Simple Method for Determining Biovar and Serovar Types of *Ureaplasma urealyticum* Clinical Isolates Using PCR–Single-Strand Conformation Polymorphism Analysis

DAVID PITCHER,1* MARGARET SILLIS,2 AND JANET A. ROBERTSON3

Respiratory and Systemic Infection Laboratory, Central Public Health Laboratory, London NW9 5HT;1 and Public Health Laboratory, Norwich NR2 3TX;2 United Kingdom, and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada3

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*Ureaplasma urealyticum* has been associated with urethritis in men, obstetric problems in women, and respiratory distress syndrome in preterm infants. *U. urealyticum* can be divided into two biovars comprising 14 serovars. Partial sequences of genes encoding the multiple-banded antigens of the cell surface are known. Using a commercially available precast DNA mutation detection gel system, we have developed a simple and reproducible PCR–single-strand conformation polymorphism analysis method for differentiating the biovars of this species that reveals five patterns among the 14 serovars and enables clinical isolates to be typed directly from broth cultures.

Ureaplasma urealyticum is a common commensal of the urogenital tract in both men and women. It has been implicated in male nongonococcal urethritis (27) and associated with a number of obstetric conditions, including chorioamnionitis and chronic respiratory distress in neonates, often with poor prognosis (1, 3). Use of polyclonal antiserum raised against whole ureaplasmal cells has identified 14 serovars (22). As shown earlier by DNA hybridization studies on serovars 1 to 8, these can be divided into two clusters or biovars. DNA homology between the biovars was less than 60% and sufficiently different to have separate species suggested for each of the biovars (5). More recently other phenotypic and genotypic traits, including results of restriction fragment analysis (7) and 16S rRNA sequencing (20), and genome size (21) have confirmed this finding. Currently, separate species status is being sought for each of the biovars (J. A. Robertson, personal communication).

Biovar 1 (parvo) consists of serovars 1, 3, 6, and 14, and biovar 2 (T960) consists of serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13 (22). For many years there has been speculation about the association of particular serovars with disease. For example, in one study in which the role of *U. urealyticum* in urinary tract disease was investigated, serotyping identified serovar 6 as the predominant type in urine samples (9). In another study, a statistical association was found between the occurrence of serovar 4 in women who had a history of recurrent abortions compared with healthy pregnant women (16). Despite these findings there has been no conclusive evidence linking a particular serovar with a disease. In part, this could reflect difficulties in interpreting serological typing results. Multiple cross-reactions are common, and clinical samples frequently contain two or more serovars that cannot casually be separated (26).

Robertson et al. (19) were unable to ascribe any correlation between serovars isolated from tissues of subjects experiencing spontaneous and therapeutic abortion.

The difficulty in interpreting the results obtained in these studies was partly due to polyclonal antisera directed at the dominant antigens of the organism. A full set of set of monoclonal antibodies (MAbs) against serovar-specific antigens has recently been developed and established that polyreactivity, a disadvantage of polyclonal serotyping, was not encountered when using MAbs (6). MAbs have been used to identify multiple-banded antigens responsible for serovar specificity on the cell surface (34). Sequence variation in the genes encoding these antigens (mba genes) has been exploited to partially differentiate serovars using combinations of restriction enzymes or DNA amplification with panels of primers (12, 15, 29). However, these methods all require multistep procedures.

Single-strand conformation polymorphism (SSCP) analysis was originally developed to detect allelic variation in human genetic disorders (18). Double-stranded DNA is denatured, and the single-stranded products are separated by gel electrophoresis under accurately controlled, nonnondenaturing conditions. During its migration through the gel, each strand assumes a folded conformation dependent on its internal base pairing and therefore on its sequence.

The migration distances of the conformers visualized as bands on the gel are reproducible and are determined by the structure. Base substitutions or deletions can alter the conformations, causing mobility shifts in the migration patterns and revealing sequence divergence in the specific genomic region amplified. In a small (100- to 200-bp) PCR product, as little as one base change can be detected by this method (2, 4).

Although SSCP analysis has not yet found wide acceptance in bacteriology, it has been used for the detection of rifampin-resistant mutants (*rpoB* gene) of *Mycobacterium tuberculosis* (28) and to subtype *Borrelia burgdorferi* isolates (16S rRNA gene) (31). Bacterial 16S ribosomal DNA (rDNA) from clinical isolates has also been identified using fluorescent primers in
a PCR followed by SSCP analysis in an automated DNA sequencer (30, 32).

Using commercially available precast gels in a horizontal electrophoresis system, we investigated the possibility of genotyping *U. urealyticum* clinical isolates from the gel patterns of single-stranded conformers of PCR-amplified products of their *mba* genes.

**MATERIALS AND METHODS**

**Strains, specimens, and culture conditions.** Stock reference cultures were obtained from the American Type Culture Collection under accession numbers ATCC 27813 (serovar 1), 27814 (serovar 2), 27815 (serovar 3), 27816 (serovar 4), 27817 (serovar 5), 27818 (serovar 6), 27819 (serovar 7), 27618 (serovar 8), 33175 (serovar 9), 33699 (serovar 10), 33695 (serovar 11), 33696 (serovar 12), 33698 (serovar 13), and 33697 (serovar 14). These were maintained in the freeze-dried state and cultured in 4 ml of U4 broth (10).

**DNA extraction.** Cells were harvested by centrifugation of broth cultures at 10,000 × g for 10 min. Pellets were washed twice in sterile phosphate-buffered saline (pH 7.4; Oxoid, Basingstoke, United Kingdom), resuspended by vigorously vortexing in 500 μl of sterilized Chelex 100 suspension (10%, wt/vol, in PCR quality water) (Bio-Rad, Hemel Hempstead, United Kingdom), and incubated in a 56°C water bath for 30 min. The suspensions were vortexed for 20 s, heated at 100°C for 8 min, and rapidly cooled on ice. Finally, samples were vortexed again, centrifuged at 10,000 × g for 5 min (17). The supernatants were transferred to 1 ml of 1 M NaOH) was added; the mixture was vortexed briefly, heated on a PCR heating block at 95°C for 5 min, and immediately cooled on ice; and 5 μl of 50% glycerol added.

**Amplification of mba gene fragment.** In PCR mixtures for SSCP analysis, the forward primer was UMS −120 (5'-TCACATTTATAGTTTGCCT-3') and UMS +46 (5'-CTTAGTAAATGCTCAAATTT-3') and UMA +46 (5'-CCTAAATGTCATAGCMAATT-3'), which were used together to account for degeneracies in bases in this region. Reaction mixtures (50 μl) contained 50 mM KCl, 2.5 mM MgCl₂, 15 mM Tris-Cl (pH 8.0), a 200 μM concentration of each deoxyribonucleotide triphosphate, 20 pmol of each primer and 1.5 U of Taq DNA polymerase (Perkin-Elmer, Warrington, United Kingdom). Chelex extract (10 μl) was added. Samples were overlaid with oil, and 45 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min were carried out, followed by a 10-min extension period. Reactions were performed in an Omnigene thermocycler (Hybaid, Ashford, United Kingdom).

**SSCP gel electrophoresis.** To 10 μl of PCR product, 10 μl denaturing buffer (1 ml of formamide containing 0.25% bromophenol blue, mixed immediately before use with 10 μl of 1 M NaOH) was added; the mixture was vortexed briefly, heated on a PCR heating block at 95°C for 5 min, and immediately cooled on ice; and 5 μl of 50% glycerol added.

The electrophoresis system was set up 1 h before the run. An SEA 2000 tank (Elchrom Scientific, Cham, Switzerland), which possesses a buffer-circulating pump, was filled with 1.5 liters of TAE buffer (30 mM Tris-acetate, 0.75 mM EDTA buffer [pH 8.2]). The external water jacket was connected to a temperature-controlled circulating water bath. The temperature of the circulating buffer was adjusted to 9°C before the MDA 26-lane gels (Elchrom Scientific) were placed in the tank. Before loading the samples, the buffer-circulating pump was turned off. Wells were loaded with 20 μl of ice-cold samples. Electrophoresis was carried out for 30 min at 48 V, the buffer pump was turned on, and electrophoresis continued for a further 17.5 h at 48 V. Gels were stained with SYBR gold (Molecular Probes, Leiden, The Netherlands) (1/10,000 in 10 mM TAE buffer) for 40 min, examined on a transilluminator at 254 nm, and photographed using a SYBR green filter and 667 Polaroid film.

The electrophoresis unit employed in this study enabled 26 samples, including controls to be run simultaneously. The approximate times required for processing 26 cultures were 1 h for DNA extraction, 15 min for preparation of the samples, 18 h for electrophoresis overnight, and 40 min for SYBR gold staining.

**RESULTS**

**SSCP grouping of reference strains.** The extraction of DNA from broth culture sediments using Chelex to suppress inhibitors of Taq DNA polymerase, when applied to both standard strain cultures and clinical isolates, yielded successful PCRs. Careful control of the electrophoresis conditions allowed identification of gel banding patterns which unambiguously differentiated biovars 1 and 2 of *U. urealyticum* directly from the broth cultures of standard strains representing the 14 established serovars of this species and enabled placement of the serovars into five *mba* gene groups. A 100-bp ladder was included to estimate gel-to-gel reproducibility and does not relate to the size of the conformers, whose migration distances are based on the shape of their folded structures rather than their sequence length (Fig. 1). Biovar 1 gave a PCR product of 173 bp from which three conformer band patterns were obtained; serovars 1 and 6 gave distinct patterns, and those of serovars 3 and 14 were identical. There was less discrimination...
among the serovars of biovar 2, where the 217-bp product gave rise to two patterns. Pattern 2A contained serovars 2, 5, 8, and 9, and pattern 2B contained serovars 4, 7, 10, 11, 12, and 13. From these results we predicted that all isolates could be typed to one of the following mba groups: biovar 1 (1, 3/14, and 6) and biovar 2 (2A and 2B).

Correlation of SSCP analysis with serotyping. First we examined the DNA from 18 coded, previously serotyped isolates of the abortion study (19). Their biovars had been predicted based on experience with serotyping, and more recently the isolates had been typed to the biovar level by 16S rRNA gene PCRs (23). PCR-SSCP band patterns for these are shown in Fig. 2. Correlation of the biovar designation between the two types of PCR (16S rRNA and mba gene sequences) and between PCRs and the serotyping results were exact except for strain RH1139 (Table 1). On the basis of serology, RH1139, indicated as carrying serovars 11 and 13 of biovar 2, was placed in biovar 1 by both types of PCR.

For 12 of the other 17 strains, the SSCP analysis and serotyped results were in agreement. The five remaining strains did not show such clear correlation. Isolate RH872 reacted with antiserum to serovar 3 but was identified as mba gene group 1 by SSCP analysis; isolate RH799, which reacted with antisera to serovars 12 and 13 (both in biovar 2), was identified correctly as group 2B by SSCP analysis, but it also reacted with serovar 9 antiserum, which is associated with biovar 2A. Similarly, isolate RH191 reacted with antisera to serovars 3 and 14 as well as 6, but only mba gene group 6 was amplified.

From the SSCP patterns two cultures of mixed biovars (RH297 and RH541) and one (RH479) of mixed mba gene groups within a single biovar were detected. In the case of isolate RH297, serotyping resulted in reactions with antisera to serovars 6 and 13, which belong to biovars 1 and 2, respectively, and which should correlate with mba gene groups 6 and 2B. However, the SSCP analysis showed genes from each biovar but biovar 2 was associated with mba gene group 2A not 2B.

In culture RH541, both serotyping and SSCP analysis identified serovar 3, but SSCP analysis also indicated the presence of biovar 2A genes, and in RH479, for which serotyping revealed only the presence of serovar 4, equivalent to the mba gene group 2B, SSCP analysis also detected the presence of group 2A.

Application of PCR-SSCP analysis to clinical isolates. Secondly, we subjected the deposits from 44 Ureaplasma broth culture isolates from clinical specimens to PCR-SSCP analysis in order to confirm that the five SSCP patterns would cover most isolates. These specimens were randomly selected and consisted of 15 cultures from the tracheal and nasopharyngeal aspirates of neonates, both full term and preterm, and 29 cultures from high vaginal swabs taken from pregnant and

![FIG. 2. PCR-SSCP patterns of serologically confirmed strains of U. urealyticum listed in Table 1. Lane 1, control serovar 1; lanes 2 to 5, RH303, RH313, RH1087, and RH872; lane 6, control serovar 3; lanes 7 to 10, RH297, RH541, RH666, and RH1139; lane 11, control serovar 6; lanes 12 to 14, RH555, RH677, and RH191; lane 15, control serovar 2; lanes 16 to 18, NIH5, T960, and RH479; lane 19, control serovar 4; lanes 20 to 23, RH122, RH507, RH539, and RH799; lane M, 100-bp ladder.](http://jcm.asm.org/)

<p>| Table 1. Correlation of blind-tested PCR-SSCP analysis-determined groups with biovar as determined by PCR of 16S rDNA and serovar and biovar as determined by serotyping |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Serovar(s) or pattern(s) as determined by:</th>
<th>Serotyping&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR</th>
<th>16S rDNA</th>
<th>Biovar</th>
<th>mba gene group</th>
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<td>1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RH313</td>
<td>—&lt;sup&gt;d&lt;/sup&gt;</td>
<td>—</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
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<td>3/14 + A</td>
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<td>1</td>
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<td>1</td>
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<td>2</td>
<td>A</td>
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<td>2</td>
<td>2</td>
<td>A</td>
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<td>B</td>
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<tr>
<td>RH799</td>
<td>9, 12, 13</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>B</td>
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</table>

<sup>a</sup> As reported by Robertson and Stemke (22).
<sup>b</sup> As reported by Jacobs et al. (11) and Robertson et al. (23).
<sup>c</sup> PCR-SSCP gel pattern groups: 1, serovar 1; 3/14, serovars 3 and 14; 6, serovar 6; 2A, serovars 2, 5, 8, and 9; 2B, serovars 4, 7, 10, 11, 12, and 13.
<sup>d</sup> Duplicate of RH303.
<sup>e</sup> Boldface type indicates discrepant results.
from nonpregnant woman (Table 2). All gave easily recognizable patterns that could be assigned to one of the above groups; no mixed groups were detected. All five groups were represented among the isolates.

### DISCUSSION

Genes encoding the multiple banded antigens (mba genes) of *U. urealyticum* have been fully sequenced for biovar 1 (serovar 3; GenBank accession no. L20329) and biovar 2 (serovar 10; GenBank accession no. U50459). The 3′ region of these genes shows marked differences in long stretches of tandem repeat units where serovar specificity is determined (34; X. Zheng, L. J. Teng, H. L. Watson, J. L. Glass, A. Blanchard, and G. H. Cassell, GenBank accession no. L20329 and U50459). Close to the 5′ end of the gene, a 45-bp deletion in the biovar 1 gene accounts for the difference in PCR product size between biovars 1 and 2 (29, 33, 34). PCRs amplifying parts of the first 400 to 500 bases of the 5′ end of the *mba* gene region including 125 bases upstream of the start codon followed by restriction endonuclease typing have enabled the partial differentiation of serovars into groups (29). More detailed sequence data were determined for this region for all 14 serovars by Kong et al. (15), who devised a stepwise system of biotyping and partial serovar identification based on PCR using two primer pairs and followed by restriction enzyme analysis of the products. Knox and colleagues (12, 13) have sequenced the *mba* 5′ regions (nucleotide positions 104 to 207) of 33 clinical isolates of *U. urealyticum* and compared them to the standard serovar sequences. Using a nested PCR with two outer and six inner primers, they defined nine subtypes within the biovar 1 isolates, including two subtypes of serovar 1, five of serovar 3, and two of serovar 6. Biovar 2 could be divided into two subtypes. In certain instances, the differences between subtypes of the same serovar was a single base difference. They also applied random amplified polymorphic DNA analysis (RAPD) to these isolates and, using seven primers, differentiated 13 RAPD subtypes. They concluded that RAPD provided the greatest discrimination as a typing method for *U. urealyticum*.

To date, significant structural and functional differences have not been identified beyond the biovar level, and neither biovars or subdivisions of them have been convincingly correlated with disease states. Unlike RAPD and restriction digest patterns of nested PCR products, PCR-SSCP analysis requires a single set of primers and a single amplification step, making it a more-efficient approach for subdividing large numbers of ureaplasmas. Groups were designated on the basis of the SSCP patterns of serovar reference strains (Fig. 1), and most of the serovars of isolates from the abortion study were broadly in agreement with their SSCP groups. The discrepant results are indicated in Table 1. One isolate (RH1139), when serotyped, belonged to biovar 2, but both 16S rDNA PCR and SSCP analysis placed it in biovar 1. The reason for this anomaly could not be explained. In two other instances where serotyping indicated mixed serovars, only one SSCP pattern was detected. These findings could represent greater precision of the SSCP analysis or changes in relative populations of subtypes in mixed cultures on later passages or the sensitivity of the methodologies.

In three instances, mixed patterns were observed on SSCP gels, but each component could clearly be identified as belonging to one of the designated groups and probably indicating mixed cultures. Although the results of gene-based methods are expected to be more easily reproducible than those of phenotyping, experience has taught us that typing cloned, laboratory-adapted strains is much easier than working with wild-type isolates (26). The serotyped isolates that were *mba* genotyped in this study emphasize this lesson. They had not been cloned; serotyping, biotyping, and PCR-SSCP analysis were not performed on identical cultures. All strains tested in this study provided unambiguous SSCP results, and the method could be used to group clinical isolates as shown in Table 2. However, because these were uncloned, randomly acquired isolates, we did not attempt to correlate the group designation with the pathogenic status of the patient. PCR-SSCP analysis did not detect any mixed groups among these specimens, although mixtures of serovars are commonly encountered on serotyping. In a mixed population of serovars, culture in broth could favor the growth of more-vigorous strains or those present at the highest initial density, and serotyping may reflect this. With PCR, there could be preferential amplification of the most abundant DNA template, resulting in a single group being detected.

Ideally, sequencing the *mba* gene would be the most accurate way of typing isolates, but it is a time-consuming, expensive, and skilled procedure and not a practical proposition for analyzing large numbers of isolates. However, PCR-SSCP analysis could be a convenient way of rapid screening prior to selecting strains for sequencing. For most diagnostic laboratories that are unable to employ sequencing, the procedure could be used routinely to analyze clinical isolates.

The two biovars of *U. urealyticum* can now be readily differentiated by PCRs based upon differences in the 16S rRNA (11, 23), the 16S-23S rRNA intergenic region (8), and the *mba* genes (29).

Genotypic methods based on the *mba* gene are effectively replacing the 14-member-serotyping scheme established with polyvalent antisera (12–15). Unlike MAbs, the hyperimmune sera used for serotyping may contain more than one antibody to each of the multiple antigens present in the whole-cell preparations used as immunogens. It has been shown, for instance, that many antisera contain antibodies to the biovar-specific urease enzyme (25). The infinitely greater complexity of antigen-antibody reactions compared to the variations in nucleotide sequences of a single gene means that complete correlation cannot be expected. In this study, we exploited sequence differences within the *mba* gene both to separate the two biovars and also to indicate subgroups consisting of one or more serovars. A single PCR will thus allow differentiation of

<table>
<thead>
<tr>
<th>Clinical sample or group</th>
<th>No. of strains belonging to indicated serovars</th>
<th>Biovar 1</th>
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<tr>
<td></td>
<td></td>
<td>3/14</td>
<td>2A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>2B</td>
</tr>
<tr>
<td>Babies (nasopharyngeal aspirates)</td>
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<td></td>
<td></td>
<td>4</td>
<td>2</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
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<td>High vaginal swabs</td>
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<tr>
<td></td>
<td></td>
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</table>
U. urealyticum into five groups. Its application to controlled clinical studies may help to further an understanding of ureaplasmal pathogenicity. We propose that the typing of U. urealyticum strains from a wide variety of sources could be achieved more rapidly, more cheaply, and in greater numbers by this technique than by previously described methods. Direct identification of these genotypes in clinical specimens is our next goal.

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REFERENCES