CHAPTER 1

UREAPLASMA SPECIES IDENTIFICATION

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Statement of Joint Authorship

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Did all the molecular work, interpreted the data and wrote the manuscript.
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Assisted in some PCR.
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Provided all the needed molecular equipment, reagents and software.
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Supervised the overall project, assisted in the research design, analysis and interpretation of data, and made a significant contribution to the manuscript.

1.1. SUMMARY

Ureaplasma urealyticum have been divided into two species *U. parvum* (previously *U. urealyticum* parvo biovar) and *U. urealyticum* (previously *U. urealyticum* T-960 biovar). In this study, we designed a series of primers, targeting the 16S rRNA gene and 16S-23S rRNA intergenic spacer regions, the urease gene subunits and adjoining regions, and the 5'-ends of the multiple-banded antigen genes (*mba*) and upstream regions, to identify the two ureaplasma species. All of the species-specific primer pairs could distinguish the two species. A selection of primer pairs was used to identify 78 clinical ureaplasma isolates from vaginal swabs of pregnant women and 185 vaginal swabs. *U. parvum* was identified in 228 (87%) of 263 isolates or specimens, *U. urealyticum* was identified in 50 (19%) and both were present in 6%. The ureaplasma species identification PCR methods will facilitate future studies of the relationship between individual ureaplasma species and human diseases.

1.2. INTRODUCTION

Human ureaplasmas had been separated into two new species, namely, *U. parvum* (previously *U. urealyticum* parvo biovar) and *U. urealyticum* (previously *U. urealyticum* T-960 biovar) (Robertson *et al*., 2002). Ureaplasmas are commensals in the genital tract, recognized causes of some diseases (Taylor-Robinson & Furr, 1997), and suspected contributors to a number of other pathological conditions (Abele-Horn *et al*., 1997). Because they are commonly found in healthy people, their pathogenic role can be difficult to prove (Ollikainen *et al*., 1998; Tully, 1993). The majority of ureaplasma isolates belong to the proposed new species *U. parvum* (Abele-Horn *et al*., 1997), which includes serovars 1, 3, 6, and 14 (Robertson *et al*., 2002; Knox *et al*., 2003). *U. urealyticum* is isolated less often but is not uncommon (Abele-Horn *et al*., 1997). *U. urealyticum* has been associated with some disease syndromes more commonly than with normal flora (Povlsen *et al*., 2002; Deguchi *et al*., 2004), but data are limited because of difficulties with conventional serotyping

methods (Povlsen *et al*., 2002; Deguchi *et al*., 2004). Rapid molecular methods for ureaplasma species identification would be of great value in studies of the epidemiology and pathogenesis of infections with *U. parvum* and *U. urealyticum* (Robertson *et al*., 2002).

Recently, PCR-based methods have been used successfully to distinguish the two ureaplasma species, but there is a need to improve their specificity and sensitivity (Robertson *et al*., 2002). Target sequences of the 16S rRNA gene (Robertson *et al*., 1993), 16S-23S rRNA intergenic spacer regions (Harasawa & Kanamoto, 1999), the urease gene subunits (Blanchard, 1990; Povlsen *et al*., 1998), and the 5'-ends of the *mba* and upstream regions (Kong *et al*., 1999a; Teng *et al*., 1995) 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 (Kong *et al*., 1999a, b). In the present study, we evaluated the specificity of a large range of primers and used a small subset to develop ureaplasma species identification assays.

1.3. MATERIALS AND METHODS

1.3.1. Bacterial strains.

Two sets of reference strains were used. Ureaplasma serovar 1 (ATCC 27813), serovar 2 (ATCC 27814), serovar 3 (ATCC 27815), serovar 4 (ATCC 27816), serovar 5 (ATCC 27817), serovar 6 (ATCC 27818), serovar 7 (ATCC 27819), serovar 8 (ATCC 27618), serovar 9 (ATCC 33175), serovar 10 (ATCC 33699), serovar 11 (ATCC 33695), serovar 12 (ATCC 33696), serovar 13 (ATCC 33698), serovar 14 (ATCC 33697) were obtained directly from the American Type Culture Collection, Manassas, VA, U.S.A. (ATCC reference strains) (Robertson *et al*., 2002). In addition, a set of reference strains of serovars 1 to 14, were kindly provided by Dr. H. L. Watson, Department of Microbiology, University of Alabama at Birmingham, Alabama (UAB reference set). These had been obtained originally

from E. A. Freundt, Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark (serovars 1-8) and J. A. Robertson, Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta, Canada (serovars 9-14) (Robertson *et al*., 2002).

Additional reference strains from 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 intergenic spacer regions.

1.3.2. Clinical isolates and specimens.

A total of 78 ureaplasma isolates obtained from vaginal swabs of pregnant women recently cultured in our laboratory, and 185 vaginal swabs obtained from pregnant women and women attending a sexually transmitted disease clinic, in which ureaplasmas had been previously detected (Kong *et al*., 1999a) were used in this study.

1.3.3. Oligonucleotide primers.

The 19 individual primers used in this study to amplify portions of three genes of all 14 serovars are shown in Table 1.1. They include 4 primers that have been previously described (Robertson *et al*., 1993; Teng *et al*., 1995) and 15 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 *mba*), the direction of the sequence (S, sense; A, antisense), and the numbered base position at which the primer sequence starts (for *mba*) (Kong *et al*., 1999b).

1.3.4. DNA preparation.

DNA preparation was performed as previously described (Kong *et al*., 1999a, b). Cells from 0.5 ml of ureaplasma broth cultures (10B) of each ureaplasma servovar were harvested from late logarithmic growth by centrifugation at 14, 000x g for 20 minutes; clinical specimens were processed directly. DNA was isolated from both cultures and clinical specimens by treatment with 500 µl of digestion buffer (10 mM Tris-HCl [pH 8.0], 0.45% Triton X-100 and 0.45% Tween 20) and proteinase K, 100 µg/ml, at 55^{o}C for 1 hour and then extraction with pheno-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). DNA was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The washed and dried pellets were hydrated in 200 µl ultrapure and sterile water.

1.3.5. PCR.

PCR was performed as previously described (Kong *et al*., 1999a, b). The 25 µl amplification reaction mixtures contained 2.5 µl of 10x PCR buffer (1x is 10 mM Tris-HCl [pH 8.8] at 25^{o}C, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100), 0.5 U of *Taq* polymerase (Finnzymes OY, Finland), 200 µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP, Boehringer Mannheim, Germany), 10 ρmol of each primer, 5 µl of sample DNA and added ultrapure sterile water to 25 µl. In each reaction, positive and negative controls were processed in parallel with the tested samples to detect false-negative results or contamination. Melting temperature (*T*m) values are shown in Table 1.1. The denaturation, annealing and elongation temperatures and times used were 96^{o}C for 10 seconds, 58-70^{o}C (according to the primer *T*m values) for 10 seconds and 72^{o}C for 30-60 seconds (according to the lengths of the amplicons), respectively, for 40 cycles using a Perkin Elmer thermocycler 9600.

Table 1.1. Primers targeting ureaplasma 3 different genes/regions.

Primers	Target	Specificity	*T*m °C[a]	GenBank numbers	Sequence[b]
UPS1	16S rRNA	*U. parvum*	61.6	AF073456	**154**ATG AGA AGA TGT AGA AAG TCG CTC**177**
UPA	"	"	65.8	"	**831**TTA GCT ACA ACA CCG ACC CAT TC**809**
UPA1	"	"	67.9	"	**833**CGT TAG CTA CAA CAC CGA CCC A**812**
UPS	"	"	80.1	"	**780**CGTAAACGAT CAT CAT TAA ATG TCG GCC CGA ATG G**814**
*UPSA	16S-23S intergenic spacers	"	71.0	AF059323	**138**AAACTCTCAAAACTAAA TAG AAT CCG ACC ATA TGA ATT TTT A**97**
U8[c]	16S rRNA	*U. urealyticum*	66.7	AF073450	**158**GAA GAT GTA GAA AGT CGC GTT TGC**181**
UUA	"	"	65.8	"	**823**CTA CAA CAC CGA CTC GTT CGA G**802**
UUS	"	"	76.5	"	777GTAAACGATCAT CAT TAA ATG TCG GCT CGA ACG AG**811**
*UUSA	16S-23S intergenic spacers	"	72.3	AF059330	**138**ACTCTCAAAACTAAAT AGA GTC CGA CCA TAT GAA CTT TTG**99**
*UPS2	urease gene clusters	*U. parvum*	65.1	AF085732	**486**GATTATATGT CAG GAT CAT CAA GTC AAT TTA G**517**
*UPA2	"	"	65.4	"	**925**GAAATTTTA AAC ATA ATG TTC CCC TTT TTA TC**894**
*UUS2	"	*U. urealyticum*	67.3	AF085724	**489**GATTATATGT CAG GAT CAT CAA ATC AAT TCA C**520**

*UUA2	"	"	68.1	"	**921**GAAATTTTAAA CAT AAT GTT CCC CTT CGT CTA**890**
*UMS-125[d] *UMS-170[e]	*mba* and upstream	*Ureaplasmas*	60.5	L20329	**1**GTA TTT GCA ATC TTT ATA TGT TTT CG**26**
UMA226[d]	"	"	64.0	"	**403**CAG/A CTG ATG TAA G/TTG CAG/A CAT TA/GA ATT C**376**
*UMS-57	"	*U. parvum*	59.9	"	**64**GATTA/GA/C T/CAA ATC TTA GTG TTC ATA TTT TTT AC**95**
UMA222	"	"	63.6	"	**396**T GTA AGT GCA GCA TTA AAT TCA ATG**372**
*UMS-61	"	*U. urealyticum*	61.2	AF055366	**103**GAAAAATA TTT GCA AAA CTA TAA ATA GAC AC**135**
UMA263[e]	"	"	70.8	"	**485**AAGTGACCT TTT GTT GTT GCG TTT TCT G**458**

Notes:

* Primers based on intergenic spacer regions

a. The melting temperatures (*T*m) values were provided by the primer synthesiser (Sigma-Aldrich, Castle Hill, NSW, Australia).

b. Numbers represent the numbered base positions at which primer sequences start and finish (numbering start point "1" refer to the start points "1" of correspondent gene GenBank accession numbers). Underlined sequences show bases added to modify previously published primers (Teng *et al*., 1994; Kong *et al*., 2000). Letters behind "/" indicate alternative nucleotides in different serovars.

c. From Robertson, *et al.*, 1993.

d. From Teng, *et al.*, 1994.

e. From Teng, *et al.*, 1995.

8 μl of PCR products were analysed by electrophoresis on 2.0% agarose gels, which were stained with 0.5 μg/ml ethidium bromide. A visible band with appropriate size on ultraviolet translumination was considered a positive result.

1.4. RESULTS

1.4.1. Specificity of *U. parvum* and *U. urealyticum* specific primer pairs.

Five primer pairs (UPS1-UPA, UPS1-UPA1, UPS-UPSA, UPS2-UPA2, and UMS-57-UMA222) were specific for and amplified all four serovars of *U. parvum*; and four (U8-UUA, UUS-UUSA, UUS2-UUA2, and UMS-61-UMA263) were specific for and amplified all ten serovars of *U. urealyticum*; UMS-125-UMA226 were specific for and amplified all 14 serovars of ureaplasmas and showed different length amplicons between *U. parvum* and *U. urealyticum*.

All the ATCC and UAB reference strains of *U. parvum* and *U. urealyticum* were correctly identified with the species-specific primers. No amplification occurred when DNA extracted from *Mycoplasma* or *Acholeplasma* species was tested with any of the above ten primer pairs.

1.4.2. *U. parvum* and *U. urealyticum* identification results for clinical isolates and clinical specimens.

Of the 78 clinical isolates, 62 (80%) were identified as *U. parvum*, 15 (19%) were identified as *U. urealyticum*, and 1 (1%) was mixed. Of 185 vaginal swabs that had been shown previously to contain ureaplasmas, 151 (82%) contained *U. parvum* only, 20 (11%) contained *U. urealyticum* only, and 14 (8%) contained both.

1.5. DISCUSSION

Recently, human ureaplasmas had been divided into two species *U. parvum* and *U. urealyticum* (Robertson *et al*., 2002). Several primer pairs have been described for the identification of the two species (previously biovars). However, they were not based on the sequences of all 14 serovars (Blanchard, 1990; Robertson *et al*., 1993) (Teng *et al*., 1994, 1995), and some lacked specificity or the ability to detect all serovars (Blanchard, 1990; Robertson *et al*., 1993). Better PCR-based methods for ureaplasma species identification are needed to facilitate studies of the relationship between ureaplasma species and disease (Cunliffe *et al*., 1996).

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 (Kong *et al*., 1999b). We believed that primers based on these regions would be more discriminatory for the identification of ureaplasma species (Kong *et al*., 1999b). These included primers UPSA and UUSA (16S-23S rRNA intergenic spacer regions), UPS2-UPA2 and UUS2-UUA2 (*ureA-ureB* and *ureB-ureC* intergenic spacer regions, respectively); and UMS-57, UMS-61 (upstream of *mba*) (Table 1.1.).

Differences between the two human ureaplasma species in the 16S rRNA genes have been described previously and were used to design species-specific primers (Robertson *et al*., 1993). The primer pair U8-P6, designed to amplify *U. urealyticum*, also amplified DNA from *M. pneumoniae* (Robertson *et al*., 1993; Sharma *et al*., 1998). We designed a new primer, UUA, which was paired with U8. This pair was specific for *U. urealyticum* and did not amplify DNA from two *M. pneumoniae* ATCC strains or the other mollicute species tested. Two new *U. parvum* specific primer pairs, UPS1-UPA and UPS-UPA, also based on the 16S rRNA gene, did not amplify DNA from either *U. urealyticum* or the other mollicute species tested.

The 16S-23S rRNA intergenic spacer regions are normally more heterogeneous than the 16S rRNA genes (Harasawa *et al*., 1993; Kong *et al*., 1999b). Primers spanning these regions UPS-UPSA for *U. parvum* and UUS-UUSA for *U. urealyticum* were specific for all serovars within the corresponding species for both ATCC and UAB reference strains.

Primers based on urease gene sequences used to differentiate *U. parvum* and *U. urealyticum* have been described previously (Blanchard, 1990). We designed two additional species-specific primer pairs, UPS2-UPA2 (*U. parvum* specific) and UUS2-UUA2 (*U. urealyticum* specific), targeting the *ureA-ureB* and *ureB-ureC* intergenic spacer regions, respectively. They were specific for all serovars within the corresponding species for both ATCC and UAB reference strains.

The *mba* contains both species- and serovar-defining regions (Zheng *et al*., 1992, 1995). Several primer sets based on 5'-ends of *mba* and upstream region sequences have been described previously for differentiating *U. parvum* and *U. urealyticum* (Teng *et al*., 1994, 1995). We noticed that UMS-125-UMA226 were specific for human ureaplasmas and could differentiate *U. parvum* and *U. urealyticum* in a single PCR reaction according to their different amplicon lengths; however it was difficult to identify mixtures of the two species, especially in clinical specimens, in which mixtures were not rare. 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*.

Having confirmed the sensitivity and specificity of the new primer pairs, we identified *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. Identification of clinical isolates and specimens confirmed the previous finding that *U. parvum* is found much more commonly (87% of isolates and specimens overall) than

U. urealyticum (19% overall) among vaginal flora (Cheng *et al.*, 1994). Both ureaplasma species were detected in 1 clinical isolate (1%) and 14 vaginal swabs (8%) (6% overall).

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 identification 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. 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.