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Detection and Characterization of Human *Ureaplasma* Species and Serovars by Real-Time PCR[▽]

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We designed primers and probes for the detection and discrimination of *Ureaplasma parvum* and *U. urealyticum* and their 14 serovars by real-time PCR. The analytical sensitivity and specificity of the multiplex species-specific PCR were determined by testing corresponding American Type Culture Collection (ATCC) type strains, 47 other microbial species, and human genomic DNA. The limits of the multiplex PCR were 2.8×10^{-2} CFU/μl PCR mixture for detecting *U. parvum* and 4.1×10^{-2} CFU/μl PCR mixture for detecting *U. urealyticum*. Clinical specificity and sensitivity were proven by comparison with culture and traditional PCR. For the detection of any *Ureaplasma* species, the clinical sensitivity and specificity of real-time PCR were 96.9% and 79.0%, respectively, using culture as a reference. Multiplex real-time PCR was also more sensitive than traditional PCR in discriminating the two *Ureaplasma* species in culture-positive subcultures. Each of the 14 monoplex serovar-specific PCR assays was specific for the corresponding ATCC type strain serovar. This new species identification PCR is specific and sensitive in the detection of *Ureaplasma* species in clinical specimens, and the serovar-specific PCR assays are the first set of complete genotypic assays to differentiate all 14 known *Ureaplasma* serovars. These assays provide quick and reliable means for investigating the epidemiology and pathogenicity of ureaplasmas at the serovar level.

Shepard provided the first descriptions of T-strain mycoplasmas, now known as ureaplasmas, in 1954 when he cultivated them *in vitro* from the urethras of men with nongonococcal urethritis (26), but the genus *Ureaplasma* was not formally designated until 1974 (28). *Ureaplasma urealyticum*, eventually shown to consist of two biovars, was considered to be the only species of this genus known to infect humans until 2002, when its two biovars were reclassified as two distinct species, *U. parvum* and *U. urealyticum*, based on genome size, 16S rRNA gene sequences, the 16S-23S rRNA intergenic region, enzyme polymorphisms, DNA-DNA hybridization, differential growth responses to manganese, and differences in the multiple-banded antigen (*mba*) genes (23). At least 14 serovars have been identified in human ureaplasmas; *U. parvum* contains serovars 1, 3, 6, and 14, while *U. urealyticum* contains the remaining 10 serovars, 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13 (23).

The two *Ureaplasma* species can be isolated individually or simultaneously from clinical specimens (35). They are common commensals colonizing the urogenital tract in healthy persons, yet they are also implicated in invasive diseases such as urethritis, postpartum endometritis, chorioamnionitis, spontaneous abortion, and premature birth, as well as low birth weight, pneumonia, bacteremia, meningitis, and chronic lung disease in prematurely born infants (35). The reasons why *Ureaplasma* spp. are commensals in some instances and produce clinically significant infections in others are still unknown, although an intact functional humoral immune system appears to be very important, as evidenced by the frequent occurrence of systemic *Ureaplasma* infections such as septic arthritis in adults and children with antibody deficiencies (35). Speculations about the association of particular *Ureaplasma* species or serovars with certain diseases have provided the rationale for investigations to develop accurate methods for determining the serovars of clinical isolates (35).

Typing methods for *Ureaplasma* serovar designation using polyclonal or monoclonal antibodies directed against whole cells or purified antigens have included growth inhibition tests (2, 27), metabolic inhibition tests (22), immunofluorescence of colonies on agar (8, 25, 29), immunoperoxidase (20), enzyme-linked immunosorbent assay (33), and immunoblot assays (36, 41). These antibody-based techniques are time-consuming, cumbersome assays that often yield results that are not reproducible, are difficult to interpret, and are inconclusive because of multiple cross-reactions and poor discriminating capacity when used with clinical samples containing two or more serovars. Lack of commercial availability and standardization generally limited serotyping to those laboratories that developed the individual serological reagents.

New rapid molecular methods based on genotypic instead of phenotypic markers are logical choices to replace the conventional antibody-based serotyping methods. PCR-based methods are also becoming an important alternative to conventional culture for the initial detection of ureaplasmas in clinical specimens and have the additional advantage of discrimination between the two *Ureaplasma* species (24). The gel-based traditional PCR assays for the detection and species identification of human ureaplasmas targeted sequences of the 16S rRNA gene and the 16S-23S rRNA intergenic spacer region (7, 9, 14, 24), the urease gene subunit (3, 19), or the multiple-banded

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antigen (*mba*) gene (13–15, 30, 31). Major issues remaining to be resolved for traditional PCR are its specificity and analytic sensitivity, as well as the potential for contamination. Real-time PCR assays targeting the urease gene (5, 17, 37), the *mba* gene (6), and the 16S rRNA gene (39) have recently been developed to distinguish the two *Ureaplasma* species. Compared to traditional PCR and culture, real-time PCR species identification assays are quantitative, as well as more rapid, specific, and sensitive and less subject to contamination (5). However, some of the previously published real-time PCR assays available for *Ureaplasma* species identification require two separate tests (5, 17) or do not have satisfactory sensitivity in one combined test (37).

To characterize the *Ureaplasma* species at the serovar level by PCR, genotyping primers based on the *mba* gene and its 5′-end upstream regions have been designed previously and partial serovar identification was achieved. In combination with direct sequencing or restriction enzyme analysis, these assays were capable of distinguishing the 4 serovars of *U. parvum* and divided the 10 serovars of *U. urealyticum* into different subgroups (5, 11, 13, 15, 32, 40). Among these PCR typing methods, only one was real-time based (5). Because of limited sequence variation in the *mba* genes, all of the PCR-based methods reported so far have lacked the capacity for the complete separation of all 14 serovars. Moreover, our personal experience with whole-genome sequencing of all 14 serovars has shown *mba* to be part of a large gene family present in many variations in different serovars and the gene is phase variable (42). Thus, while the *mba* gene is still, in some cases, a valid and in fact necessary target for serovar-specific PCRs, attention must be given to the locations of phase-variable forms of these genes so that PCR amplicons do not span recombination sites within these genomes.

We have evaluated the complete genome sequences of all 14 *Ureaplasma* serovars and designed primers and probes targeting new specific regions for species and serovar identification by real-time PCR assays. One species-specific multiplex PCR and 14 serovar-specific monoplex PCRs were developed and assessed for analytical and clinical specificity and sensitivity.

MATERIALS AND METHODS

Bacterial strains and clinical specimens. The 14 type strains of the *Ureaplasma* serovars obtained from the American Type Culture Collection (ATCC) and used to validate the species and serovar identification assays are described in Table 1. Additional live microorganisms or their DNA used to validate assay specificity are also described in Table 1.

Two groups of clinical specimens or microorganisms were used in this investigation. They consisted of one group of 132 consecutive neonatal nasal or endotracheal aspirate specimens obtained between 2004 and 2007 for diagnostic purposes and a second group of 194 consecutive *Ureaplasma*-positive broth subcultures of neonatal nasal or endotracheal aspirates obtained for diagnostic purposes between 1994 and 2003. Bacterial subcultures and clinical specimens were kept frozen at −80°C until processed for species and serovar identification PCR assays. Cultures were performed as described by Waites et al. (34).

DNA preparation for PCR. Genomic DNA was extracted by the proteinase K method as described previously (4). If inhibition occurred, proteinase K-digested samples were purified using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). Prepared DNA samples were stored at −80°C.

PCR primers and probes. Primer/probe sets were designed using Roche LightCycler Probe Design Software 2.0 (Table 2). A multiplex real-time PCR assay was designed to differentiate the two *Ureaplasma* species simultaneously. The *U. parvum* primer/probe set anneals to the 477-bp UP063 gene (NP_077893), which

TABLE 1. Species used to validate real-time PCR assays

Name	Source
Ureaplasma species	
U. parvum serovar 1	ATCC 27813
U. parvum serovar 3	ATCC 27815
U. parvum serovar 6	ATCC 27818
U. parvum serovar 14	ATCC 33697
U. urealyticum serovar 2	ATCC 27814
U. urealyticum serovar 4	ATCC 27816
U. urealyticum serovar 5	ATCC 27817
U. urealyticum serovar 7	ATCC 27819
U. urealyticum serovar 8	ATCC 27618
U. urealyticum serovar 9	ATCC 33175
U. urealyticum serovar 10	ATCC 33699
U. urealyticum serovar 11	ATCC 33695
U. urealyticum serovar 12	ATCC 33696
U. urealyticum serovar 13	ATCC 33698
Human species	
Acholeplasma laidlawii PG 8	ATCC 23206
Acholeplasma oculi	ATCC 27350
Mycoplasma amphoriforme	Clinical isolate
Mycoplasma buccale	ATCC 23636
Mycoplasma faucium	ATCC 25293
Mycoplasma fermentans PG 18	ATCC 19989
Mycoplasma genitalium PG37	ATCC 33530
Mycoplasma hominis PG21	ATCC 23114
Mycoplasma lipophilum	ATCC 27790
Mycoplasma orale	ATCC 23714
Mycoplasma penetrans	ATCC 55252
Mycoplasma pirum BER p9	Clinical isolate
Mycoplasma salivarium PG 20	ATCC 23064
Mycoplasma spermatophilum	ATCC 49695
Nonhuman *Mycoplasma* species	
M. arthritidis PG 27	ATCC 23192
M. collis	ATCC 35278
M. hyorhinis	Clinical isolate
M. muris	ATCC 33757
M. neurolyticum	ATCC 15049
M. primatum	ATCC 15497
M. pulmonis A	ATCC 19612
Other bacterial species	
Bordetella pertussis	ATCC BAA-589D
Burkholderia cepacia	ATCC 25416
Chlamydophila pneumoniae	Clinical isolate
Corynebacterium diphtheriae	ATCC 10701
Enterobacter cloacae	Clinical isolate
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 25922
Haemophilus influenzae	ATCC 23533
Haemophilus parainfluenzae	ATCC 7901
Helicobacter pylori	Clinical isolate
Klebsiella pneumoniae	ATCC 33495
Legionella pneumophila	ATCC 33152
Moraxella catarrhalis	ATCC 25240D
Neisseria gonorrhoeae	ATCC 49226
Proteus mirabilis	ATCC 2798
Pseudomonas aeruginosa	ATCC 27853
Staphylococcus aureus	ATCC 29213
Streptococcus agalactiae	ATCC 13813
Streptococcus pneumoniae	ATCC 49619
Streptococcus pyogenes	ATCC 19615
Streptococcus salivarius	ATCC 13419
Candida species	Clinical isolate
Viruses	
Adenovirus	Clinical isolate
Buffalopox virus BP4	Clinical isolate
Herpes simplex virus type 1	Clinical isolate
Herpes simplex virus type 2	Clinical isolate
Human genomic DNA	Roche Diagnostics

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TABLE 2. Primers and probes for *Ureaplasma* species-specific and serovar-specific PCR assays

Species or serovar	Target	Primer or probe	Sequence (5′–3′)	Amplicon size (bp)
U. parvum	UP063	UP063#1F	TGCGGTGTTTGTGAACT	152
		UP063#1R	TGATCAAACTGATATCGCAATTATAGA	
		UP063#1 probe 1	TGGTTTAACGTGTTTTTGAAGTGCTACAAAA T-FL[c]	
		UP063#1 probe 2	LC Red 640-CCCATTTCAGCCATGGTGCCATCA	
U. urealyticum	UUR10_0680	UU127#1F	GGATTTGTTAGATATCGTCAAGG	
		UU127#1R	TCATCTTTTAAAGCTCCACATTATTAGT	152
		UU127#1 probe 1	AACACGAGTATGGATGAAATCAAAATCATCA AA-FL	
		UU127#1 probe 2	LC Red 705-AATAACGGTGGTTCAGCTATTTG AGTATGAGC	
Serovar 1	MBA[a] (UPA1_G0002, gcontig_1106501367851)[d]	UP1F1-2	GATAATTTGAATTATCAAACAGAAAAAGTG	116
		UP1R1-2	TGTTCTTTACCTGGTTGTTGT	
		UP1 probe 1	FL-SPC[e]-GAACCAAAAGAAAATGGTGGAGAA CAACC	
Serovar 2	Putative lipoprotein (UUR2_0261, gcontig_1118448734166)	UU2_F_1	GCTTGTTGATAAGCAAAAGGATTAC	76
		UU2_R_1	GTTTTGGTTCTGGATTGTTTGAC	
Serovar 3	CHP[b] (UPA3_0391/UU376, NC_010503)	UP3F1-2	AATAATGCAAATTATGATGTTAAATTAACC	63
		UP3R1-2	TGTTTCTATGTTAACATTTAACAATTTAGC	
Serovar 4	Intergenic (gcontig_1105428130534)	Uu04_1F	CTAAAGCGTGCTTAATGTGT	162
		Uu04_1R	GTTAGTTTGGGAACCACCT	
		Uu04_1 probe 1	TGTTATTATTATAGAAATTGTTGTAAGAATC AAAG-FL	
		Uu04_1 probe 2	LC Red 610-ATAATTTCGTATAATGTCATGTTG TTCACCTCCTT	
Serovar 5	ATP-dependent RNA helicase (UUR5_G0006, gcontig_1105469453572)	Uu05-3F	ATTATGAAAAATTAAACTCTCATTACTCG	121
		Uu05-3R	TTCTCATATTGAAAAGAAAATGAATGCTC	
		Uu05-3 probe	FL-SPC-AGGAAGAATAAAACATTTTAATTTAT ATCCACGAA	
Serovar 6	MBA (UPA6_A0411, gcontig_1105428157138)	UP6F1	GAACTTTGAAACAGCTCCG	60
		UP6R1	CCTGGTTCTTTACCTGGTTCTTTA	
Serovar 7	MBA (UUR7_0421, gcontig_1104407586372)	UU7_F_1	CAAACAAAGCATTAACAGCTTCAAAA	58
		UU7_R_1	TTATTAACTTCTTCCTTTGTTGTAAGTGTAGC	
Serovar 8	Intergenic (gcontig_1118436613429)	UU8_F_1	AGTTTTAATTATTTCGTTGTTTAAGTAGC	60
		UU8_R_1	AGTTTTTACGTGGTAAGTGGTT	
Serovar 9	FtsK/spoIIIE family protein (UUR9_0160, gcontig_1105462525206)	UU9_F_1	CGAAGCGGAAGTCGCAGGT	51
		UU9_R_1	TATTGCCACACCAGCCAGCA	
Serovar 10	MBA (UUR10_0418, NC_011374)	UU10_F_4	TGCATCAACATCGCTAAATTCA	95
		UU10_R_4	ATACGCCTACTCCGACT	
		UU10_P_4	FL-SPC-TTGGTGTAGGTGTTGGTTGTGG	
Serovar 11	Intergenic (gcontig_1105428123667)	UU11_F_1	AACCTTATCAATTCTATTAATCATAGTTCA	126
		UU11_R_1	TGAAAACAAAAAACACGCTCC	
		UU11_P_1	FL-SPC-AAACCAAACTAACACATTAAGCACGC	
Serovar 12	CHP (UUR12_A0390, gcontig_1105428175482)	Uu12_1F	AATTGGTCAAACAACATATCGTG	167
		Uu12_1R	ATTTAACACGATTAACATCTTCACGTTTA	
		Uu12_1 probe 1	TTTGCGAAGCATTACCGCCAAATAC-FL	
		Uu12_1 probe 2	LC Red 705-GGCTTAGTTATGAACCAGCTGTT ATTAATATAGCG	
Serovar 13	F_1: CHP; R_1: putative SSB protein (gcontig_1106518107722)	13_F_1	CCAAGACCAGCACCTACTGCC	90
		13_R_1	AGAAAACGAGGAGGAATAAATAATGGATT	
Serovar 14	Intergenic (gcontig_1106438328052)	UP14F1	TCCACACTACGGTAAGTAGTTT	60
		UP14R1	CGCGCAGACCCTTGAATA	

[a] MBA, multiple-banded antigen.
[b] CHP, conserved hypothetical protein.
[c] FL, fluorescein.
[d] Target names in parentheses are National Center for Biotechnology Information names.
[e] SPC, Simple Probe Chemistry.

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TABLE 3. Genome sequences of ATCC type strains and GenBank accession numbers

Ureaplasma strain	GenBank accession no.	Genome size (bp)	Complete genome	No. of contigs
U. parvum serovar 1 ATCC 27813	NZ_ABES00000000	753,674	No	12
U. parvum serovar 3 ATCC 27815	NC_010503	751,679	Yes	1
U. parvum serovar 6 ATCC 27818	NZ_AAZQ00000000	772,971	No	5
U. parvum serovar 14 ATCC 33697	NZ_ABER00000000	749,965	No	8
U. urealyticum serovar 2 ATCC 27814	NZ_ABFL00000000	870,038	No	9
U. urealyticum serovar 4 ATCC 27816	NZ_AAYO00000000	835,413	No	4
U. urealyticum serovar 5 ATCC 27817	NZ_AAZR00000000	884,046	No	18
U. urealyticum serovar 7 ATCC 27819	NZ_AAYP00000000	894,015	No	20
U. urealyticum serovar 8 ATCC 27618	NZ_AAYN00000000	881,828	No	2
U. urealyticum serovar 9 ATCC 33175	NZ_AAYQ00000000	947,165	No	10
U. urealyticum serovar 10 ATCC 33699	NC_011374	874,478	Yes	1
U. urealyticum serovar 11 ATCC 33695	NZ_AAZS00000000	886,725	No	9
U. urealyticum serovar 12 ATCC 33696	NZ_AAZT00000000	844,514	No	4
U. urealyticum serovar 13 ATCC 33698	NZ_ABEV00000000	846,596	No	5

encodes a conserved hypothetical protein that is identical in all four *U. parvum* type strain serovars. The *U. urealyticum* primer/probe set anneals to a 15,072-bp open reading frame that is almost perfectly (>99.97%) conserved in all 10 *U. urealyticum* type strain serovars (serovar 10, GenBank ID ACI60066.1). To design the serovar-specific primers and probes, the genomes of the 14 ATCC type strains that had been previously sequenced (Table 3) were computationally searched to identify 25- to 35-bp candidate regions that are unique to each genome. Each of the candidate region or locus sequences in a genome was then searched against all of the other genomes, and only those that had <80% identity matches were kept. The Roche LightCycler Probe Design Software 2.0 was used to analyze the regions around some of the unique 30-bp regions, and primers and probes were generated. Each possible primer was checked against all 14 *Ureaplasma* genomes using BLAST, and fewer than 1 in 20 of the unique small regions yielded acceptable serovar-specific primers based on computational analysis. The resulting primers and probes shown in Table 2 were tested with the LightCycler using the 14 ATCC type stains. The specificity of eight serovars (serovars 2, 3, 6, 7, 8, 9, 13, and 14) came from the two primers, while the other six were from probes, either Simple Probe Chemistry (SPC) probes (serovars 1, 5, 10, and 11) or fluorescence resonance energy transfer (FRET) probes (serovars 4 and 12). Serovar 5 primers may also amplify the other nine *U. urealyticum serovars*; however, the amplicon is much larger (668 bp versus 121 bp in serovar 5) and the probe has a large unmatched loop (547 bp), so this should not affect specificity. A short extension time that only allows complete amplification of the serovar 5 fragment but not the others enables the distinction. A melting curve analysis will show the differences of the products if unexpected amplification has occurred. Urease-based primers and ^{32}P-labled probes for *Ureaplasma* species identification by traditional PCR were described previously (10). All primers were synthesized by Invitrogen (Carlsbad, CA), and probes were manufactured by Roche Diagnostics.

PCR conditions. Real-time PCR assays were performed on a Roche LightCycler 2.0 instrument. An asymmetric PCR condition was used for the multiplex species identification PCR; the PCR master mix contained less of the forward primers (0.3 μM UP063#1F and 0.2 μM UU127#1F) than of the reverse primers (0.5 μM). The other components were 0.15 μM each UP probe, 0.2 μM each UU probe, 0.5 U of uracil-DNA glycosylase (UNG), 3 mM $MgCl_2$, and 4 μl of 5× Multiplex DNA Master HybProbe buffer (Roche Diagnostics). A volume of 2 μl of the specimen was added to the master mix to reach a total reaction volume of 20 μl. The PCR program was 10 min preincubation at 40°C and 95°C, followed by 45 cycles of amplification at 95°C for 15 s, 55°C for 10 s with a single measurement, and 72°C for 9 s. Melting curves were generated by 95°C for 0 s, 65°C for 30 s, and 95°C with a slope rate of 0.1°C/s in continuous-acquisition mode. Finally, the machine was cooled down to 40°C for 30s. The PCR conditions for 14 monoplex serovar-specific PCRs are summarized in Tables 4 and 5. All 20-μl reaction volumes contained 0.5 U of UNG and 2 μl of specimen. The serovar 1, 10, and 11 PCRs were asymmetric. Every PCR program included a preincubation procedure and the final cooling procedure described above. Touchdown programs were designed for serovars 3, 5, and 8 to ensure specificity. All PCR runs included a positive control that was the designated species or serovar being evaluated and a negative control (water). For species identification PCR assays, external amplification controls (DNA mixture from serovars 3 and 10) were also included in each run. Conditions for urease gene-based traditional PCR have been described previously (4, 10, 19).

Measurement of analytical sensitivity of PCR assays. DNA concentrations (ng/μl) of specimens purified by the QIAamp kit were measured by NanoDrop Spectrophotometer ND-1000 (NanoDrop, Wilmington, DE) and transformed into molecules/μl, which is equal to genomes/μl, using the calculated molecular mass of *U. parvum* serovar 3 (ATCC 27815), which is 4.65×10^8 Da, based on a 0.75-Mb genome length, and that of *U. urealyticum* serovar 10 (ATCC 33699), which is 5.40×10^8 Da, based on a 0.87-Mb genome length. Tenfold serial dilutions of the QIAamp-purified DNA of *U. parvum* (serovar 3, ATCC 27815) and *U. urealyticum* (serovar 10, ATCC 33699) were used to determine the analytical sensitivity of the PCR assay in terms of numbers of molecules/μl. Tenfold serial dilutions of broth cultures of *U. parvum* (serovar 3, ATCC 27815) and *U. urealyticum* (serovar 10, ATCC 33699) were purified by the proteinase K method and used to determine the analytical sensitivity of the PCR assay in terms of numbers of CFU/μl.

RESULTS

Analytical specificity and sensitivity of *Ureaplasma* species-specific multiplex PCR. Using genomic DNA of each of 14 ATCC *Ureaplasma* serovar type strains as the template, our

TABLE 4. Master mix components for serovar-specific monoplex PCR assays

Serovar	Concn (μM) of:				PCR buffer[a]	$MgCl_2$ concn (mM)[b]	UNG amt (U)
	Forward primer	Reverse primer	Probe 1	Probe 2			
1	0.3	0.5	0.2		2		0.5
2	0.5	0.5			1	3.0	0.5
3	0.25	0.25			1	3.0	0.5
4	0.5	0.5	0.2	0.2	2		0.5
5	0.4	0.5	0.2		2		0.5
6	0.4	0.4			1	3.0	0.5
7	0.25	0.25			1	3.0	0.5
8	0.25	0.25			1	3.0	0.5
9	0.25	0.25			1	3.0	0.5
10	0.3	0.5	0.15		2		0.5
11	0.2	0.5	0.2		2		0.5
12	0.5	0.5	0.2	0.2	2		0.5
13	0.4	0.4			1	3.0	0.5
14	0.5	0.5			1	3.0	0.5

[a] 1, 2 μl LightCycler FastStart DNA Master SYBR green I; 2, 4 μl LightCycler FastStart DNA Master^{PLUS} HybProbe.
[b] Final $MgCl_2$ concentration.

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TABLE 5. Amplification programs for monoplex serovar-specific PCR assays

Serovar	Amplification program	Melting range (°C)
1	95°C, 1 s; 55°C, 10 s, single acquisition; 72°C; 4 s; 45 cycles	50–70
2	95°C, 0 s; 57°C; 4 s; 72°C, 2 s, single acquisition; 45 cycles	65–95
3	Amplification I, 95°C, 0 s; 57°C, 5 s; 15 cycles; amplification II, 95°C, 0 s; 57°C, 3 s; 2nd target, 53°C, 1°C/step, delay 1 cycle; single acquisition; 45 cycles	65–85
4	95°C, 10 s; 55°C, 6 s, single acquisition; 72°C, 5 s; 45 cycles	50–80
5	Amplification I, 95°C, 2 s; 57°C, 6 s; 72°C, 2 s; 10 cycles; amplification II, 95°C, 5 s; 57°C, 8 s; ramp rate, 5°C/s; 2nd target, 54°C, 1°C/step, delay 1 cycle; single acquisition; 72°C, 3 s; 45 cycles	50–70
6	95°C, 0 s; 58°C, 2 s; 72°C, 1 s, single acquisition; 40 cycles	63–90
7	95°C, 0 s; 55°C, 1 s, single acquisition; 40 cycles	65–95
8	Amplification I, 95°C, 0 s; 66°C, 5 s; 15 cycles; amplification II, 95°C, 0 s; 66°C, 2 s; 2nd target, 58°C, 1°C/step, delay 1 cycle; 72°C, 1 s, single acquisition; 40 cycles	66–95
9	95°C, 0 s; 62°C, 1 s, single acquisition; 33 cycles	70–95
10	95°C, 2 s; 56°C, 5 s, single acquisition; 72°C, 2 s; 45 cycles	55–75
11	95°C, 2 s; 53°C, 10 s; ramp rate, 5°C/s, single acquisition; 72°C; 4 s; 50 cycles	54–75
12	95°C, 10 s; 59°C, 5 s, single acquisition; 72°C, 6 s; 45 cycles	50–80
13	95°C, 0 s; 57°C, 2 s; 72°C, 2 s, single acquisition; 40 cycles	63–90
14	95°C, 0 s; 55°C; 4 s; 72°C, 2 s, single acquisition; 45 cycles	70–90

multiplex real-time PCR differentiated *U. parvum* and *U. urealyticum* in two different channels without any cross-reactions. All of the serovars within the two respective species were recognized (Fig. 1). Species-specific amplification was successful in the presence of high copy numbers of the other *Ureaplasma* species (1:100 ratio; data not shown). No cross-reactivity with any of the other microorganisms listed in Table 1 was detected. The detection limit of the *U. parvum* assay was 5.2 DNA molecules/μl (2.8×10^{-2} CFU/μl PCR mixture). For *U. urealyticum*, the limit was 24 DNA molecules/μl (4.1×10^{-2} CFU/μl PCR mixture). These experiments were repeated three times with similar results.

Comparison of species-specific multiplex PCR with culture and traditional PCR. The species-specific multiplex PCR assay was evaluated in two groups of archived clinical specimens/subcultures in order to include direct comparisons with culture and traditional PCR. Among the 132 clinical specimens in group 1, there were 32 (24.2%) that were culture positive for *Ureaplasma* spp. and 100 (75.8%) that were culture negative. The multiplex species identification PCR detected 52 *Ureaplasma*-positive samples and identified 80 as negative. Thus, PCR detected 20 (15.2%) more positive samples than did culture. There was only one culture-positive specimen that was negative by real-time PCR, and the number of organisms in that specimen was <10 CFU/ml. Although PCR is generally considered to be more sensitive than culture, using culture as the reference standard, real-time PCR had a sensitivity of 96.9% and a specificity of 79.0% for the detection of *Ureaplasma* spp.

Among 194 *Ureaplasma*-positive subcultures from clinical specimens, traditional PCR detected 176 positives and 18 negatives, whereas the multiplex real-time PCR detected 188 positives and 6 negatives. Thus, the multiplex real-time PCR assay

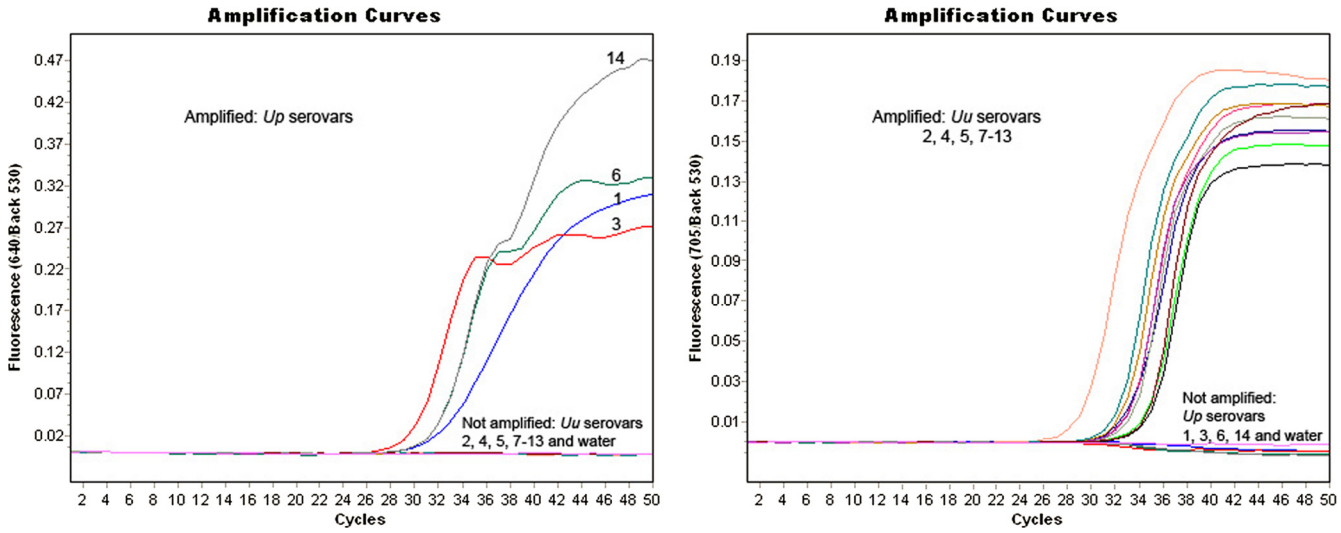

FIG. 1. Amplification curves for *Ureaplasma* species-specific multiplex real-time PCR assays tested against 14 ATCC serovar type strains demonstrating simultaneous distinction between *U. parvum* (*Up*) and *U. urealyticum* (*Uu*) and detection of all serovars of each species.

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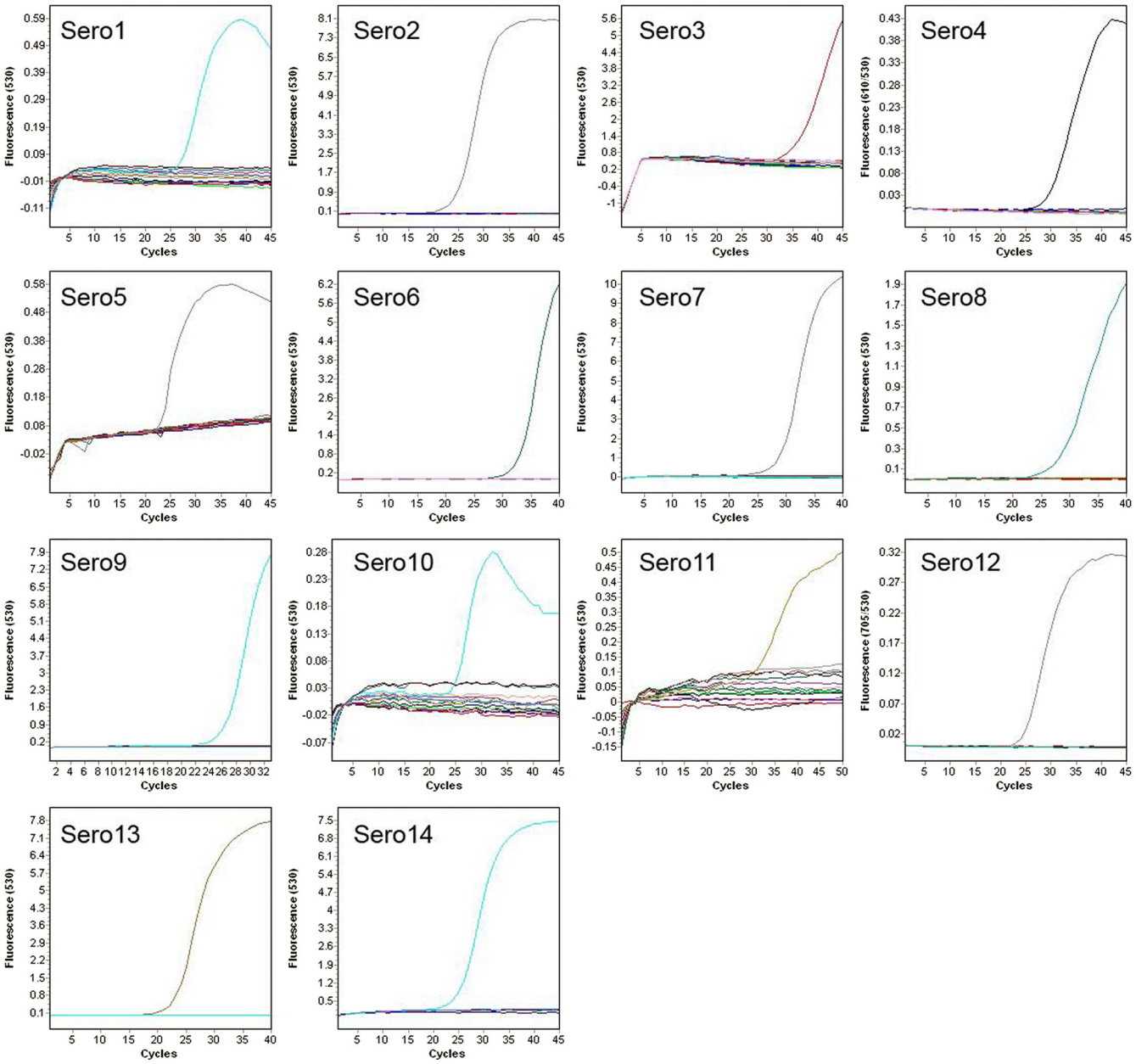

FIG. 2. Amplification curves for monoplex serovar-specific PCR assays tested individually with all 14 *Ureaplasma* sp. serovar (Sero) type strains demonstrating specific amplification of each.

reduced false-negative results among culture-positive specimens from 9.3% to 3.1% compared to traditional PCR. Among the 175 subcultures positive by both PCR methods, 116 (66.3%) contained only *U. parvum*, 54 (30.9%) contained only *U. urealyticum*, and 5 (2.9%) contained both species by traditional PCR. By multiplex species identification PCR, 115 (65.7%) contained only *U. parvum*, 48 (27.4%) contained only *U. urealyticum*, and 12 (6.9%) contained both species. Overall, real-time PCR recognized six more *U. parvum* isolates and one more *U. urealyticum* isolate than traditional PCR. Using traditional PCR as the reference standard, real-time PCR had a diagnostic sensitivity of 98.3% for *U. parvum* and 96.6% for *U. urealyticum*; while the specificity of detection was 88.9% for *U. parvum* versus 99.1% for *U. urealyticum*. The DNA used for this study was originally isolated in 2004, so it is possible that degradation occurred over a 5-year period, which would account for the fact that some culture-positive specimens were PCR negative when retested for the present evaluation.

Analytical specificity and sensitivity of serovar-specific real-time PCR assays. Real-time PCR assays specific for 14 *Ureaplasma* serovars were developed for the ATCC type strains, and the specificity of each PCR was tested against the 13 other serovar type strains. Each individual PCR assay amplified the designated serovar but none of the other 13 serovars (Fig. 2). The primer/probe concentration, $MgCl_2$ concentration, annealing temperature, and annealing time of each PCR pro-

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gram were carefully optimized to make the assay specificity possible. For PCRs with primers only (serovars 2, 3, 6, 7, 8, 9, 13, and 14), the primer concentrations were 0.25 to 0.5 μM; for PCRs that required a combination of primers and probes, asymmetric PCR conditions were used (except for serovars 4 and 12). The annealing time of all programs was kept as short as possible, especially for the eight primer-only programs, which could be as short as 1 s (serovars 7 and 9). The annealing temperatures were kept as high as possible to ensure the specificity of each PCR. Touchdown programs containing a short amplification step under stringent conditions prior to the major amplification program were developed for serovars 3, 5, and 8 to improve specificity. After amplification, a melting curve analysis was performed for each PCR providing additional proof of differentiation power. The detection limits of the serovar-specific PCR assays ranged from 15 (serovar 6) to 3,461 (serovar 9) DNA molecules/μl of PCR mixture. No evaluation was performed directly on broth subcultures to determine the limit of CFU detection as was done for the species-specific multiplex PCR assay, and these assays were not tested directly for specificity against other microbial species since their utility is primarily in the testing of isolates or clinical specimens known to contain *U. parvum* or *U. urealyticum*.

DISCUSSION

Taking advantage of the genomic sequence data now available for all 14 *Ureaplasma* serovars, our multiplex real-time PCR assay for the simultaneous detection and discrimination of the two *Ureaplasma* species targets two conserved gene sequences, each present only in *U. parvum* or *U. urealyticum*. This differs from previously reported assays for *Ureaplasma* species identification that targeted small differences within genes conserved in both species. The specificity of this assay was ensured computationally and experimentally verified by BLAST analysis and running the assay against a broad range of other microorganisms.

Compared to culture and traditional PCR, our findings using a single-step multiplex assay format agreed with those of Cao et al. (6), who found the real-time PCR to be the most sensitive in the detection of *Ureaplasma* species. The detection threshold of our assays is well below the level necessary to detect ureaplasmas in clinical specimens, and our multiplex PCR detected *Ureaplasma* spp. in 15.2% more clinical specimens than did culture in a direct comparison using optimum cultivation methods developed and validated in our laboratory over several years (34). Even though culture is often considered the reference method for the detection of ureaplasmas in clinical specimens, improved PCR assays may be inherently more sensitive and capable of detecting the organisms in very low numbers. Thus, the PCR-positive and culture-negative specimens we encountered are likely to be true positives rather than false negatives. Whether a PCR assay is more sensitive than culture for the detection of ureaplasmas in clinical specimens depends on the PCR target, the assay conditions, and the method of culture used. Numerous studies have shown clearly that PCR assays can be superior to culture (1, 4, 16, 35, 38). However, even though PCR has the added potential advantages of providing *Ureaplasma* species identification and same-day turnaround without the necessity of maintaining organism viability, culture is relatively simple and can often provide results in 24 to 48 h. Compared to traditional PCR, the new multiplex real-time PCR greatly reduced the false-negative rate in the detection of *Ureaplasma* species as defined by culture positivity, making this assay even more attractive than traditional PCR in this setting.

Yi et al. (37) developed a real-time PCR assay to simultaneously detect and discriminate between the two *Ureaplasma* species in a single test with one pair of common primers for both species and two species-specific TaqMan probes. This assay, however, lacked analytical sensitivity, and its clinical sensitivity was lower than that of traditional PCR. In contrast, our multiplex real-time PCR found a total of six more *U. parvum* strains alone or in combination than with our traditional PCR and one additional *U. urealyticum* strain. No other published studies have provided a quantitative comparison of species differentiation using real-time PCR versus traditional PCR.

Molecular genotyping methods based on the *mba* gene and its 5′ end have been explored as a replacement for the antibody-based phenotyping methods (5, 11–15, 18) that have been in use for almost 3 decades since the original description of the 14-member serotyping scheme by Robertson and Stemke in 1982 (21). However, using the limited sequence data available for previous genotyping methods, only partial serovar identification was possible. The four serovars of *U. parvum* were readily differentiated from each other by traditional PCR with different sets of primers (13) or by two multiplex real-time PCRs (5). However, to distinguish the 10 serovars of *U. urealyticum* is still challenging. Using single-stranded conformation polymorphism analysis, the 10 serovars were divided into two groups, A (serovars 2, 5, 8, and 9) and B (serovars 4, 7, 10, 11, 12, and 13) (18). By PCR and sequencing, Kong et al. (14) classified the 10 serovars into three subgroups, 1 (serovars 2, 5, 8, and 9), 2 (serovars 4, 10, 12, and 13), and 3 (serovars 7 and 11). The same investigators then provided better discrimination by dividing the 10 serovars into five MBA genotypes with the added individual separation of serovars 9 and 10, i.e., genotypes A (serovars 2, 5, and 8), B (serovar 10), C (serovars 4, 12, and 13), D (serovar 9), and E (serovars 7 and 11) (13). This approach, using just the sequence of an *mba* gene specific to each serovar, should be reconsidered in light of the whole genome sequences that show that all *Ureaplasma* serovars encode multiple members of the phase-variable *mba* gene family.

In designing our serovar-specific real-time PCR assays, we sought to avoid the *mba* gene family because of its ubiquity within the ureaplasmas and because of its phase variability. This intent was thwarted by the high interserovar identity among the 14 serovars. The average difference among the 4 *U. parvum* genomes is 0.56%, and among the 10 *U. urealyticum* genomes, the average is 0.63%. While it is straightforward to design PCRs for the discrimination of any pair of serovars, the discrimination of 1 serovar from the other 13 is often very challenging. We sought to have all assays employ primers and probes; however, in many cases, this was not possible. We were even forced, in several cases, to target *mba* genes or *mba* paralogous gene family genes to obtain serovar identification. In those instances, we made sure our PCR targets did not span sites at which chromosome rearrangements took place during phase variation. Those sites could be identified by analyzing

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whole-genome shotgun sequencing assemblies. By computationally searching thousands of unique loci in each serovar and testing with 14 ATCC type stains, we created PCRs specific to all 14 serovars without any cross-reactions. Many PCRs predicted to be serovar specific failed when tested and were abandoned. Ultimately, each serovar primer/probe set we used contains at least one serovar-specific primer or probe. In the cases where the set could cross-react, leading to a significantly larger amplicon, the PCR conditions were adjusted to prevent it. The strategy to multiplex the 14 PCR assays, which should be more efficient, was not used because each PCR condition was unique and combination with another assay compromised the specificity and sensitivity of one or both assays (data not shown).

In conclusion, our new multiplex real-time PCR for the detection and discrimination of *Ureaplasma* species is robust and ready to replace the traditional PCR for clinical diagnostic purposes and is a suitable alternative to culture. We have also shown for the first time that all 14 serovar type strains of *Ureaplasma* spp. can be clearly differentiated from each other by using our novel real-time PCR technology, which overcomes many of the limitations that hampered the utilization of serologically based typing methods. This should greatly facilitate future investigations of ureaplasmas at the species and serovar levels under clinical conditions to assess differential pathogenicity. Our data regarding the monoplex serovar-specific PCR assays were limited to evaluations using individual type strains representing each serovar. We did not evaluate clinical specimens directly, nor have we applied this technology thus far to clinical isolates. The value of this assay as a diagnostic typing method may ultimately depend on the degree of genetic variability that exists among clinical isolates and the extent of horizontal gene transfer that may influence serovar designation. The analytical sensitivities we obtained suggest that our assay should meet the requirements for direct testing of clinical specimens that may contain serovars alone or in combination. Proof of this will require additional clinical studies, as will the evaluation of how well these assays perform with clinical isolates of unknown serovar status.

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