPA2[c]	*U. parvum*	60	921 AAC ATA ATG TTC CCC TTT TTA TC 899	
	UUS2[c]	*U. urealyticum*	60	501 CAG GAT CAT CAA ATC AAT TCA C 522	
	UUA2[c]	*U. urealyticum*	60	919 CAT AAT GTT CCC CTT CGT CTA 899	
5′ Ends of MBA genes and upstream regions	UMS-125[c]	*Ureaplasma* spp.	66	−151 GTA TTT GCA ATC TTT ATA TGT TTT CG −125	16, 17
	UMS-57[c]	*U. parvum*	62	−82 (T/C)AA ATC TTA GTG TTC ATA TTT TTT AC −57	
	UMA222	*U. parvum*	64	245 GTA AGT GCA GCA TTA AAT TCA ATG 222	
	UMS-170[c]	*Ureaplasma* spp.	66	−195 GTA TTT GCA ATC TTT ATA TGT TTT CG −170	17
	UMS-61[c]	*U. urealyticum*	58	−83 TTT GCA AAA CTA TAA ATA GAC AC −61	
	UMA263	*U. urealyticum*	52	281 TTT GTT GTT GCG TTT TCT G 263	17
	UMA269	Serovars 3 and 14	64	293 CTA AAT GAC CTT TTT CAA GTG TAC 269	
	UMA269′	Serovars 1 and 6	64	293 CCA AAT GAC CTT TTG TAA CTA GAT 269	
	UMS-54[c]	Serovar 6	60	−77 CTT AGT GTT CAT ATT TTT TAC TAG −54	
	UMS-83[c]	Serovar 1	64	−107 TACT GTA GAA ATT ATG TAA GAT TGC −83	
	UMA219	Subtypes 1 and 2	76	245 GTA ATT GCA ACA TGG AAT TCA GCT TCG 219	
	UMA219′	Subtype 3	74	245 GTA ATT GCA ACA TGG AAT TCA GTT TCA 219	
	UMS-112[c]	Subtypes 1 and 3	62	−137 GAT TAA ACA AAA TCT TAA TGT TGT TA −112	
	UMS-112′[c]	Subtype 2	64	−137 GAT TAA ACA AAA TCT TAA TGT TGT TG −112	
	UMA194	Subtypes 1 and 3	68	220 CGT TTA ATG CTT TTT TAT CAT TTT CAG 194	
	UMA194′	Subtype 2	66	220 CGT TTA ATG CTT TTT TAT CAT TTT CAT 194	

[a] Melting temperature (T_m) values were calculated with the following formula: T_m = [4× numbers of (G+C)] + [2× numbers of (A+T)].
[b] Numbers represent the base positions at which primer sequences start and finish.
[c] Based on intergenic regions.

UMS-170–UMA263, and UMS-61–UMA263, for which the annealing temperature was 55°C.

Subtyping of *U. urealyticum* by direct sequencing. We previously showed (9) that the 10 serovars of the new species *U. urealyticum* could be divided into three subtypes based on sequence differences in amplified fragments of the 5′ ends of the MBA genes at six positions between −112 and 251. With primers UMS-170 and UMA263, amplified fragments of reference serovars, clinical isolates, and specimens were sequenced by use of an ABI 373A sequencing machine with Applied Biosystems *Taq* DyeDeoxy Terminator Cycle-Sequencing Ready Reaction Kits according to the manufacturer's instructions. All of the *U. urealyticum* isolates and specimens were sequenced with primer UMS-170; some were also sequenced with primer UMA263 to confirm the results.

Algorithm for identification and subtyping of *U. parvum* and *U. urealyticum*. In order to develop a practical identification and subtyping scheme, we designed an algorithm (Fig. 1) for clinical use and then applied it to all known *Ureaplasma* serovars in the ATCC and UAB reference sets and to clinical isolates and clinical specimens.

RESULTS

PCRs. No amplification occurred when DNA extracted from *Mycoplasma* or *Acholeplasma* species was tested with any of 17 primer pairs. Five primer pairs (UPS1-UPA, UPS1-UPA1, UPS-UPSA, UPS2-UPA2, and UMS-57–UMA222) were specific for and amplified all 4 serovars of *U. parvum*, and four (U8-UUA, UUS-UUSA, UUS2-UUA2, and UMS-61–UMA263) were specific for and amplified all 10 serovars of *U. urealyticum*.

Serovars of *U. parvum* could be identified with new primer pairs as follows: UMS-83–UMA269′ amplified only serovar 1; UMS-125–UMA269 amplified only serovar 3 or 14; and UMS-54–UMA269′ amplified only serovar 6. The three subtypes of *U. urealyticum* could be identified with new primer pairs as follows: UMS-61–UMA219 amplified subtypes 1 and 2 only; UMS-61–UMA219′ amplified subtype 3 only; UMS-112–UMA194 amplified subtypes 1 and 3 only; and UMS-112′–UMA194′ amplified subtype 2 only. Nine primer pairs were used in combination to identify *Ureaplasma* species and serovars or subtypes as shown in Fig. 1.

Specificity of *U. parvum* and *U. urealyticum* identification and subtyping primers. All the ATCC and UAB reference strains of *U. parvum* and *U. urealyticum* were correctly identified with the species- and subtype-specific primers as shown in the algorithm (Fig. 1).

***U. parvum* and *U. urealyticum* identification and subtyping results for clinical isolates and clinical specimens.** Of the 78 clinical isolates, 62 (79.5%) were identified as *U. parvum*, 15 (19.2%) were identified as *U. urealyticum*, and 1 (1.3%) was mixed. Of 185 vaginal swabs that had been shown previously to contain *Ureaplasma* species, 151 (81.6%) contained *U. parvum* only, 20 (10.8%) contained *U. urealyticum* only, and 14 (7.6%) contained both. Results of subtyping of clinical isolates and *Ureaplasma* species in vaginal swabs are shown in Table 2.

DISCUSSION

In the past, *U. urealyticum* was divided into two biovars by various phenotypic and genetic methods (9). Recently, we reported new data supporting the proposal that these two biovars be designated separate species, namely, *U. parvum* (previously *U. urealyticum* biovar 1) and *U. urealyticum* (previously *U. urealyticum* biovar 2) (9). Several primer sets have been described for the identification of biovars of the old species *U. urealyticum* (or the new species *U. parvum* and *U. urealyticum*). However, they were not based on the sequences of all 14 serovars (2, 12, 13, 16, 17), and some lacked specificity or the ability to detect all serovars (2, 12). Better PCR-based methods for both identification and subtyping are needed to facilitate studies of

A. Identification of *U. parvum* and *U. urealyticum.*

Amplification primers	UMS-57/UMA222	UMS-170/UMA263
Result (band size)	326/327bp	476bp
Interpretation	*U. parvum*	*U. urealyticum*

B. Subtyping of *U. parvum* and *U. urealyticum.*

1) U. parvum

Amplification primers	UMS-83/UMA269′	UMS-125/UMA269	UMS-54/UMA269′
Result (band size)	398bp	442bp	369bp
Interpretation	serovar 1	serovars 3/14	serovar 6

2) U. urealyticum

a) by PCR

Amplification primers	UMS-61/UMA219	UMS-61/UMA219′
Result (band size)	328bp	328bp
Interpretation	subtypes 1/2	subtype 3
Amplification primers	UMS-112/UMA194	UMS-112′/UMA194′
Result (band size)	356bp	356bp
Interpretation	subtypes 1/3	subtype 2

b) by direct sequencing of amplicons using primers UMS-170 and UMA263

base – 29, A; base – 112, A; base 194, C: subtype 1 (serovars 2, 5, 8, 9);

base – 29, G; base – 112, G; base 194, A: subtype 2 (serovars 4, 10, 12, 13);

base – 29, A; base – 112, A; base 194, C: subtype 3 (serovars 7, 11);

base 219, T; base 223, A; base 251, T: subtype 3 (serovars 7, 11);

base 219, C; base 223, G; base 251, C: subtypes 1/2 (serovars 2, 5, 8, 9; 4, 10, 12, 13).

FIG. 1. Algorithm for identification and subtyping of *U. parvum* and *U. urealyticum*. For identification of *U. parvum* and *U. urealyticum* (A), the result (band size) was 326 bp for *U. parvum* serovars 1 and 3/14 and 327 bp for *U. parvum* serovar 6.

the relationship between *Ureaplasma* species or subtype and disease (4).

Many of the primer targets used in this study were based on our previous observation that the heterogeneity of the intergenic spacer regions is greater than that within the genes (9). We believed that primers based on these regions would be more discriminatory for the identification and subtyping of *Ureaplasma* species (9). These included primers UPSA-UUSA (16S rRNA-23S rRNA gene spacer regions); UPS2-UPA2 and UUS2-UUA2 (*ureA-ureB* and *ureB-ureC* gene spacer re-

TABLE 2. Serovars and subtypes of *U. parvum* and *U. urealyticum* among clinical isolates and vaginal swabs

Sample	*U. parvum*								*U. urealyticum*				
	n	No. (%) of samples with the following serovar(s):							*n*	No. (%) of samples with the following subtype(s) (serovars):			
		1	3/14[b]	6	1 and 3/14[c]	1 and 6[c]	3/14 and 6[c]	1, 3/14, and 6[c]		1 (2, 5, 8, and 9)	2 (4, 10, 12, and 13)	3 (7 and 11)	1 and 3[c]
Clinical isolates	63	17 (27)	31 (49)	10 (16)	1 (2)	3 (5)	1 (2)		16	5 (31)	10 (63)		1 (6)
Vaginal swabs[a]	165	53 (32)	59 (36)	26 (16)	11 (7)	10 (6)	3 (2)	3 (2)	34	11 (32)	21 (62)	2 (6)	

[a] Previously shown to contain *Ureaplasma* species.
[b] Serovars 3 and 14 are indistinguishable by the methods used here.
[c] Presumed to be mixed.

gions, respectively); and UMS-57, UMS-61, UMS-83, UMS-54, UMS-112, and UMS-112′ (upstream of the MBA genes) (Table 1).

Differences between the two proposed new species in the 16S rRNA genes have been described previously and used to design biovar-specific (or species-specific) primers (12). The primer pair U8-P6, designed to amplify *U. urealyticum* (biovar 2), also amplified DNA from *M. pneumoniae* (12), which is found not infrequently in the genital tract (14). We designed a new primer, UUA, which was paired with U8. This pair was specific for *U. urealyticum* (biovar 2) and did not amplify DNA from two *M. pneumoniae* ATCC strains or other *Mycoplasma* species tested. Two new *U. parvum* (biovar 1)-specific primer pairs, UPS1-UPA and UPS-UPA, also based on the 16S rRNA gene, did not amplify DNA from either *U. urealyticum* (biovar 2) or the *Mycoplasma* species tested.

The 16S rRNA and 23S rRNA gene spacer regions are normally continuous with but more heterogeneous than the 16S rRNA genes (6, 9). Primers spanning these regions—UPS-UPSA for *U. parvum* (biovar 1) and UUS-UUSA for *U. urealyticum* (biovar 2)—were specific for all serovars within the corresponding species (biovar) for both ATCC and UAB reference strains.

Primers based on urease gene sequences and used to differentiate *U. parvum* and *U. urealyticum* have been described previously (2, 13). We designed two additional species-specific primer sets, UPS2-UPA2 (*U. parvum* specific) and UUS2-UUA2 (*U. urealyticum* specific), targeting the *ureA-ureB* and *ureB-ureC* gene spacer regions, respectively. They were specific for all serovars within the corresponding species (biovar) for both ATCC and UAB reference strains.

The MBA genes contain both species- and serovar-defining regions (19, 20). Several primer sets based on MBA sequences have been described previously for differentiating *U. parvum* and *U. urealyticum* (9, 16, 17). To improve sensitivity and specificity and to provide more choice, we designed additional species-specific primers based on our sequencing results. The primer pair UMS-57–UMA222 was specific for *U. parvum*, and UMS-61–UMA263 was specific for *U. urealyticum*.

Although the 5′ ends of the MBA genes are highly conserved among the 10 serovars of *U. urealyticum*, we were able to distinguish three subtypes based on sequence data (9). There is more sequence variation in the 5′ ends of the MBA genes of the four serovars of *U. parvum*, which allow them to be separated into three groups. Primer pairs with specificity for individual serovars or subtypes of *U. parvum* and *U. urealyticum* were used in combination and supplemented, when necessary, by sequencing of UMS-170–UMA 263 amplicons to characterize human ureaplasmas, as shown in Fig. 1.

Having confirmed the sensitivity and specificity of the new primer pairs, we designed an algorithm (Fig. 1) for the identification and subtyping of *U. parvum* and *U. urealyticum*, using a selection of the most suitable primers. Its utility was evaluated with ATCC and UAB reference strains, stored clinical isolates, and *Ureaplasma*-positive clinical specimens. The results suggested that the algorithm would be particularly suitable for use in epidemiological studies. Identification and subtyping of clinical isolates and specimens confirmed the previous finding that *U. parvum* is found much more commonly (87% of isolates or specimens overall) than *U. urealyticum* (19%) among vaginal flora (3). Both *Ureaplasma* species were detected in 1 clinical isolate (1%) and 14 vaginal swabs (8%) (6% overall). Among the *U. parvum* strains detected, serovars 3/14 (48%) and 1 (43%) were found more commonly than serovar 6 (23%); 8% of isolates and 16% of vaginal swabs (14% overall) contained two or more subtypes. *U. urealyticum* subtypes 1 (34%) and 2 (62%) were found more commonly than subtype 3 (6%). Only one clinical isolate contained mixed *U. urealyticum* subtypes.

In summary, we have designed a series of new primer pairs based on previously reported sequences of three important ureaplasma genes and adjoining regions and modified some previously published primers to improve their sensitivity and specificity. Our initial evaluation of the algorithm for the identification and subtyping of *U. parvum* and *U. urealyticum*, using a selected set of primers with ATCC and UAB reference strains, clinical isolates, and clinical specimens, confirmed their specificity and sensitivity. Further evaluation of their sensitivity for direct examination of clinical specimens is required. However, we believe that, in the future, they will assist in studies of the epidemiology, pathogenicity, and clinical significance of *Ureaplasma* species in humans and will provide significant advantages over conventional serotyping methods.

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