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Ureaplasma urealyticum Biovar Specificity and Diversity Are Encoded in Multiple-Banded Antigen Gene

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***Ureaplasma urealyticum* is a commensal organism of the lower genital tract of females, but in a subpopulation of individuals, it can invade the upper genital tract. It is a significant cause of chorioamnionitis and neonatal morbidity and mortality. There are 14 recognized serovars of *U. urealyticum*; these can be divided into two distinct clusters or biovars. Biovar 1 is composed of serovars 1, 3, 6, and 14. Biovar 2 is composed of serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13. We previously identified a surface antigen, the multiple-banded (MB) antigen, which contains both serovar-specific and cross-reactive epitopes. Genotypic characterization of the C-terminal region of the MB antigen of serovar 3 indicates that serovar specificity and MB antigen size variation reside in that domain. In the present study, we used PCR analysis with primers derived from the serovar 3 MB antigen gene DNA sequence to determine if the MB antigen gene was present in the remaining 13 reference serovars as well as in invasive clinical isolates. The results indicated that not only was the MB antigen gene present in all serovars but that the genes' 5′ regions were markers of biovar specificity and diversity. Further analysis of this region should reveal the phylogenetic relationship among serovars of *U. urealyticum* and, possibly, their invasive potential.**

Ureaplasma urealyticum is a common commensal organism of the urogenital tract of sexually mature females. However, ureaplasmal infection of the chorioamnion is strongly associated with chorioamnionitis, premature birth, and perinatal morbidity and mortality (4, 5). *U. urealyticum* is the single most common microorganism isolated from the central nervous system and lower respiratory tract of newborn infants (5), particularly those born prematurely. Because of the frequency with which *U. urealyticum* occurs in healthy asymptomatic individuals, it has been suggested that only certain subgroups of the species are truly disease associated. However, the development of reliable typing reagents is necessary to explore this possibility.

There are 14 recognized serovars of *U. urealyticum*; these can be divided into two distinct clusters or biovars. Biovar 1 (or parvo biovar) is composed of serovars 1, 3, 6, and 14. Biovar 2 (or T960 biovar [19]) is composed of 10 serovars, which are numbered 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13. Members of the two biovars differ phenotypically in their susceptibilities to manganese (17). They can also be differentiated by DNA-DNA hybridization (7), restriction fragment length polymorphism (10, 15, 16), one- and two-dimensional gel electrophoresis (22), genomic sizes (18), and PCR amplification of specific genes (1, 19).

We recently cloned and sequenced the multiple-banded (MB) antigen gene of the *U. urealyticum* serovar 3 reference strain (28). This is the predominant antigen recognized by patients infected with *U. urealyticum* (23). Nucleotide sequence analysis predicts that the MB antigen contains a signal peptide and acylation site in the N-terminal region, while the C-terminal region is composed of multiple six-amino-acid (encoded by 18 nucleotides) tandem repeats, which contain serovar-specific epitopes. Alteration of the copy number of the repeating units results in MB antigen size variation (28). In contrast to the repeat region, nucleotide sequencing and PCR analysis of different serovar 3 MB antigen size variants suggested that the 5′ region is conserved among serovar 3 size variants (28). Thus, we hypothesized that this conserved 5′ section of the serovar 3 gene encoding the N-terminal region of the MB antigen may also have homologous counterparts in the other 13 serovars. The goal of the present study was to test this hypothesis by using PCR primers derived from the upstream region and the 5′ region of the MB antigen gene of serovar 3. All 14 reference serovars plus 20 well-characterized invasive isolates were found to contain an MB antigen gene, and we found that members of the two biovars could be distinguished by the sizes of the amplification products. Although evidence presented elsewhere (28) indicates that serovar specificity is determined by the composition of the C-terminal region of MB antigens, we show here that the heterogeneity detected in the sequence of the 5′ region of the MB antigen gene of the different serovars allowed us to divide the 14 serovars into several subgroups on the basis of restriction endonuclease digestion of the PCR products.

MATERIALS AND METHODS

Organisms. The reference strains of *U. urealyticum* serovars 1 through 8 used in the present study were obtained from E. A. Freundt (Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark), and serovars 9 through 14 were obtained from J. A. Robertson (Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta, Canada). *Mycoplasma pneumoniae* Eaton, *Mycoplasma fermentans*, *Mycoplasma hominis*, *Mycoplasma buccale*, *Mycoplasma faucium*, *Mycoplasma orale*, *Mycoplasma salivarium*, *Mycoplasma genitalium*, *Mycoplasma pirum*, *Mycoplasma primatum*, *Mycoplasma arthritidis*, *Mycoplasma muris*,

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TABLE 1. Sequences of oligonucleotide primers

Primer	Sequence (5′ to 3′)
UMS−125 (sense)	GTATTTGCAATCTTTATATGTTTTCG
UMS51 (sense)	CTGAGCTATGACATTAGGTGTTACC
UMA226 (antisense)	CAGCTGATGTAAGTGCAGCATTAAATTC
UMA427 (antisense)	ACCTGGTTGTGTAGTTTCAAAGTTCAC

Mycoplasma neurolyticum, *Mycoplasma collis*, *Mycoplasma pulmonis*, *Acholeplasma laidlawii*, and *Acholeplasma oculi* were obtained from J. G. Tully of the National Institute of Allergy and Infectious Diseases.

Ten clinical isolates from cerebrospinal fluid of newborn infants and 10 isolates from chorioamnions were serotyped by immunoblotting with monoclonal antibodies (MAbs) as described previously (29). However, in the present study a newly developed serovar 6-specific MAb instead of rabbit anti-serovar 6 serum was used as described previously (29). One cerebrospinal fluid isolate (isolate C4), whose serotype was unknown in our previous study (29), was identified as serovar 6 with the new MAb.

Sample preparations for PCR. Rapid sample preparation prior to DNA amplification was done as described previously (2). Briefly, 1 ml of the cultures was centrifuged (12,000 × *g*, 4°C, 20 min). The pellet was resuspended in 100 μl of proteinase K solution (0.25 mg/ml). These cell suspensions were incubated for 1 h at 60°C prior to incubation at 95°C for 10 min. Five microliters of the deproteinized samples was added to the 50-μl amplification reaction mixture.

PCRs. The primers used for the PCRs were derived from the nucleotide sequence of the serovar 3 MB antigen gene (Table 1) (28). The oligonucleotide nomenclature was as follows: molecules were named UMS or UMA for ureaplasma MB antigen coding sense primers or antisense primers, respectively. The number following UMS or UMA refers to the location on the MB antigen gene map corresponding to the 3′-most base of the oligonucleotide. PCRs with primers UMS−125 and UMA226 were designated UM-1. Reactions with UMS51 and UMA427 were designated UM-2. The locations of the oligonucleotides used as PCR primers were mapped on the MB antigen gene as indicated in Fig. 1.

The amplification reaction mixtures contained 50 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.), 200 μM (each) deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; Perkin-Elmer), 0.5 μM (each) primer, and 5 μl of sample. The PCR was carried out in a DNA thermal cycler (MJ Research Inc., Watertown, Mass.) with 35 cycles of denaturation (94°C, 20 s), annealing (62°C, 1 min), and extension (72°C, 1 min) and a final extension step (72°C, 5 min).

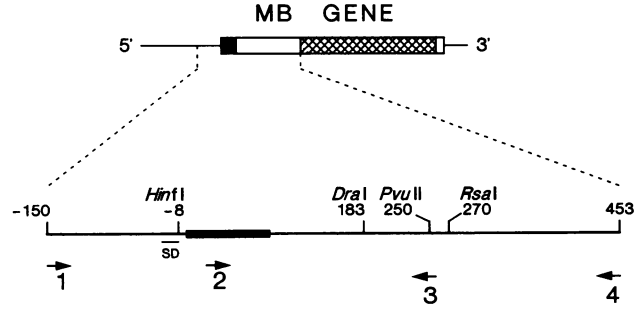

FIG. 1. Schematic map of the *U. urealyticum* serovar 3 MB antigen gene and positions of PCR primers. The number included in the PCR primer name refers to the location of the MB antigen gene map corresponding to the 3′-most base of the oligonucleotide. Arrows indicate the positions and orientations of the PCR primers. The repeat region of the gene (cross-hatched), which comprises 67% of the 3′-end of the gene, and the signal peptide (black) are marked; SD, Shine-Dalgarno sequence. Primer 1, UMS−125; primer 2, UMS51; primer 3, UMA226; primer 4, UMA427.

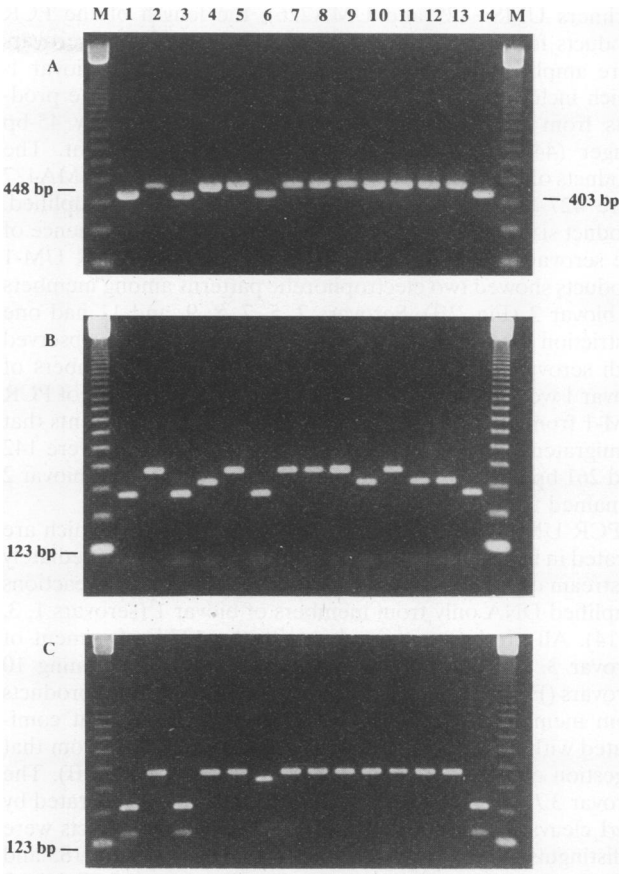

FIG. 2. Electrophoretic analysis of the PCR UM-1 products obtained with 14 serovar reference strains of *U. urealyticum* (lanes 1 through 14, respectively). (A) Untreated PCR products. (B and C) PCR products after digestion with *Dra*I or *Hin*fI, respectively. M, molecular size standards (123-bp ladder obtained from Bethesda Research Laboratories, Gaithersburg, Md.).

Primers and PCR conditions for urease gene amplification were as described previously (1).

Analysis of amplified samples. PCR products were analyzed electrophoretically on 2% agarose gels stained with ethidium bromide (20). The products of some PCR amplifications were digested with restriction enzymes according to the manufacturer's instructions (Promega, Madison, Wis.).

RESULTS

PCR UM-1 defined a DNA fragment from 150 bp upstream from the MB antigen gene start codon to a position 253 bp into the coding sequence. These locations are based on the nucleotide sequence of serovar 3 and are therefore only estimates of the locations for the other serovars. Using these primers

(primers UMS−125 and UMA226), the length of the PCR products for serovar 3 was 403 bp. All 14 reference serovars were amplified (Fig. 2A). The PCR products from biovar 1, which includes serovar 3, all comigrated. However, the products from members of biovar 2 were approximately 45-bp longer (448 bp) than the 403-bp biovar 1 fragment. The products of PCR UM-2 with primer pair UMS51 and UMA427 were 427 bp, and serovars 1, 3, 6, and 14 were amplified. Product size calculations were based on the DNA sequence of the serovar 3 reference strain. *Dra*I digestion of PCR UM-1 products showed two electrophoretic patterns among members of biovar 2 (Fig. 2B). Serovars 2, 5, 7, 8, 9, and 11 had one restriction digestion pattern; a different pattern was observed with serovars 4, 10, 12, and 13. *Dra*I digests of members of biovar 1 were identical. *Hin*fI digestion of the products of PCR UM-1 from serovars 1, 3, and 14 yielded DNA fragments that comigrated with serovar 3 digestion products, which were 142 and 261 bp in length, while serovar 6 and members of biovar 2 remained undigested (Fig. 2C).

PCR UM-2 used primers UMS51 and UMA427, which are located in the signal peptide region and the region immediately upstream of the repeat sequence, respectively. These reactions amplified DNA only from members of biovar 1 (serovars 1, 3, 6, 14). All products comigrated with the 427-bp fragment of serovar 3. No product was detected from the remaining 10 serovars (Fig. 3A). Digestion with *Dra*I of PCR UM-2 products from members of biovar 1 all yielded fragments that comigrated with the 157- and 270-bp fragments obtained from that digestion of *U. urealyticum* serovar 3 products (Fig. 3B). The serovar 3 *Dra*I site is at position 183. Fragments generated by *Rsa*I cleavage of serovar 3 and 14 PCR UM-2 products were indistinguishable; for serovar 3, those fragments were 183 and 244 bp (Fig. 3B). Additionally, *Pvu*II digestion of PCR UM-2 products from serovars 3 and 14 generated identically sized DNA fragments (data not shown).

Using PCRs UM-1 and UM-2, we determined the biovar designations of 20 clinical isolates. The serovar designations of those samples had been determined previously by using MAbs; 16 were typeable with currently available MAbs, and 4 could not be typed. The biovars determined from the results of PCR agreed with the immunologically predicted biovars for the 16 isolates with known serovars (Table 2). PCRs with primers from the urease gene (the sequence was published previously [1]) were also performed on these 20 isolates, and the results correlated exactly with those of PCRs UM-1 and UM-2 (Table 2). Restriction digestion of the MB antigen gene PCR products from the 16 clinical strains with known serovars yielded electrophoretic patterns that were identical to those obtained for the corresponding reference serovars. The PCR results for the remaining four isolates with unknown serovars suggested that two of them (isolates C8 and 9128) were biovar 2, while the other two (isolates C3 and 0015) were biovar 1. None of these four isolates reacted with any of the MAbs directed to serovars 1, 3, 2/5, 6, 8, 9, 10, and 3/14. The restriction digestion patterns (*Dra*I, *Hin*fI, and *Rsa*I) of these two isolates (isolates C3 and 0015) were indistinguishable from the pattern of the serovar 1 reference strain (data not shown).

We determined the sensitivity and species specificity of the PCR conditions described above. The detection limits for PCR UM-1 were estimated to be approximately 30 color changing units. The PCR UM-1 and PCR UM-2 primers and conditions were also tested for their reactivities with 17 other mycoplasma species including phylogenetically closely related species such as *M. pneumoniae* (24). No DNA from any of the other mycoplasma species was amplified in PCR UM-1 or PCR UM-2.

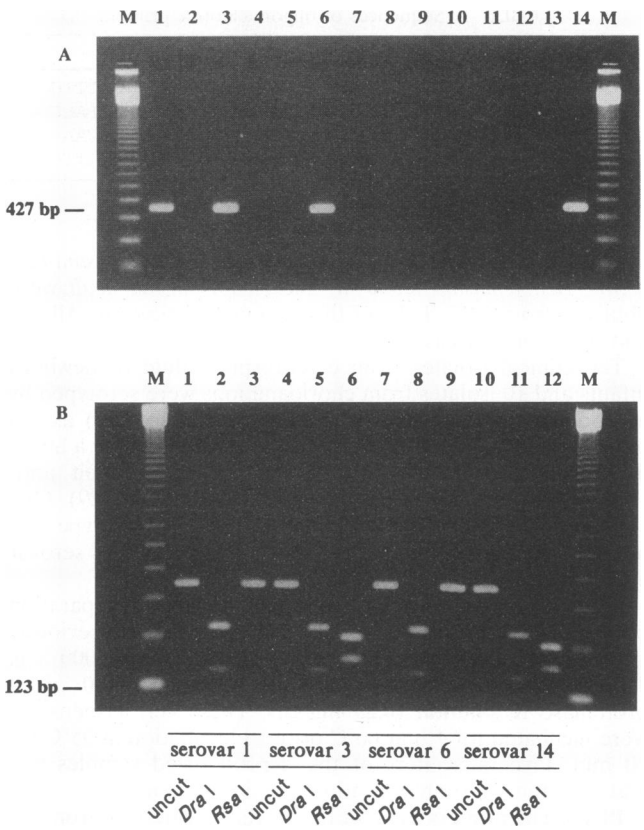

FIG. 3. Electrophoretic analysis of the PCR UM-2 products. (A) Untreated PCR products obtained from all 14 serovar reference strains of *U. urealyticum*. (B) Restriction endonuclease digests of PCR UM-2 products. Only the serovars whose MB antigen gene fragments were amplified are shown in the gel; those serovars comprised biovar 1. Lanes contain the following samples: serovar 1 (lanes 1 through 3), serovar 3 (lanes 4 through 6), serovar 6 (lanes 7 through 9), and serovar 14 (lanes 10 through 12). The DNA amplified from each of the four serovars was digested with two different endonucleases: undigested products (lanes 1, 4, 7, and 10) and DNA fragments after digestion with *Dra*I (lanes 2, 5, 8, and 11) and *Rsa*I (lanes 3, 6, 9, and 12). M, molecular size standards (123-bp ladder).

DISCUSSION

Studies of surface antigens that contain repetitive structures found in both prokaryotic and eukaryotic pathogens have focused more on the repeat regions than on the nonrepeat regions of these proteins (11–13, 25–27). The repeat regions usually contain important antigenic determinants and are of importance in understanding disease pathogenesis and immunogenesis (9, 12, 13, 25), and these protein domains are also typically associated with antigenic variation and increased genetic plasticity (11, 12, 25–28). Conversely, characterization of the nonrepeat regions, which are less variable among genes from related species and subspecies, might provide both more useful diagnostic tools and better methods for phylogenetic analysis of related organisms than would analysis focused on repeat regions. For example, with the goal of developing a broadly protective antistreptococcus vaccine, instead of using

TABLE 2. Serovar and biovar analysis by using immunoblot and biovar analysis with PCR for 20 clinical isolates

Isolate no.	Immunoblot serovar	PCR UM-1 biovar	Amplification of DNA by PCR UM-2[a]	Amplification of urease gene[a]
C1	3	1	+	−
C2	1	1	+	−
C3	Unknown	1	+	−
C4	6	1	+	−
C5	3	1	+	−
C6	10	2	−	+
C7	8	2	−	+
C8	Unknown	2	−	+
C9	10	2	−	+
C10	6	1	+	−
1414	3	1	+	−
1687	3	1	+	−
1815	10	2	−	+
0015	Unknown	1	+	−
1649	6	1	+	−
2564	10	2	−	+
9128	Unknown	2	−	+
4020	6	1	+	−
6539	6	1	+	−
7518	8	2	−	+

[a] Amplification of DNA is reported by a plus sign; failure to detect amplified DNA is reported with a minus sign.

the highly variable repeat region, a conserved region from the M-protein gene of group A streptococcus has been expressed by using a recombinant vaccinia virus. This vaccine protects mice against challenge by several different group A streptococcal types (9). Conserved regions of a giardia gene have been used as a PCR amplification target for identification and diagnostic purposes (8).

Our previous investigations indicated that the region in the serovar 3 MB antigen gene that encoded the N-terminal nonrepeat domain of the antigen was conserved among different variants, while the lengths of the gene domains coding for the C-terminal repeat regions were variable and were associated with the size variation of this antigen (28). In the present study, we used PCR to show the presence of a specific fragment of the 5′ region in all 14 reference serovars as well as in each of 20 clinical isolates representing 5 different serovars. These results strongly suggest that, unlike some other serovar determinants, which are present only among certain isolates (6), the MB antigen gene exists among all the reference serovars and is likely to exist among all isolates of the organism.

The present results also demonstrated that biovar specificity resides in the 5′ region. With PCR UM-1, organisms of different biovars produced distinct PCR products, allowing differentiation between the two biovars. The differences were also consistent for the 16 clinical isolates with known serovars. Since PCR with primers UMS51 and UMA226, which both map in the coding region of the MB antigen gene, resulted in amplification of DNA fragments of the same size among all 14 serovars (data not shown), it is likely that the length differences between the two biovars were in the 5′-upstream regions. Using the primers UMS51 and UMA427 (PCR UM-2), DNA was amplified only from members of biovar 1. The PCR UM-2 experiment as well as PCR with primers UMS−125 and UMA427, which amplified members of biovar 1 only (data not shown), suggested that the UMA427 region contains biovar-specific sequences. Interestingly, although our results place serovar 6 into biovar 1, the lack of sensitivity to *Hin*fI is a biovar 2 characteristic. This result suggests that even before the nucleotide sequence is available so that the phylogenetic relationships of the *U. urealyticum* serovars can be predicted, restriction enzyme digestion analysis of PCR products may permit further subdivision of the *U. urealyticum* serovars. Importantly, when we eventually obtain nucleotide sequence data for the genes of all the *U. urealyticum* serovars, we may find that restriction endonuclease digestion of the PCR products could be an effective method for determining the serovars of clinical isolates.

These PCR results revealed that the difference in sequences between two biovars occurred not only among reference strains but also among clinical isolates. In addition, the results also support our previous finding that the N-terminal regions are conserved among the MB antigens of the same serovar. Importantly, in an illustration of the diagnostic potential of PCR for *U. urealyticum* typing, clinical isolates that could not be typed with currently available immunologic reagents were biotyped. For the two isolates (isolates C8 and 9128) which possessed biovar 2 PCR phenotypes, the serovars could not be identified with available serovar-specific MAbs which identify serovars 1, 2, 3, 5, 6, 8, 9, 10, and 14. Therefore, it is likely that they may belong to serovar 4, 7, 11, 12, or 13. The PCR products from the remaining two isolates with unidentifiable serovars (isolates C3 and 0015) exhibited biovar 1 patterns. Although we could not confirm immunologically the serovars of these two isolates, restriction endonuclease digestion of their PCR products showed that they were identical to the PCR products of the serovar 1 reference strain. We offer several explanations for this. First, although the MB antigen gene exists in these two strains, the gene products may not be expressed. Therefore, our serovar 1-specific MAb, MAb 3C4.6, was not reactive. However, because it encodes an apparently important antigen, it would be surprising if it were not expressed in clinical pathogenic isolates. Second, and alternatively, the antigen recognized by MAb 3C4.6 is not the MB antigen gene product. In this scenario, these two isolates expressed the MB antigen; however, it was not recognized by MAb 3C4.6. Third, and perhaps the most likely explanation, is that, although MAb 3C4.6 is serovar specific, it may recognize a nonconserved epitope. Additional serovar 1 MAbs will be required to evaluate further these two isolates immunologically.

There are two published nucleotide sequences from *U. urealyticum* that have been used as PCR targets for biovar differentiation: the 16S rRNA gene sequences (19) and the urease structural genes (1). Robertson et al. (19) determined the 16S rRNA nucleotide sequence of the serovar 3 reference strain, and they exploited the differences in the variable regions of 16S rRNA genes to provide primers for biovar-specific PCRs. In their study, one set of conditions amplified DNAs from all biovar 1 isolates, while another set of conditions and primers amplified gene fragments from members of biovar 2 only. However, the PCR of Robertson et al. (19) for biovar 2 also reacted with several phylogenetically closely related species: *M. pneumoniae*, *M. fermentans* incognitus, and animal ureaplasmas. Blanchard and colleagues (1, 3) tested several pairs of primers for PCR using urease gene sequences. One of those amplifications of the urease gene that used oligonucleotides located upstream and within the 11.2-kDa unit amplified serovars 2, 4, 5, 7, 8, 9, and 13, and consequently, that method classified serovars 10 and 12 into biovar 1 (1). This result conflicts with those of most reports, in which these two serovars are usually classified as biovar 2 on the basis of their susceptibilities to manganese (17), protein analysis on polyacrylamide gels (14), Southern blot analysis with rRNA probes

(16), and PCR based on 16S rRNA gene sequences (19). However, when Blanchard (1a) performed PCR with the aforementioned urease gene primers on the 14 reference strains obtained from our laboratory, serovars 10 and 12 were classified into biovar 2. In the present study, our PCR results clearly classified serovars 1, 3, 6, and 14 into biovar 1 and the remainder of the serovars into biovar 2. For the clinical isolates, biovar information obtained with our primers concurred with the results obtained by using primers based on the urease gene. Importantly, unlike the biovar determination methods that use PCR amplification of the 16S rRNA and urease genes, which classify samples on the basis of the presence or absence of DNA amplification, our PCR UM-1 detected all serovars and simultaneously differentiated two biovars by the length of their amplification products.

Subdivisions within the genus *Ureaplasma* have been based largely on the host species and antigenic heterogeneity. *U. urealyticum* is the designation for all ureaplasmas isolated from humans. The two distinct biovars of the species are identified by various methods. It is noteworthy that the experiments with PCR analysis of different genes (including rRNA, urease, and MB antigen genes) could differentiate the two biovars, thus showing that biovar specificity exists among the three functionally unrelated genes. These phenomena may reflect the phylogenetic relationship among organisms taxonomically classified as *U. urealyticum*. In fact, the question of whether the two clusters should be separated taxonomically has been raised (19). Our results support the separation of the two biovars into separate subgroups.

It has been postulated that certain serovars of *U. urealyticum* are more likely to be associated with invasive disease. However, serotyping studies of clinical isolates that sought to link virulence with specific serovars have yielded conflicting results (5). Furthermore, the currently available methods of serotyping are both impractical and often unreliable for routine diagnosis (21). Our preliminary data obtained by using 14 reference serovars and a limited number of clinical isolates demonstrated that biovar-specific PCR could differentiate the two biovars without the need for time-consuming cultivation of *U. urealyticum* isolates. The PCR that we described in this report may provide a simple and rapid alternative method to culture for the detection and biotyping of *U. urealyticum* isolates.

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At the time of the study, L.-J.T. was on official leave from the School of Medical Technology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China.

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