

## INVESTIGATIVE REPORT

Investigation on Biovars and Genotypes of *Ureaplasma urealyticum* in the Cervix in a Chinese Gynecologic Check-up Population and Sex Workers

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*Ureaplasma urealyticum* is a causative agent of non-gonococcal urethritis and is implicated in the pathogenesis of several other diseases. However, *U. urealyticum* is also frequently found in the normal genitourinary tract. To characterize the distribution pattern of biovars and genotypes in normal physical check-up women and in sex workers, cervical swabs taken from 261 physical check-up clients and 98 sex workers were cultured. Positive cultures were further bityped and genotyped by PCR. The data indicate that a) *U. urealyticum* is more frequently isolated in sex workers than in physical check-up women ( $p < 0.001$ ); b) infection with only one genotype (genotype 1, 3 or 6) of biovar 1 is frequently found in physical check-up women; c) biovar 2 infection and mixed infection caused by more than one genotype of biovar 1 are more prevalent in sex workers than in physical check-up women ( $p < 0.001$  and  $p < 0.01$ , respectively); d) no difference in distribution of genotype 1, 3 and 6 of biovar 1 is found between sex workers and physical check-up women ( $p = 0.396$ ); e) the PCR method described here is relatively simple, rapid and specific for the bityping between biovar 1 and 2 and genotyping of genotypes 1, 3, 6, and 14 in biovar 1. **Key words:** biovar; genotype; *Ureaplasma urealyticum*.

(Accepted December 4, 2002.)

Acta Derm Venereol 2003; 83: 175–178.

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*Ureaplasma urealyticum* is a causative agent of non-gonococcal urethritis and is implicated in the pathogenesis of several other diseases including chorioamnionitis, chronic urinary symptoms in women, preterm birth and chronic neonatal lung diseases (1, 2). However, *U. urealyticum* is also commonly found in the healthy human genitourinary tract. According to several studies, the finding rate of *U. urealyticum* in the female cervix or vagina is from 40% to 80% (3). Its pathogenic role in some individuals is therefore difficult to prove. Whether antibiotic treatment of such individuals is necessary needs to be considered.

*U. urealyticum* is divided into two biovars and 14

genotypes. The majority of human isolates of *U. urealyticum* belong to biovar 1, which includes genotypes 1, 3, 6 and 14. Biovar 2 is less commonly isolated and includes the remaining 10 genotypes. Biovar 1 of *U. urealyticum* has been suggested to represent a new species called *U. parvum*. Although isolation of *U. urealyticum* from clinical samples is relatively easy because of the development of commercial selective media, further genotyping is still difficult because of the problems associated with the traditional genotyping methods. A rapid molecular method for the identification of biovars and genotypes of the microorganism would be of great value in studies of epidemiology and pathogenesis of infections with *U. urealyticum*.

## MATERIALS AND METHODS

*U. urealyticum* strains

The reference strains used were *U. urealyticum* genotype 1 (ATCC 27813), genotype 2 (ATCC 27814), genotype 3 (ATCC 27815), genotype 4 (ATCC 27816), genotype 5 (ATCC 27817), genotype 6 (ATCC 27818), genotype 7 (ATCC 27819), genotype 8 (ATCC 27618), genotype 9 (ATCC 33175), genotype 10 (ATCC 33699), genotype 11 (ATCC 33695), genotype 12 (ATCC 33696), genotype 13 (ATCC 33698) and genotype 14 (ATCC 33697).

*Clinical specimens*

Cervical swabs from 98 female sex workers (mean age  $24.8 \pm 5.7$ ) and 261 female clients (mean age  $39.2 \pm 7.3$ ) attending the Physical Check-up Clinic of Peking University First Hospital were collected (Table I). Cultures were performed immediately using a commercial *U. urealyticum* selective culture kit (BioMerieux, France). DNA from positive cultures was prepared for the biovar and genotype identification.

Table I. Characteristics of sex workers and physical check-up clients.

	Sex workers	Physical check-up clients
Total number	98	261
Mean age (years)	$24.8 \pm 5.7$	$39.2 \pm 7.3$
Range (years)	16–42	26–72
<20 years ( <i>n</i> )	26	0
21–30 years ( <i>n</i> )	57	29
31–40 years ( <i>n</i> )	14	125
41–50 years ( <i>n</i> )	1	95
>51 years ( <i>n</i> )	0	12

### Oligonucleotide primers

Biovar specific primers UMS125 (5'-GTA TTT GCA ATC TTT ATA TGT TTT CG), UMA226 (5'-CAG CTG ATG TAA GTG CAG CAT TAA ATT C) based on a previously published sequence (4) were used for the amplification of the 5' end of MBA (multiple-banded antigen) gene. Genotype specific primers for biovar 1 were designed according to the slight differences of the 5' end of the MBA gene among genotypes 1, 3, 6, and 14 (5). The sequences of these primers were as follows: UMSu1 (5'-TTA CTG TAG AAA TTA TGT AAG ATT GC), UMSu3 (5'-ACT GTA GAA ATT ATG TAA GAT TAC C), UMSu6 (5'-CTT AGT GTT CAT ATT TTT TAC TAG), UMSu14 (5'-ATT ACT GTA GAA ATT ATG TAA GAT TAA), UMA269 (5'-CTA AAT GAC CTT TTT CAA GTG TAC), and UMA269' (5'-CCA AAT GAC CTT TTG TAA CTA GAT).

### DNA preparation

The preparation included 0.5 ml bacterial cultures, 1.0 ml lysis buffer (4 M guanidine thiocyanate, 50 mM Tris-HCl pH8.0, 20 mM EDTA pH8.0, 1% Triton X-100) and 5 µl silica (sigma) mixed for 5 min at room temperature. Silica was washed three times with 0.5 ml washing buffer (200 mM NaCl, 10 mM EDTA pH8.0, 40 mM Tris-HCl pH 8.0, 50% ethanol). DNA was eluted from silica with 30 µl TE (10 mM Tris-HCl pH8.0, 1mM EDTA pH8.0).

### PCR

The PCR mixture contained 2.5 µl of 10× PCR buffer, 0.25 µl Taq polymerase (5 u/µl), 1 µl dNTP (2.5 mM), 2 µl of primer (5 pmol/µl each) and 2 µl of sample DNA in a total volume of 25 µl. In each reaction, positive and negative controls were processed in parallel with the tested samples to detect any possible inhibition or contamination.

The PCR condition used was 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min for 32 cycles. The PCR product was analyzed by electrophoresis on a 2.0% agarose gel containing 0.5 µg/ml ethidium bromide. A visible band of appropriate size on UV transillumination was considered to be a positive result.

### Identification of biovars and genotypes by PCR

Biovar specific primers UMS125 and UMA226 were used to amplify DNA from reference (ATCC) strains. Genotype specific primers of biovar 1 were used to amplify genotypes 1, 3, 6 and 14. Clinical samples were cultured for *U. urealyticum* first. DNA samples from positive isolates were tested using primers UMS125 and UMA226 and divided into two biovars. Biovar 1 positive samples were further examined and divided into genotypes 1, 3, 6 and 14.

## RESULTS

### Biotyping and genotyping of *U. urealyticum*

To identify the usefulness of biotyping and genotyping of *U. urealyticum* by PCR, we used DNA samples from the 14 reference strains as the PCR template. The amplified size of the 5' end of the MBA gene by PCR using primers UMS125 and UMA226 were 403 bp (or 404 bp for genotype 6) for biovar 1 strains and 448 bp for biovar 2 strains. Biovar specific primers allowed a clear distinction between biovar 1 (genotypes 1, 3, 6 and 14) and biovar 2 (genotypes 2, 4, 5, 7, 8, 9, 10, 11,

12 and 13) (Fig. 1). In genotyping of biovar 1 reference strains, the sizes of PCR products were 399 bp from genotype 1 (using primers UMSu1 and UMA269'), 397 bp from genotype 3 (using primers UMSu3 and UMA 269), 369 bp from genotype 6 (using primers UMSu6 and UMA269'), and 400 bp from genotype 14 (using primers UMSu14 and UMA269) (Fig. 2). These 4 pairs of primers were specific for the identification of genotypes 1, 3, 6 and 14. There was never any cross-reaction of positive PCR results between the two biovars and among the 4 genotypes.

### Detection of *U. urealyticum* from the physical check-up clients and the sex workers

Cervical swabs from 261 physical check-up clients were cultured and tested. Of these, 159 women (60.9%) were

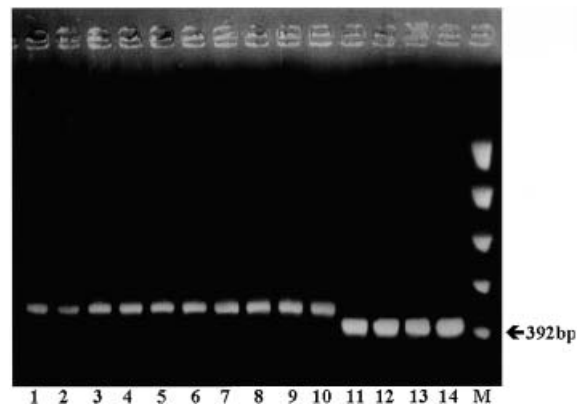


Fig. 1. Results of PCR amplification of MBA genes of all 14 genotypes of *U. urealyticum* with primers UMS125 and UMA226. Positive results are shown by 403 bp band for genotype 1, 3, 6, 14 (lanes 11–14) and 448 bp band for genotype 2, 4, 5, 7, 8, 9, 10, 11, 12, 13 (lanes 1–10). M: size marker.

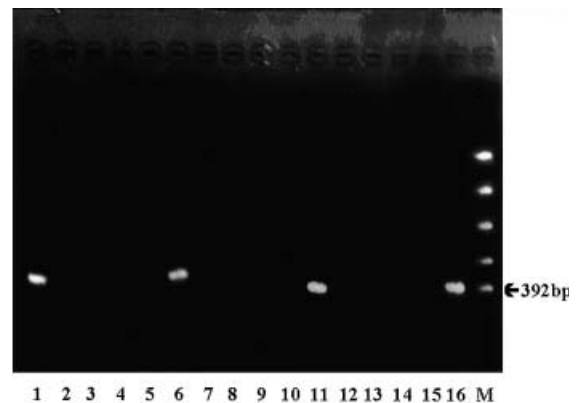


Fig. 2. Results of PCR amplification of MBA (multiple-banded antigen) genes of genotypes 1, 3, 6 and 14 of *U. urealyticum* with genotype-specific primers respectively. Positive results are shown by 399 bp band for genotype 1 (lanes 1–4); 397 bp band for genotype 3 (lanes 5–8); 369 bp band for genotype 6 (lanes 9–12); and 400 bp for genotype 14 (lanes 13–16) in PCR reactions of genotypes 1, 3, 6 and 14 reference strains with genotype specific primers, respectively. M: size marker.

positive, with biovar 1 from 151 women (95%) and biovar 2 from 8 women (5%). Genotyping of the *U. urealyticum* from the 151 positive cases with biovar 1 was as follows: genotype 1 in 30 (19.9%); genotype 3 in 62 (41.1%); genotype 6 in 44 (29.1%); genotype 14 in 2 (1.3%) and mixed infection caused by more than one genotype in 13 (8.6%). The biovar 1 genotypes 1, 3 and 6 were the dominant strains seen in the physical check-up population. Most of them were infected with only one strain of *U. urealyticum* (91.4%). No relationship was found between the presence of *U. urealyticum* isolates and the clinical manifestations of cervical erosion, cervicitis or bacterial vaginosis (data not shown).

Cervical swabs from 98 female sex workers were cultured and tested. Eighty-nine cases (90.8%) harbored *U. urealyticum*, of whom biovar 1 was isolated from 64 cases (71.9%), biovar 2 from 18 cases (20.2%) and biovar 1+2 from 7 cases (7.9%). Genotyping of the biovar 1 from the 71 positive cases showed genotype 1 in 17 cases (23.9%), genotype 3 in 22 (31.1%), genotype 6 in 15 (21.1%) and mixed infection in 17 cases (23.9%).

#### Comparison of biotyping and genotyping of *U. urealyticum* between physical check-up women and sex workers

Although these two groups were not age-matched and sex workers were significantly younger than physical check-up clients, the key issue we considered in this study was the high-risk sex exposure since *U. urealyticum* infection is closely related to sexual activity after sexual maturity. We therefore compared these two groups to elucidate the situation concerning *U. urealyticum* infection in sex workers and physical check-up women.

Infection of more than one genotype of biovar 1 was defined as mixed infection, and that of one genotype of biovar 1 was defined as single infection. The comparison of biotype and genotype between the physical check-up women and sex workers is presented in Table II. *U. urealyticum* was isolated much more commonly from sex workers than from the physical check-up women ( $p < 0.001$ ). The isolation rates of biovar 2 and mixed infection were significantly higher in sex workers than in the physical check-up women ( $p < 0.001$  and  $p < 0.01$  respectively). There was no statistical difference in the distribution of genotypes 1, 3, and 6 of biovar 1 between the sex workers and physical check-up women.

## DISCUSSION

The isolation rate of *U. urealyticum* in neonatal babies has been reported to be about 50% (6), the colonization rate decreasing rapidly after 3 months. *U. urealyticum* was not pathogenic to infants under normal circumstances. The isolation rate was less than 10% in children and teenagers without sexual experience. After adolescence

Table II. Comparison of biovar and genotype of *U. urealyticum* between sex workers and physical check-up women.

	Group n (%)		p value <sup>a</sup>
	Sex workers	Physical check-up women	
Culture			
Negative	9 (9.2)	102 (39.1)	<0.001
Positive	89 (90.8)	159 (60.9)	
Biovar <sup>b</sup>			
1	71 (74.0)	151 (95.0)	<0.001
2	25 (26.0)	8 (5.0)	
Infections <sup>c</sup>			
Single	54 (76.1)	138 (91.4)	<0.01
Mixed	17 (23.9)	13 (8.6)	
Genotype <sup>d</sup>			
1	17 (31.5)	30 (22.1)	0.396
3	22 (40.7)	62 (45.6)	
6	15 (27.8)	44 (32.4)	

<sup>a</sup>  $\chi^2$  test.

<sup>b</sup> Culture positive cases were divided into biovars. Seven cases in the sex workers' group were found to carry both biovar 1 and biovar 2.

<sup>c</sup> Biovar 1 positive cases were divided into genotypes. More than one genotype of biovar 1 found in a case was defined as mixed infection.

<sup>d</sup> Two cases of genotype 14 were excluded from the physical check-up women.

and puberty, the isolation rate increased gradually. In pregnant women, the isolation rate of *U. urealyticum* in the genital tract was about 60%. In this study, 60.9% of cases in the physical check-up group carried *U. urealyticum*, of which 95% were biovar 1 and 5% were biovar 2. All genotypes of biovar 1, i.e., genotypes 1, 3, 6 and 14, were found in this group, but genotype 14 was isolated only occasionally. The distribution pattern of genotypes is consistent with that found in other studies, in which *U. urealyticum* isolates were genotyped using traditional methods (7, 8). For example, Naessens et al. (8) showed that more than 90% of isolations were identified as biovar 1, of which 52.2% were genotype 3, 30.3% were genotype 6, and 9.5% were genotype 1. The physical check-up clients we observed in this study were mostly civil servants with a good education and in good economic circumstances. Following the traditional Chinese conception of sex, these women were expected to be conservative in their sexual habits. There was no statistical association between the isolation of *U. urealyticum* and genital tract symptoms and signs, and most strains of *U. urealyticum* found in physical check-up women were genotypes 1, 3 and 6 of biovar 1. Therefore, these genotypes of *U. urealyticum* may be considered as normal bacterial flora. Few isolates of biovar 2 were found.

Compared with the physical check-up clients, the sex workers had a lower standard of education, lived in poor sanitary conditions and seldom used condoms during sex trade. The colonization rate of *U. urealyticum* and the distribution pattern of biovars and genotypes in sex workers were different from those in physical

check-up women. Although the isolation rate of *U. urealyticum* was high (60.9%) in physical check-up women, the rate was even higher (90.8%) in sex workers ( $p < 0.001$ ). This implies that *U. urealyticum* is more likely to be found in the genital tract and may sometimes become pathogenic in this group. The rate of biovar 2 isolation (26.0%) was approximately 5 times higher than that in physical check-up women (5.0%) ( $p < 0.001$ ). The rate of mixed infection caused by more than one genotype of biovar 1 (23.9%) was approximately 3 times higher than that in physical check-up women (8.6%) ( $p < 0.01$ ). In any case, there was no statistical difference in the distribution of genotypes 1, 3 and 6 of biovar 1 between sex workers and physical check-up women.

Selective media for *U. urealyticum* are currently widely used in the clinic and show excellent sensibility and specificity. However, more information about biovars and genotypes is required for clinical diagnosis and treatment because of the high isolation rate in healthy women. Traditional methods in biotyping and genotyping of *U. urealyticum* are time-consuming and not suitable for clinical practice. With the understanding of gene differences between biovars, we can readily distinguish between the two biovars of *U. urealyticum* using various molecular methods (9–11). However, genotyping by molecular method has not been routinely used due to the lack of information about gene differences among genotypes. Multiple-banded antigen (MBA) is a major antigen recognized by the host during *U. urealyticum* infection and is probably an important virulence determinant. MBA contains the species-specific and serotype-specific epitopes (12). The 5' end of the MBA gene is relatively well conserved and encodes the transmembrane domain. The sequences of the MBA gene of the 14 genotypes are known. By using the slight nucleic acid differences among genotypes, genotypes of biovar 1 can readily be distinguished by PCR. In this study, we developed a method of rapid preparation of *U. urealyticum* DNA from culture broth and that of biotyping and genotyping based on PCR, which can be widely used in the study of epidemiology and pathogenesis of *U. urealyticum* infection. The PCR method described here is relatively simple, rapid, practical and specific, and can also be used in the biotyping and genotyping of *U. urealyticum* in clinical laboratories. Kong et al. (13) divided biovar 2 into 3 subtypes further. Since biovar 2 is related to high-risk sex exposure in our study, a simple and accurate method for the genotyping of biovar 2 is required for clinical diagnosis.

In conclusion, biotyping and genotyping of *U. urealyticum* are useful in clinical diagnosis and epidemiological studies. One of the genotypes of *U. urealyticum* biovar 1, genotype 1, 3 or 6, is

frequently found in cervix of physical check-up women. Biovar 2 infection and mixed infection caused by more than one genotype of biovar 1 are more prevalent in sex workers than in physical check-up women ( $p < 0.001$  and  $p < 0.01$ , respectively). However, no statistical difference was found in the distribution of genotypes 1, 3 and 6 between sex workers and physical check-up women.

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