CHAPTER 1

UREAPLASMA SPECIES IDENTIFICATION

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Statement of Joint Authorship

**Kong, F.** (candidate)
Did all the molecular work, interpreted the data and wrote the manuscript.

**Ma, Z.**
Assisted in some PCR.

**James, G.**
Provided all the needed molecular equipment, reagents and software.

**Gordon, S.**
Assisted in culture of related reference strains and some clinical isolates.

**Gilbert G. L.** (supervisor)
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).
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°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°C, 1.5 mM MgCl₂, 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 pmol 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 (Tm) values are shown in Table 1.1. The denaturation, annealing and elongation temperatures and times used were 96°C for 10 seconds, 58-70°C (according to the primer Tm values) for 10 seconds and 72°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.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Target</th>
<th>Specificity</th>
<th>Tm °C(^a)</th>
<th>GenBank numbers</th>
<th>Sequence(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPS1</td>
<td>16S rRNA</td>
<td>U. parvum</td>
<td>61.6</td>
<td>AF073456</td>
<td>154ATG AGA AGA TGT AGA AAG TCG CTC177</td>
</tr>
<tr>
<td>UPA</td>
<td>“</td>
<td>“</td>
<td>65.8</td>
<td>“</td>
<td>831TTA GCT ACA ACA CCG ACC CAT TC809</td>
</tr>
<tr>
<td>UPA1</td>
<td>“</td>
<td>“</td>
<td>67.9</td>
<td>“</td>
<td>833CGT TAG CTA CAA CAC CGA CCC A812</td>
</tr>
<tr>
<td>UPS</td>
<td>“</td>
<td>“</td>
<td>80.1</td>
<td>“</td>
<td>780CGTAAACGAT CAT CAT TAA ATG TCG GCC CGA ATG G814</td>
</tr>
<tr>
<td>*UPSA</td>
<td>16S-23S intergenic spacers</td>
<td>“</td>
<td>71.0</td>
<td>AF059323</td>
<td>138AAACTCTCAAAACTAAA TAG AAT CCG ACC ATA TGA ATT TTT A97</td>
</tr>
<tr>
<td>U8(^c)</td>
<td>16S rRNA</td>
<td>U. urealyticum</td>
<td>66.7</td>
<td>AF073450</td>
<td>158GAA GAT GTA GAA AGT CGC GTT TGC181</td>
</tr>
<tr>
<td>UUA</td>
<td>“</td>
<td>“</td>
<td>65.8</td>
<td>“</td>
<td>823CTA CAA CAC CGA CTC GTT CGA G802</td>
</tr>
<tr>
<td>UUS</td>
<td>“</td>
<td>“</td>
<td>76.5</td>
<td>“</td>
<td>777GTAACGATCAT CAT TAA ATG TCG GCT CGA ACG AG811</td>
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<td>*UUSA</td>
<td>16S-23S intergenic spacers</td>
<td>“</td>
<td>72.3</td>
<td>AF059330</td>
<td>138ACTCTCAAAACTAAAT AGA GTC CGA CCA TAT GAA CTT TTG99</td>
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<tr>
<td>*UPS2</td>
<td>urease gene clusters</td>
<td>U. parvum</td>
<td>65.1</td>
<td>AF085732</td>
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<tr>
<td>*UPA2</td>
<td>“</td>
<td>“</td>
<td>65.4</td>
<td>“</td>
<td>925GAAATTTTA AAC ATA ATG TTC CCC TTT TTA TC894</td>
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<td>*UUS2</td>
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<td>U. urealyticum</td>
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<td>AF085724</td>
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<td>Species</td>
<td>Tm</td>
<td>Accession</td>
<td>Sequence</td>
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<td>-----------</td>
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<tr>
<td>*UUA2</td>
<td></td>
<td></td>
<td>68.1</td>
<td></td>
<td>GAAATTTTAAA CAT AAT GTT CCC CTT CGT CTA890</td>
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<tr>
<td>*UMS-125d</td>
<td>*</td>
<td>Ureaplasma</td>
<td>60.5</td>
<td>L20329</td>
<td>GTA TTT GCA ATC TTT ATA TGT TTT CG26</td>
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<tr>
<td>*UMS-170e</td>
<td>*</td>
<td>mba and upstream</td>
<td>60.5</td>
<td>L100329</td>
<td>GTA TTT GCA ATC TTT ATA TGT TTT CG26</td>
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<tr>
<td>UMA226d</td>
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<td></td>
<td>64.0</td>
<td></td>
<td>CAG/A CTG ATG TAA G/T</td>
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<tr>
<td>*UMS-57</td>
<td></td>
<td>U. parvum</td>
<td>59.9</td>
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<tr>
<td>UMA222</td>
<td></td>
<td></td>
<td>63.6</td>
<td></td>
<td>GTA TTT GCA ATC TTT ATA TGT TTT CG26</td>
</tr>
<tr>
<td>*UMS-61</td>
<td></td>
<td>U. urealyticum</td>
<td>61.2</td>
<td>AF055366</td>
<td>GTA TTT GCA ATC TTT ATA TGT TTT CG26</td>
</tr>
<tr>
<td>UMA263e</td>
<td></td>
<td></td>
<td>70.8</td>
<td></td>
<td>GTA TTT GCA ATC TTT ATA TGT TTT CG26</td>
</tr>
</tbody>
</table>

Notes:
* Primers based on intergenic spacer regions
a. The melting temperatures (Tm) 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 “f” indicate alternative nucleotides in different serovars.
e. From Teng, et al., 1995.
GBS and ureaplasmas: Ureaplasma species identification

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.