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Molecular genotyping of human *Ureaplasma* species based on multiple-banded antigen (MBA) gene sequences

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***Ureaplasma urealyticum* has been divided into 14 serovars. Recently, subdivision of *U. urealyticum* into two species has been proposed: *U. parvum* (previously *U. urealyticum* parvo biovar), comprising four serovars (1, 3, 6, 14) and *U. urealyticum* (previously *U. urealyticum* T-960 biovar), 10 serovars (2, 4, 5, 7–13). The multiple-banded antigen (MBA) genes of these species contain both species and serovar/subtype specific sequences. Based on whole sequences of the 5′-ends of MBA genes of *U. parvum* serovars and partial sequences of the 5′-ends of MBA genes of *U. urealyticum* serovars, we previously divided each of these species into three MBA genotypes. To further elucidate the relationships between serovars, we sequenced the whole 5′-ends of MBA genes of all 10 *U. urealyticum* serovars and partial repetitive regions of these genes from all serovars of *U. parvum* and *U. urealyticum*. For the first time, all four serovars of *U. parvum* were clearly differentiated from each other. In addition, the 10 serovars of *U. urealyticum* were divided into five MBA genotypes, as follows: MBA genotype A comprises serovars 2, 5, 8; MBA genotype B, serovar 10 only; MBA genotype C, serovars 4, 12, 13; MBA genotype D, serovar 9 only; and MBA genotype E comprises serovars 7 and 11. There were no sequence differences between members within each MBA genotype. Further work is required to identify other genes or other regions of the MBA genes that may be used to differentiate *U. urealyticum* serovars within MBA genotypes A, C and E. A better understanding of the molecular basis of serotype differentiation will help to improve subtyping methods for use in studies of the pathogenesis and epidemiology of these organisms.**

Keywords: *Ureaplasma parvum*, *Ureaplasma urealyticum*, MBA gene, subtyping

INTRODUCTION

Human ureaplasmas are recognized causes of urethritis (Hewish *et al*., 1986; Cracea *et al*., 1985; Taylor-Robinson *et al*., 1985), and have been associated with complications of pregnancy and prematurity (Abele-Horn *et al*., 1997; Robertson *et al*., 1986; Kundsin *et al*., 1996; Hannaford *et al*., 1999). However, as common genital tract commensals (Viarengo *et al*., 1980; Cracea *et al*., 1985), their pathogenic roles in individual cases are difficult to confirm (Zheng *et al*., 1992).

Abbreviation: MBA, multiple-banded antigen.

The former species *Ureaplasma urealyticum* contained 14 serovars (Razin & Yogev, 1986; Robertson & Stemke, 1982). In the proposed new taxonomy, *Ureaplasma parvum* (previously *U. urealyticum* parvo biovar), contains four and *U. urealyticum* (previously *U. urealyticum* T-960 biovar), 10 serovars (Kong *et al*., 1999b). The relationship between serovars and disease syndromes needs to be studied further (Grattard *et al*., 1995; Naessens *et al*., 1988). However, this has been limited by technical difficulties and cross-reactions associated with serotyping (Wiley & Quinn, 1984; Quinn *et al*., 1981; Robertson & Stemke, 1979; Stemke & Robertson, 1985), even when monoclonal antibodies were used (Naessens *et al*., 1998).

Better understanding of the genetic basis of the

Table 1. Oligonucleotide primers used in this study

Primer	Specificity	T_m (°C)	Sequence
UMS-125	*Ureaplasma* spp.	66	−**151**GTA TTT GCA ATC TTT ATA TGT TTT CG-**125**
UMS-57	*U. parvum*	66	−**84**(A/G)(A/C)(T/C)AA ATC TTA GTG TTC ATA TTT TTT AC-**57**
UMSPS1*	*U. parvum*	68	**319**CCT CGT GAA CCA AAA CCT AAT G**340**
UMSPS2*	*U. parvum*	66	**367**GGA TTA ATC AAG ACT TCA GGT TTG**390**
UMA1213	UP 3/14	68	**1239**CTA AAG TAA TTA TTT TCC AGT AGT TTC**1213**
UMA1586	UP 3/14	72	**1613**GAT AAT CAT TCA TCT TCT CTT AAT TGT C**1586**
UMS3S*	UP 3	68	−**107**TTA CTG TAG AAA TTA TGT AAG ATT ACC-**81**
UMS14S*	UP 14	68	−**109**AAT TAC TGT AGA AAT TAT GTA AGA TTA AT-**81**
UMA269	UP 3/14	66	**293**AA CTA AAT GAC CTT TTT CAA GTG TAC**269**
UMA314A*	UP 3/14	68	**463**GTT GTT CTT TAC CTG GTT GTG TAG**440**
UMA314A′*	UP 3/14	68	**465**TG/TG TTG TTC TTT ACC TGG TTG TGT A**441**
UMS-54	UP 6	66	−**77**AAT CTT AGT GTT CAT ATT TTT TAC TAG-**54**
UMA6A*	UP 6	66	**464**CCT GGT TCT TGA GTT TTC GGA G**443**
UMA6A′*	UP 6	68	**468**TTT ACC TGG TTC TTG AGT TTT CGG**445**
UMS-83	UP 1	66	−**107**TTACT GTA GAA ATT ATG TAA GAT TGC-**83**
UMA1A*	UP 1	70	**469**TTT CTT TTG GTT CTT CAG TTT TTG AAG**443**
UMA1A′*	UP 1	68	**471**ATT TTC TTT TGG TTC TTC AGT TTT TGA**445**
UMA269′	UP 1/6	66	**293**ACCA AAT GAC CTT TTG TAA CTA GAT**269**
UMS-170	*Ureaplasma* spp.	66	−**195**GTA TTT GCA ATC TTT ATA TGT TTT CG-**170**
UMS-61	*U. urealyticum*	66	−**83**TATA TTT GCA AAA CTA TAA ATA GAC AC-**61**
UMSUS*	*U. urealyticum*	66	**163**GTT TAC GAC ATT GAA AAT TTC GAT G**187**
UMAUA*	*U. urealyticum*	62	**466**GGG (G/T)(A/T)G TT(G/T) (A/C/T)AC CA(C/T) T(G/T)C CTG GTT**443**
UMSUS1*	*U. urealyticum*	62	**378**AAC TGC ATC T(C/T)T AG(C/T) ATT ACC T**399**
UMSUS2*	*U. urealyticum*	62	**397**CCT GAT AAT TT(G/T) AAT TAT CAA ACA G**421**
UMAUA1*	*U. urealyticum*	66	**1537**GCC CAA TTC ATA GGC TAT TAA TTG**1514**
UMAUA2*	*U. urealyticum*	64	**1546**AAA AAA ATA GCC CAA TTC ATA GGC**1523**
UMA2A1*	UU A/B	66	**451**TTC CTG GTT TTG TTT CAA AAC CTA T**427**
UMA2A2*	UU A/B	66	**454**CAC TTC CTG GTT TTG TTT CAA AAC**431**
UMA2A*	UU A	66	**479**CCA CTT CCT GGT TTT GTA GTT TC**457**
UMA10A*	UU B	68	**479**CCA CTT CCT GGT TGT GTA GTT GA**457**
UMA4A1*	UU C	68	**452**TT GCC TGG TTG TGT TTC GAA CTC**430**
UMA4A2*	UU C	68	**454**CAT TGC CTG GTT GTG TTT CGA AC**432**
UMA9A1*	UU D	66	**460**CTG GAG TTG GTG TAG GCG CAT**440**
UMA9A2*	UU D	66	**462**TTC TGG AGT TGG TGT AGG CGC**442**
UMA7A1	UU E	74	**245**GTA ATT GCA ACA TGG AAT TCA GTT TCA**219**
UMA7A2*	UU E	66	**458**GGT TCT GGT GTA TGA GTG CTT TT**436**
UMA7A3*	UU E	66	**461**GTT GGT TCT GGT GTA TGA GTG C**440**

*Primers designed specifically for this study. All others have been previously published (see text for references).

The melting temperatures (T_m) of primers were calculated by the formula: $T_m = 4 \times$ no. of (G + C) + $2 \times$ no. of (A + T).

conventional ureaplasma serotype classification will assist in development of a practical molecular serotyping system and allow further investigation of the pathogenic potential of individual subtypes/serovars (Robertson & Stemke, 1982; Kong *et al.*, 1999a). In our previous study, we sequenced three genes and adjoining regions of all 14 ureaplasma serovars and studied the phylogenetic relationships between them (Kong *et al.*, 1999b). We showed that the sequences of the 16S rRNA genes and 16S–23S rRNA intergenic spacer regions, the urease gene subunits *ureA*, *ureB*, partial *ureC* and adjoining regions – upstream of *ureA*, *ureA–ureB* spacer, and the *ureB–ureC* spacer – were generally conserved for serovars within each of the two proposed new species. Only the 5′-end regions of the MBA genes showed heterogeneity between the four serovars of *U. parvum* and the 10 serovars of *U. urealyticum*.

It has been suggested, previously, that the repetitive region of the MBA gene would contain serovar-specific sites (Watson *et al.*, 1990; Zheng *et al.*, 1996). In this study, we sequenced partial repetitive regions of the MBA genes of all 14 ureaplasma serovars, to determine whether these genes could provide further evidence to support the present serotype classification and improve our previous molecular subtyping system for *U. parvum* and *U. urealyticum* (Kong *et al.*, 1999a, 2000).

METHODS

Bacterial strains. Two sets of reference strains of all 14 serovars of *U. parvum* and *U. urealyticum* were used as previously described (Kong *et al.*, 1999b). One set was obtained directly from the American Type Culture Collection (ATCC reference strains) and the other was kindly provided by Dr H. L. Watson, Department of Microbiology, University of Alabama at Birmingham, AL, USA (UAB reference strains).

Oligonucleotide primers. The oligonucleotide primers used in this study are shown in Table 1. Previously published oligonucleotide primers UMS-125, UMA1213, UMA1586 (Zheng *et al.*, 1995), UMS-57 (Kong *et al.*, 2000), and new primers designed by us – UMSPS1, UMSPS2, UMAUA – based on previously published sequences (Zheng *et al.*, 1999; GenBank accession nos U50459, U50460, U50461) were used to sequence the repetitive regions of the MBA genes of the four *U. parvum* serovars. Previously published oligonucleotide primers UMS-170 (Teng *et al.*, 1995), UMS-61 (Kong *et al.*, 2000), and new primers designed by us – UMSUS, UMSUS1, UMSUS2, UMAUA, UMAUA1, UMAUA2 – based on the previously published sequences (Kong *et al.*, 1999b; Zheng *et al.*, 1999; GenBank accession nos U50459, U50460, U50461) were used to sequence the 5′-end and the repetitive regions of the MBA genes of all the 10 *U. urealyticum* serovars.

Additional new primers – UMS3S, UMA314A, UMA314A′, UMS14S, UMA1A, UMA1A′, UMA6A, UMA6A′ – based on sequences determined in this and previous studies (Kong *et al.*, 1999b), and previously published primers designed by us UMS-83, UMS-54, UMA269 and UMA269′ were designed specifically to amplify and differentiate MBA genes of four *U. parvum* serovars 3, 14, 1 and 6. New primers UMA2A1, UMA2A2; UMA2A; UMA10A; UMA4A1, UMA4A2; UMA9A1, UMA9A2; UMA7A2 and UMA7A3, based on the sequences obtained in this study, and the previously published primer UMA7A1 (Kong *et al.*, 2000) were designed to amplify and differentiate MBA genotypes A/B, MBA genotype A, MBA genotype B, MBA genotype C, MBA genotype D and MBA genotype E (Table 1).

DNA preparations and PCR. DNA preparations and PCR systems were used as previously described (Kong *et al.*, 1999a, b). To amplify the repetitive regions of the MBA genes of *U. parvum* serovars for sequencing, a nested PCR was developed, using UMS-125/UMA1586 as outer primers and UMS-57/UMA1213 (for serovars 3 and 14) and UMS-57/UMAUA (for all four serovars of *U. parvum*) as inner primers. Nested PCR was also used to amplify the 5′-ends and repetitive regions of the MBA genes of the 10 *U. urealyticum* serovars for sequencing. The outer primers were UMS-170/UMAUA2 and inner primers were UMS-61/UMAUA and UMSUS/UMAUA2 (or UMSUS/UMAUA1) (Table 1).

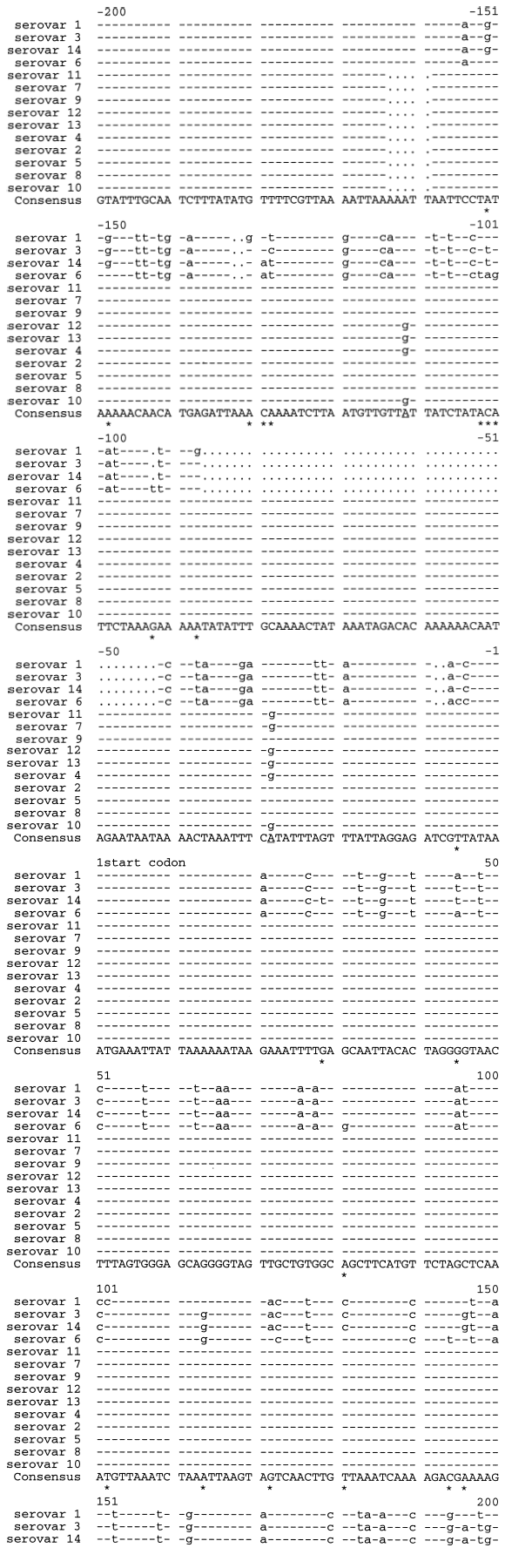
The denaturation, annealing and elongation temperatures and times used for the first step PCR were 95 °C for 30 s, 50 °C for 30 s and 72 °C for 3 min, respectively, for 30 cycles. For the second step PCR, the denaturation, annealing and elongation temperatures and times used were 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, for 30 cycles. For the serovar-/MBA genotype-specific PCR, the denaturation, annealing and elongation temperatures and times used were 95 °C for 30 s, 55–62 °C (according to the T_m value) for 30 s and 72 °C for 1 min, respectively, for 40 cycles.

Table 2. Primer pairs used to differentiate all 14 serovars of *U. parvum* and *U. urealyticum* and summary of PCR results showing sizes of bands (amplicons)

See Table 1 for primer sequences. UP 3: *U. parvum* serovar 3; UP 14: *U. parvum* serovar 14; UP 1: *U. parvum* serovar 1; UP 6: *U. parvum* serovar 6. UU A/B: *U. urealyticum* MBA genotypes A and B, includes serovars 2, 5, 8 and 10; UU A: *U. urealyticum* MBA genotype A, includes serovars 2, 5 and 8; UU B: *U. urealyticum* MBA genotype B, includes serovar 10; UU C: *U. urealyticum* MBA genotype C, includes serovars 4, 12 and 13; UU D: *U. urealyticum* MBA genotype D, includes serovar 9; UU E: *U. urealyticum* MBA genotype E, includes serovars 7 and 11. See Figs 4 and 5 for PCR results for UMS-83 UMA269′ and UMS-61 UMA7A1, respectively.

Primer pairs	Specificity (subtype/serovar)	Amplicon size (bp)
UMS3S UMA269	UP 3	400
UMS3S UMA314A	UP 3	570
UMS3S UMA314A′	UP 3	572
UMS14S UMA269	UP 14	402
UMS14S UMA314A	UP 14	572
UMS14S UMA314A′	UP 14	574
UMS-83 UMA1A	UP 1	576
UMS-83 UMA1A′	UP 1	578
UMS-83 UMA269′	UP 1	400
UMS-54 UMA6A	UP 6	544
UMS-54 UMA6A′	UP 6	548
UMS-54 UMA269′	UP 6	370
UMS-61 UMA2A1	UU A/B	539
UMSUS UMA2A1	UU A/B	289
UMS-61 UMA2A2	UU A/B	537
UMSUS UMA2A2	UU A/B	292
UMS-61 UMA2A	UU A	562
UMSUS UMA2A	UU A	317
UMS-61 UMA10A	UU B	562
UMSUS UMA10A	UU B	317
UMS-61 UMA4A1	UU C	535
UMSUS UMA4A1	UU C	290
UMS-61 UMA4A2	UU C	537
UMSUS UMA4A2	UU C	292
UMS-61 UMA9A1	UU D	543
UMSUS UMA9A1	UU D	298
UMS-61 UMA9A2	UU D	545
UMSUS UMA9A2	UU D	300
UMS-61 UMA7A1	UU E	328
UMS-61 UMA7A2	UU E	541
UMSUS UMA7A2	UU E	296
UMS-61 UMA7A3	UU E	544
UMSUS UMA7A3	UU E	299

Primer pairs used to amplify and differentiate serovars of *U. parvum* and subtypes of *U. urealyticum* are shown in Table 2. PCR products (12·5 µl) were analysed by electrophoresis on 2·0 % agarose gels, which were stained with 0·5 µg ethidium bromide ml^{-1}. For sequencing, PCR products of appropriate size that produced visible bands on UV illumination were further purified. For identification of individual sub-



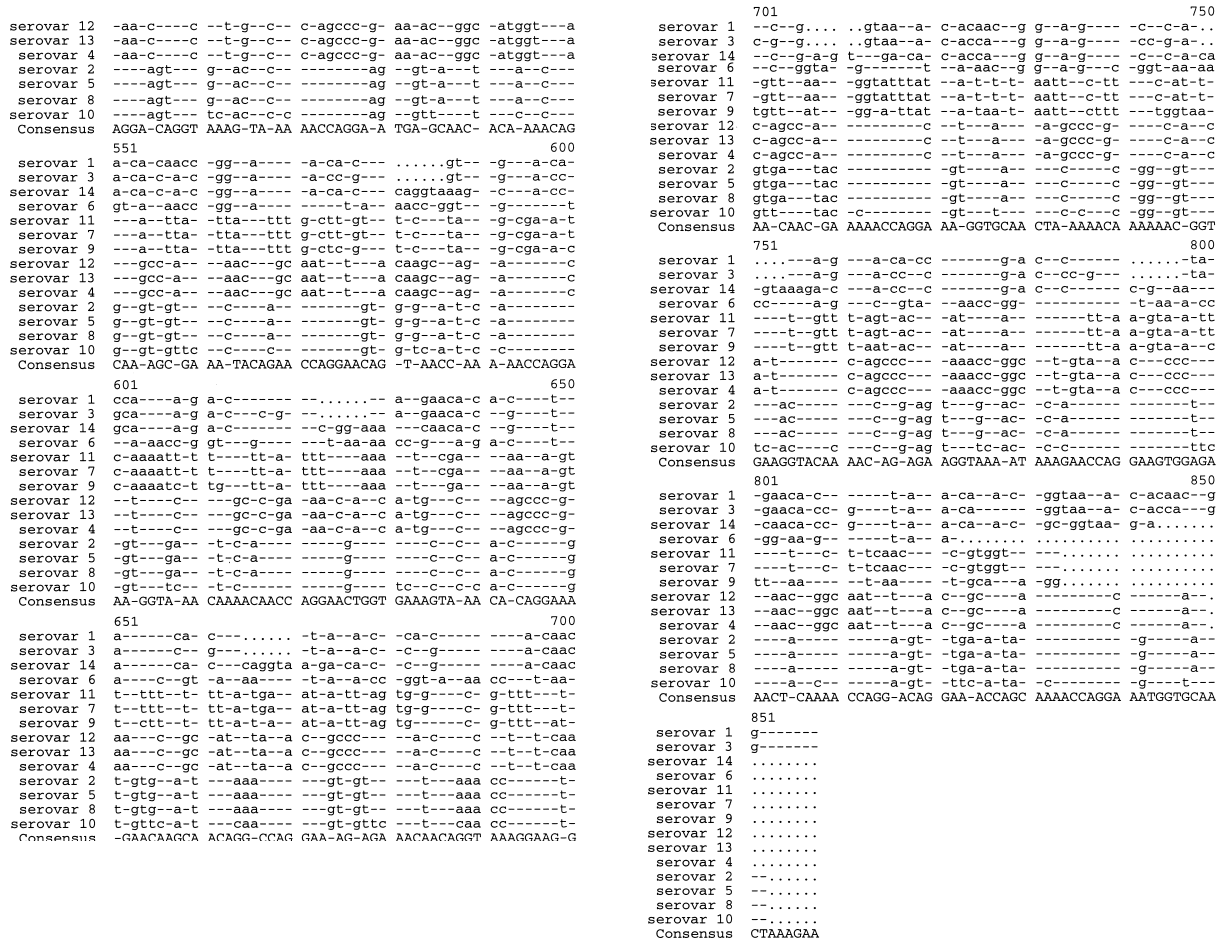


Fig. 1. Multiple sequence alignment of the MBA gene DNA sequences of 14 serovars of *U. parvum* and *U. urealyticum* (ATCC strains). *45 sites of nucleotide differences between *U. parvum* serovars; underlining indicates 22 sites of nucleotide differences between *U. urealyticum* serovars.

types, the presence of PCR amplicons of expected length on ultraviolet transillumination were accepted as positive.

Sequencing and phylogenetic analysis. The PCR products of the 5′-end and the repetitive regions of the MBA genes of all the 14 ureaplasma serovars were sequenced using Applied Biosystems (ABI) *Taq* DyeDexoy terminator cycle-sequencing kits according to standard protocols. Primers UMSPS1 (or UMSPS2) were used as sequencing primers for the amplicons of UMS-57/UMA1213 (for serovars 3 and 14), and UMS-57/UMAUA (for serovars 1 and 6); UMSUS, UMSUS1 (or UMSUS2) were used as sequencing primers for the amplicons of UMS-61/UMAUA, and UMSUS/UMAUA2 (or UMSUS/UMAUA1).

The amino acid sequences were derived by converting nucleotide sequences using Translate program from the Readseq program groups provided in WebANGIS, ANGIS (Australian National Genomic Information Service), 3rd version (mycoplasma translation codes were used). Multiple sequence alignments were performed using PILEUP and PRETTY programs from the Multiple Sequence Analysis program group, provided in WebANGIS, ANGIS (Australian National Genomic Information Service), 3rd version.

Phylogenetic relationships based on the MBA gene DNA sequences for the 14 serovars of *U. parvum* and *U. urealyticum* (ATCC strains) were studied using CLUSTAL for alignment and PHYLIP to construct the phylogenetic tree. The tree was formed using *Chlamydia trachomatis* (GenBank accession no. AE001315) as outgroup and was bootstrapped with 100 replications.

Nucleotide sequence accession numbers. The sequence data used in the paper are in GenBank/EMBL/DDBJ with the following accession numbers: AF056982, AF056983, AF056984; AF055358, AF055359, AF055360, AF055361, AF055362, AF055363, AF055364, AF055365, AF055366, AF055367 (Kong *et al.*, 1999a, b).

RESULTS

PCR and sequencing

As predicted, the inner primer pair UMS-57/UMA1213 produced amplicons only from serovars 3 and 14 and UMS-57/UMAUA produced amplicons from all four serovars of *U. parvum*. From *U. urealy-*

ticum, inner primers UMS-61/UMAUA amplified portions of all 10 serovars whereas UMSUS/UMAUA2 (or UMSUS/UMAUA1) amplified portions of seven (all except serovars 9, 7 and 11).

Comparative study of the nucleotide sequences and amino acid sequences of the 5′-end region and partial repetitive regions of MBA genes

There were base differences at 45 (45/601 = 7·5 %) sites at the 5′-end of MBA genes (−200–450) among the four serovars of *U. parvum* (Kong *et al.*, 1999b) (Fig. 1). There were amino acids differences at 19 (19/150 = 12·6 %) sites at the N-terminus of MBA (1–159) among the four serovars of *U. parvum* (Fig. 2). There were base differences at 22 (22/634 = 3·5 %) sites at the 5′-end of MBA genes (−200–439) among the 10 serovars of *U. urealyticum* (Fig. 1) and amino acid differences at 9 (9/146 = 6·2 %) sites at the N-terminus of MBA (1–146) (Fig. 2).

Nucleotide and amino acid sequences of the MBA gene repetitive units of *U. parvum* and *U. urealyticum* are shown in Table 3. They begin in the vicinity of nucleotide 451 (Fig. 1) and amino acid 151 (Fig. 2). There were differences between sequences from all 4 *U. parvum* serovars. Sequences from serovars 2, 5 and 8 were identical and grouped as MBA genotype A. The serovar 10 sequence was the same length but differed from MBA genotype A by 3/24 nucleotide bases and 2/8 amino acids; this serovar was classified as MBA genotype B. Serovar 4, 12 and 13 sequences were longer than those of MBA genotypes A and B, but identical with each other and were grouped together as MBA genotype C. No repetitive units were identified for serovars 9, 7 and 11 (Table 3). However, there were differences between serovar 9 and serovars 7/11 in 58 (58/391 = 14·8 %) nucleotide bases in the region 440–833 and 14 (14/634 = 2·2 %) in the region −200–439 at the 5′-end of MBA genes (Fig. 1). There were corresponding differences between serovar 9 and serovars 7/11 in 31 amino acids (31/130 = 23·8 %) in the regions of 151–283 and 6 (6/146 = 4·1 %) in the region 1–146 at the N-terminus of MBA (Fig. 2). These differences defined two additional MBA genotypes, D (serovar 9) and E (serovars 7 and 11).

The phylogenetic trees of the 14 serovars of *U. parvum* and *U. urealyticum*

Phylogenetic tree, based on the nucleotide sequences of the 5′-ends and partial repetitive regions of MBA genes is shown in Fig. 3. Serovars 3 and 14 of *U. parvum* are most closely related, with serovars 1 and 6 more distant. The 10 serovars of *U. urealyticum* form five MBA genotypes as outlined above. MBA genotypes A, C and E are separate clusters of three, three and two serovars, respectively, with the MBA genotype B (a single serovar) located between MBA genotypes A and C; MBA genotype D (also a single serovar) is located between MBA genotypes C and E (Fig. 3).

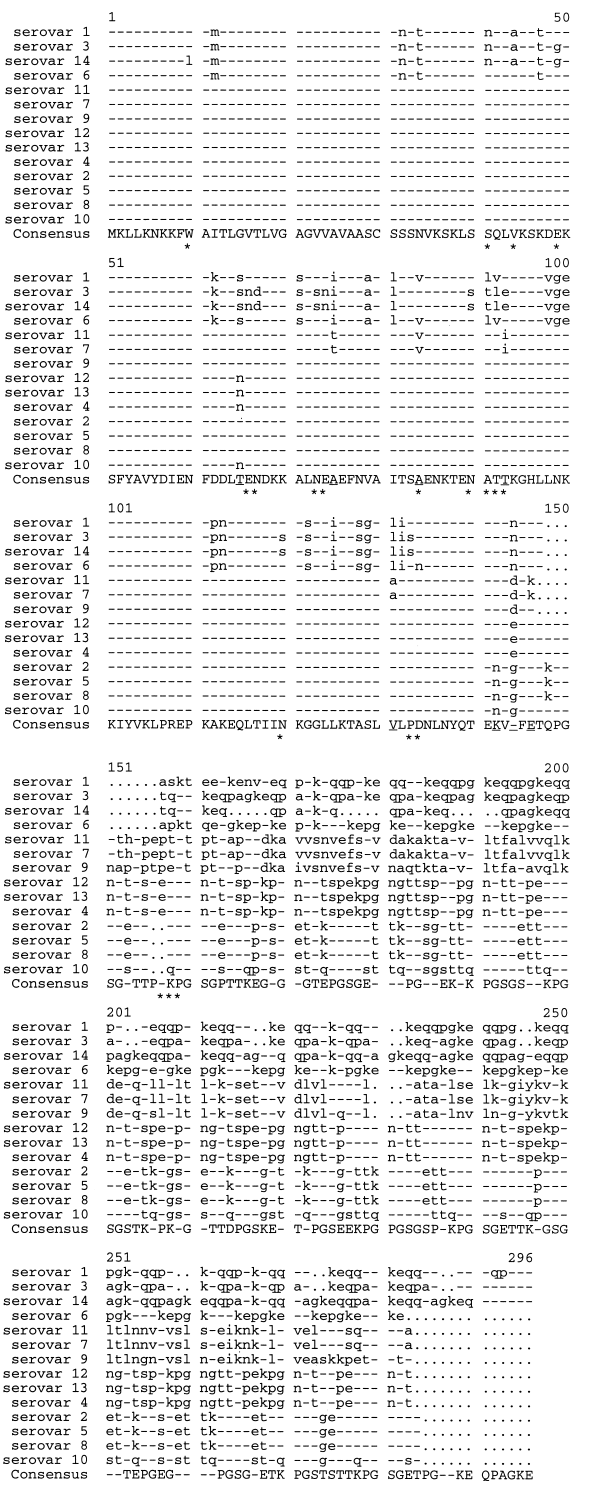


Fig. 2. Multiple sequence alignment of the MBA amino acid sequences of 14 serovars of *U. parvum* and *U. urealyticum* (ATCC strains). *19 sites of amino acid differences between *U. parvum* serovars; underlining indicates nine sites of amino acid differences between *U. urealyticum* serovars.

The specificity of *U. urealyticum* subtyping primers

All the ATCC and UAB reference strains of *U. parvum* and *U. urealyticum* were correctly identified by the

Table 3. Nucleotide and amino acid sequences of repetitive units of the MBA genes of 14 serovars of *U. parvum* and *U. urealyticum*

UP: *U. parvum*; UU: *U. urealyticum*. UU MBA genotype A: includes serovars 2, 5 and 8; UU MBA genotype B: includes serovar 10; UU MBA genotype C: includes serovars 4, 12 and 13; UU MBA genotype D: includes serovar 9; UU MBA genotype E: includes serovars 7 and 11.

Serovar/subtype	Nucleotide sequence	Amino acid sequence
UP Serovar 1	CAA CAA CCA GGT AAA GAA	QQPGKE
UP Serovar 3	CAA CCA GCA GGT AAA GAA	QPAGKE
UP Serovar 6	GGT AAA GAA CCA	PGKE
UP Serovar 14	CAA CAA CCA GCA GGT AAA GAA	Q QPAGKE
UU MBA genotype A	ACA AAA CCA GGA AGT GGT GAA ACT	TKPGSGET
UU MBA genotype B	ACA CAA CCA GGA AGT GGT TCA ACT	TQPGSGST
UU MBA genotype C	ACA AGC CCA GAA AAA CCA GGC AAT GGT ACA	TSPEKPGNGT
UU MBA genotype D	—	—
UU MBA genotype E	—	—

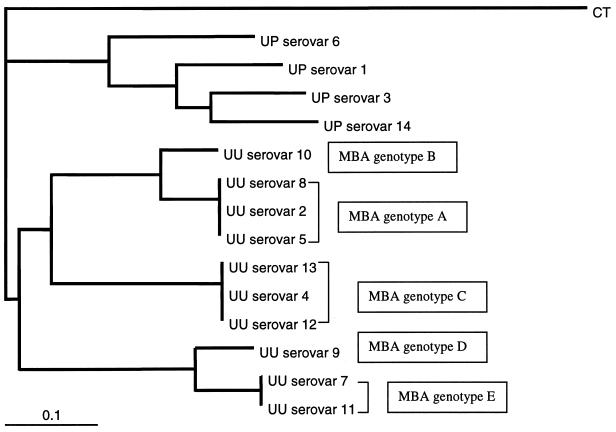


Fig. 3. Phylogenetic tree for 14 serovars of *U. parvum* (UP) and *U. urealyticum* (UU) (ATCC strains) based on the MBA gene DNA sequences. CLUSTAL was used for alignment, and PHYLIP was used for constructing the phylogenetic tree. The tree was formed using *Chlamydia trachomatis* (GenBank no. AE001315) as outgroup and was bootstrapped with 100 replications.

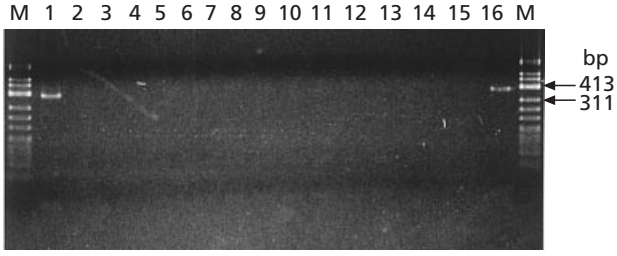


Fig. 4. Results of PCR amplification of the 5′-end of MBA genes of all 14 serovars of *U. parvum* and *U. urealyticum* using primers UMS-83 and UMA269′. *U. parvum* consists of serovars 1, 3, 6 and 14; *U. urealyticum* consists of serovars 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13. Lanes: M, molecular mass markers ϕX174 DNA/*Hin*fI; 1 and 16, *U. parvum* serovar 1 ATCC strain and UAB reference strain; 6 and 7, *U. parvum* serovar 6 ATCC strain and UAB reference strain; 2–5, *U. urealyticum* serovar 2, *U. parvum* serovar 3, *U. urealyticum* serovars 4 and 5 ATCC strains; 8–15, *U. urealyticum* serovars 7–13 and *U. parvum* serovar 14 ATCC strains.

serovar-/MBA genotype-specific primers. The results of PCR for all the serovars of *U. parvum* and *U. urealyticum*, using the 33 subtype-specific primer pairs to amplify the 5′-end of the MBA genes are summarized in Table 2 and representative examples are shown as Figs 4 and 5.

Our primary evaluation showed that the serovars and MBA genotypes (corresponding with serovars) of *U. parvum* and *U. urealyticum* were identified, specifically, using primer pairs as shown in Table 2.

DISCUSSION

Various phenotypic and molecular methods have been described previously to distinguish the two main groups of human ureaplasmas (formerly two biovars of *U. urealyticum*, now proposed species *U. parvum* and *U. urealyticum*) (previously summarized by Kong *et al.*, 1999b). Our previous study showed that homology between sequences of the 16S rRNA genes, 16S–23S rRNA intergenic spacer regions and urease gene subunits of serovars within each proposed species was high and these regions could not be used for further subtyping (Kong *et al.*, 1999b, 2000). However, sequence differences between the partial 5′-end regions of the MBA genes allowed each species to be divided into three genotypes (Kong *et al.*, 2000). It had been suggested previously that the repetitive region of the MBA gene should also contain serovar-specific definition sites (Zheng *et al.*, 1996). Therefore, in this study, we sequenced the whole 5′-end regions of the MBA genes of the 10 serovars of *U. urealyticum* and partial repetitive regions of the MBA genes for all 14 ureaplasma serovars. Our aim was to define sequence differences that would allow further molecular identi-

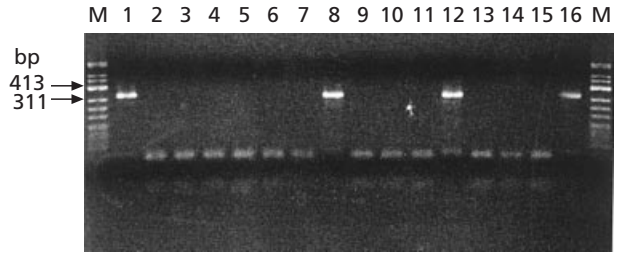


Fig. 5. Results of PCR amplification of the 5′-end of MBA genes of all 14 serovars of *U. parvum* and *U. urealyticum* using primers UMS-61 and UMA7A1. *U. parvum* consists of serovars 1, 3, 6 and 14; *U. urealyticum* consists of serovars 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13. Lanes: M, molecular mass markers ϕX174 DNA/*Hin*fI; 1 and 8, *U. urealyticum* serovar 7 UAB reference strain and ATCC strain; 12 and 16, *U. urealyticum* serovar 11 ATCC strain and UAB reference strain; 2–7, *U. parvum* serovar 1, *U. urealyticum* serovar 2, *U. parvum* serovar 3, *U. urealyticum* serovars 4, 5 and *U. parvum* serovar 6 ATCC strains; 9–11, *U. urealyticum* serovars 8, 9 and 10 ATCC strains; 13–15, *U. urealyticum* serovars 12, 13 and *U. parvum* serovar 14 ATCC strains.

fication of serovars or additional MBA genotypes of *U. parvum* and *U. urealyticum* (Kong *et al.*, 2000).

Our previous studies showed only three base differences between sequences of the 5′-end of MBA gene of *U. parvum* serovars 3 and 14 (Kong *et al.*, 1999a, b). In this study we showed more numerous differences in nucleotide and amino acid sequences of the repetitive units, between *U. parvum* serovars, which allowed all of them, including serovars 3 and 14, to be differentiated. Based on our previous study of partial 5′-end sequences of the MBA genes of *U. urealyticum* (Kong *et al.*, 1999b), serovar 10 is closely related to serovars 4, 12 and 13. However, differences in nucleotide and amino acid sequences immediately upstream of the repetitive regions and in the repetitive units themselves, allowed serovar 10 to be separated from serovars 4/12/13. Similarly, serovar 9 was closely related to serovars 2, 5 and 8, based on sequences of the 5′-end of the MBA genes (Kong *et al.*, 1999b) but deletion of the repetitive region in serovar 9 allowed it to be differentiated from serovars 2/5/8. This finding is supported by the recent development of a monoclonal antibody against *U. urealyticum* serovar 9, that cross-reacts minimally only with serovar 2 (Naessens *et al.*, 1998a).

The present study also showed that there were 22 bases at the 5′-end of the MBA genes of the 10 serovars of *U. urealyticum*, upstream of the repetitive regions, which helped to differentiate the five MBA genotypes. More than half of these differences were between MBA genotype E (serovars 7/11) and the other four MBA genotypes. Serovars 7/11 were similar to serovar 9 in that the repetitive sequences were deleted. However, their sequences differed by 14 bases at the 5′-end of MBA genes and 58 within sequences that corresponded with those of the repetitive regions of MBA genes of the other serovars (Fig. 1). Serovars in MBA genotypes A (serovars 2, 5 and 8), C (serovars 4, 12 and 13) and E (serovars 7 and 11) could not be differentiated further on the basis of these sequences.

Reliable differentiation between the serovars of *U. urealyticum* using phenotypic methods is difficult. Antigenic cross-reactions between serovars 2 and 5 (Wiley & Quinn, 1984; Quinn *et al.*, 1981), 4 and 8 (Quinn *et al.*, 1981), and 8, 2 and 4 (Robertson & Stemke, 1979) have been described. Because of variable strain selection from frequently mixed cultures, the reproducibility of serovar determination between primary and secondary plating of isolates was only 83%; it increased only to 87% on multiple, secondary cultures (Stemke & Roberston, 1985). The fact that genetic differences between some serovars are minor has been confirmed by demonstration that a single amino acid difference between the MBA of serovars 3 and 14 of *U. parvum* accounts for epitope differences that can be distinguished using type-specific monoclonal anibodies (Zheng *et al.*, 1996). Arbitrarily primed PCR, using a pairwise combination of primers, was able to differentiate only a few of the 10 serovars of *U. urealyticum* (Grattard *et al.*, 1995).

Further work is required to identify other genes or other regions of the MBA genes that may be used to differentiate *U. urealyticum* serovars within MBA genotypes A, C and E. However, on the basis of our data, we suggest that genetic and antigenic differences between some serovars are so minor that further subdivision into serovars might be artificial and/or unnecessary. These data provide a better understanding of the molecular basis of serotype differentiation. Based on the phylogenetic analysis, we designed a series of serovar-/MBA genotype-specific primer pairs to subtype each *Ureaplasma* species and are developing a more practical subtyping system that can be used for further study of the relationship between subtypes and diseases.

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