***Ureaplasma urealyticum* Biovar Determination in Women Attending a Family Planning Clinic in Guiné-Bissau, Using Polymerase Chain Reaction of the Multiple-Banded Antigen Gene**

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Although *Ureaplasma urealyticum* is commonly found in the genital tract of asymptomatic women, it has been suggested that only certain subgroups of this microorganism are disease associated. Vaginal specimens were collected to determine the distribution of *U. urealyticum* biovars and to estimate their possible association with age, absence of lactobacilli, and tetracycline resistance. Of the 94 women studied, 40 (43%) carried *U. urealyticum* and were biotyped by polymerase chain reaction (PCR). Twenty-nine (73%) strains presented with parvo biovar, 10 (25%) with T960 biovar, and one (2.5%) with both biovars. Parvo biovar was

predominant in all age groups and appears to be more frequent in women 20–25 years of age (41%), whereas T960 was common in women 30–35 years of age (22%). In this study, *U. urealyticum* was not associated with changes in vaginal flora, although the inverse apparently was true for *Mycoplasma hominis*. However, T960 biovar was more associated with the loss of lactobacilli than was parvo biovar. The number of T960 biovar strains that presented tetracycline (40%) or multiple (100%) resistance was higher than that of parvo biovar strains (27% and 69%, respectively). J. Clin. Lab. Anal. 16:71–75, 2002. © 2002 Wiley-Liss, Inc.

Key words: parvo biovar; T960 biovar; lactobacilli; antibiotic; resistance

INTRODUCTION

Ureaplasma urealyticum is a common commensal microorganism in the genital tract of sexually mature women (1–4). However, this agent is strongly associated with genitourinary syndromes and with complications in pregnancy such as chorioamnionitis, preterm birth, respiratory disease, meningitis, pneumonia, and bronchopulmonary dysplasia. It is the most common microorganism isolated from the central nervous system and the lower respiratory tract of newborn infants (5–10). 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 disease associated (11–14).

There are 14 widely accepted serovars of *U. urealyticum*, which can be divided into two distinct clusters, or “biovars” (15–17): biovar 1 or parvo biovar, and biovar 2 or T960. The first cluster includes four serovars (1, 3, 6, and 14), and the second the remaining 10 serovars (17,18).

The two *U. urealyticum* biovars may be differentiated by restriction fragment length polymorphism (RFLP), polypep-

tide patterns from one- or two-dimensional gel electrophoresis, sensitivity to Mn²⁺, enzyme profiles, DNA-DNA homology, restriction endonuclease patterns, DNA modification system, and genome size. They can also be differentiated by PCR amplification of specific genes, such as the 16S rRNA, urease, and multiple-banded antigen genes (3,4,11,15).

The assay employed in the present work used primers directed against the 5' region of the MB antigen gene (as described previously by Teng et al. (11)). This is the predominant antigen recognized by patients infected with *U. urealyticum* (11–13,17,19,20). The advantages of this assay

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are that it not only detects all 14 serovars of *U. urealyticum* in a single PCR reaction, but it also differentiates biovar 1 from biovar 2 strains (403 vs. 448 bp, respectively) and does not detect any product from the other 17 mycoplasma species, including those that are closely phylogenetically related (11,12,16,17,21).

The aims of this study were to: 1) determine the distribution of the two biovars among patients attending a family-planning clinic in Bissau Guiné-Bissau; 2) estimate possible associations with age, sexual activity, and changes in vaginal flora (absence of lactobacilli); and 3) examine the relationship between tetracycline resistance and the two biovars.

MATERIAL AND METHODS

Reference Strains

Serotype standard strains 3 and 8 were obtained from the American Type Culture Collection (ATCC) and used as a positive control.

Clinical Specimens

Ninety-four women attending a family-planning clinic in Guiné-Bissau were included in the study. After informed consent was obtained, they all answered a standard questionnaire and provided a medical history. A clinical observation was performed and vaginal swabs were taken from every woman.

Culture of *U. urealyticum*

The vaginal swabs were placed in 2SP transport medium until they were transported to the laboratory. Then 200 µl of inoculated transport media were added to 1.8 ml of Shepard 10 B broth (22) and 20 µl of this mixture were inoculated in an Agar A7 plate (22,23). Incubation was at 37°C in aerobic atmosphere for broths, and 5% CO₂ for plates. Broths showing a pH shift (color change from yellow to red) were treated for PCR. Plates were read to confirm the presence of brown colonies, which are typical of *U. urealyticum*. Antibiotic susceptibility was ascertained using the Mycoplasma IST Kit (BioMérieux, Marcy-L'Etoile, France).

DNA Preparation

Samples were prepared as previously described (2,24). Briefly, 250 µl of each positive culture were centrifuged (12,000 g for 20 min at 4°C). The pellet was first resuspended in 50 µl of solution A (10 mM Tris-HCl, pH 8.3, containing 100 mM KCl and 2.5 mM MgCl₂) and then in 50 µl of solution B (10 mM Tris-HCl, pH 8.3, containing 2.5 mM MgCl₂, 1% Tween 20, 1% Triton X-100, and 120 µl/ml proteinase K). Cell suspensions were then incubated for 60 min at 60°C and heated at 95°C for 10 min, and finally 5 µl were taken from each sample for PCR. The remaining DNA solutions were frozen at -80°C.

PCR

The conditions used to test the study specimens were as follows: 50 µl reaction mixtures were set up, which consisted of 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 µl of each deoxynucleotide triphosphate (dATP, dCTP, dTTP, and dGTP (GIBCO BRL, Gaithersburg, MD)), 2.5 U Taq DNA polymerase (Pharmacia Biotech, Upsala, Sweden), and 30 pmol of each primer (UMS 125 (GTA TTT GCA ATC TTT ATA TGT TTT CG) and UMA 226 (CAG CTG ATG TAA GTG CAG CAT TAA ATT C)), synthesized by GIBCO BRL. The reaction mixtures were covered with mineral oil and subjected to an initial denaturation at 95°C for 4 min; then 40 cycles at 95°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec; and a final elongation at 72°C for 3 min. Negative and positive controls were also included (11,16,17).

PCR Products Detection

After PCR was performed, 15 µl of each sample were subjected to electrophoresis through a 1.5% agarose gel and stained with 0.3 µg/ml ethidium bromide in TBE (all Pharmacia Biotech) at 80 V for 1 hr 30 min. A DNA product band of 403 bp (parvo biovar) or 448 bp (T960 biovar) in size, as visualized by UV illumination, was considered positive.

RESULTS

Of the 94 women examined, 40 (43%) were colonized by *U. urealyticum*. All specimens found to be positive for *U. urealyticum* were biotyped. Twenty-nine (72.5%) presented with parvo biovar, 10 (25%) with T960 biovar, and one (2.5%) with both biovars. Figure 1 shows some of the amplified products.

When the *U. urealyticum* biovars were related to age (Fig. 2), we observed that four of 12 women (33%) less than 20

Fig. 1. PCR results obtained with primers UMS-125 and UMA-226. Lane 2: Molecular weight marker (50 bp marker from GIBCO BRL). Lane 3: Negative control. Lane 4: Parvo biovar positive control (403 bp). Lane 5: T960 biovar positive control (448). Lanes 7–10, 12, and 13: Positive samples for parvo biovar. Lanes 6 and 11: Positive samples for T960 biovar.

Fig. 2. *U. urealyticum* biovars distribution by age.

years of age were colonized with *U. urealyticum* biovar 1, while one (8%) carried biovar 2. *U. urealyticum* biovar 1 was present in seven of 17 women (41%) 20–25 years of age, biovar 2 in two (12%), and biovars 1 and 2 in one (6%). Four of 14 women (29%) 25–30 years of age were colonized with *U. urealyticum* biovar 1, and three (21%) had *U. urealyticum* biovar 2. Three of nine patients (33%) 30–35 years of age were shown to be colonized with *U. urealyticum* biovar 1, while two (22%) carried *U. urealyticum* biovar 2. In four of 19 women (21%) over 35 years of age, *U. urealyticum* biovar 1 was isolated, while two (11%) had *U. urealyticum* biovar 2. Parvo biovar was the most frequently found in all age groups.

Changes in vaginal flora (absence of lactobacilli) and its relationship with genital mycoplasmas were also studied (Table 1). *Mycoplasma hominis* was detected in three of 20 females (15%) with normal flora, and in 11 of 49 (22%) with abnormal flora (absence of lactobacilli). *U. urealyticum* was present in five of 20 women (25%) without alterations in vaginal flora, while three in 49 (6%) had absence of lactobacilli. Thirty-five percent (7/20) of women who carried both mycoplasmas had normal vaginal flora, whereas 45% (22/49) of females with absence of lactobacilli had both mycoplasmas.

The association between absence of lactobacilli and *U. urealyticum* biovars was also studied (Table 2).

Parvo biovar was found in 10/12 (83%) of women who had normal vaginal flora, and in 19/28 (68%) of patients with absence of lactobacilli. T960 biovar was found in 2/12 (17%) of females with lactobacilli and in 8/28 (29%) of those who did not have lactobacilli.

Figure 3 shows the relationship between biovars and tetracycline resistance. Of the 29 women colonized with *U. urealyticum* parvo biovar, eight (27%) were resistant to tetracycline, while four (14%) presented intermediate resistance

TABLE 1. Relationship between vaginal flora changes and genital mycoplasmas

Vaginal flora	Genital mycoplasmas		
	<i>M. hominis</i>	<i>U. urealyticum</i>	Both
Normal	3/20 (15%)	5/20 (25%)	7/20 (35%)
Absence of lactobacilli	11/49 (22%)	3/49 (6%)	22/49 (45%)

TABLE 2. Relationship between absence of lactobacilli and *U. urealyticum* biovars

Vaginal flora	<i>U. urealyticum</i> biovars		
	Parvo biovar	T960	Both
Normal	10/12 (83%)	2/12 (17%)	0/12 (0%)
Absence of lactobacilli	19/28 (68%)	8/28 (29%)	1/28 (3.5%)

and 17 (59%) were susceptible to this antibiotic. Of the 10 women in whom *U. urealyticum* T960 biovar was identified, four (40%) were resistant to tetracycline, one (10%) had intermediate resistance, and five (50%) were susceptible. Full resistance was observed in the woman who had both biovars (data not shown).

Resistance to more than one antibiotic was also observed. Of the 29 females with biovar 1, 20 (69%) showed resistance to more than one antibiotic. Women with biovar 2 or both biovars (100%) presented resistance to more than one antibiotic studied (tetracycline, erythromycin, and ofloxacin).

DISCUSSION

Although a relationship between *U. urealyticum* and specific clinical syndromes was hypothesized previously (24,27–29), it has not been clearly demonstrated. In this study, we decided to determine the distribution of the *U. urealyticum* biovars—parvo and T960—among women in Guiné-Bissau, and to estimate their possible association with various factors such as age, absence of lactobacilli, and tetracycline resistance. As in previous studies (25–27), parvo biovar was the most frequently found. The frequency of parvo biovar was maximal in the 21–25 years age group. It decreased slowly after that, while the frequency of T960 biovars was lower in the same age group. This is also in accordance with previous observations (2) that parvo biovar is found more frequently in younger women. We also tried to establish a relationship between genital mycoplasmas and *U. urealyticum* biovars with absence of lactobacilli. Although not statistically significant, an association was found between *M. hominis* and absence of lactobacilli. This observation was not surprising, since *M. hominis* is a well known agent of bacterial vaginosis (BV)

Fig. 3. Relationship between *U. urealyticum* biovars and tetracycline resistance.

(30), and the absence of lactobacilli is an indicator of BV. No explicit relationship was observed between *U. urealyticum* and the absence of lactobacilli, which indicates that the absence of lactobacilli among women colonized by both microorganisms was probably exclusively due to *M. hominis*. Other authors have associated *U. urealyticum* with BV, but no studies have been undertaken relating the presence of *U. urealyticum* to the absence of lactobacilli (26,31). Further studies are required to determine whether *U. urealyticum* is really associated with BV or only with the absence of lactobacilli. Furthermore, it should be clarified whether this association is with *U. urealyticum* only, when both *M. hominis* and *U. urealyticum* are found in the same woman. Although in our study *U. urealyticum* was not found to be associated with absence of lactobacilli, we decided to evaluate whether one of the biovars was more frequently associated with that condition than the other. In our study, T960 was found to be the most frequent biovar associated with absence of lactobacilli.

Both biovars of *U. urealyticum* presented high levels of resistance to tetracycline. However, T960 was found to be the most resistant. This observation is important, since tetracycline is the antibiotic of choice for the treatment of infections caused by these microorganisms. Resistance to multiple antibiotics was common in both biovars, with all T960 strains being multiple-resistant to the antibiotics tested. These results are similar to those obtained by Abele-Horn et al. (2). In conclusion, the results obtained in this study were mostly in accordance with those published by others (2,26,27,29). Parvo biovar was the most frequently found biovar, while T960 appears to be more disease associated. Since the absence of lactobacilli related to a high pH, and *M. hominis* grows better at pH = 7.4, further studies should be undertaken to clarify the role of vaginal pH on the growth of this organism.

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REFERENCES

- Cunliffe NA, Fergusson S, Davidson F, Lyon A, Ross PW. Comparison of culture with the polymerase chain reaction for detection of *Ureaplasma urealyticum* in endotracheal aspirates of preterm infants. *J Med Microbiol* 1996;45:27–30.
- Abele-Horn M, Wolff C, Dressel P, Pfaff F, Zimmermann A. Association of *Ureaplasma urealyticum* biovars with clinical outcome for neonates, obstetric patients, and gynaecological patients with pelvic inflammatory disease. *J Clin Microbiol* 1997;35:1199–1202.
- Harasawa R, Kanamoto Y. Differentiation of two biovars of *Ureaplasma urealyticum* based on 16S-23S rRNA intergenic spacer region. *J Clin Microbiol* 1999;37:4135–4138.
- Echahidi F, Muyldermans G, Lauwers S, Naessens A. Development of monoclonal antibodies against *Ureaplasma urealyticum* serotypes and their use for serotyping clinical isolates. *Clin Diagn Lab Immunol* 2000;7:563–567.
- Taylor-Robinson D, Furr PM. Update on sexually transmitted mycoplasmas. *Lancet* 1998;351(Suppl III):12–15.
- Davis JK, Crouse DT, Robertson JA, Davidson MK, Brown MB, Reyes L. The role of *Ureaplasma urealyticum* in human reproductive and neonatal diseases. *Infect Dis Rev* 1999;1:200–207.
- Knox C, Timms P. Comparison of PCR, nested PCR, and random amplified polymorphic DNA PCR for detection and typing of *Ureaplasma urealyticum* in specimens from pregnant women. *J Clin Microbiol* 1998;36:3032–3039.
- Kong F, Zhenfang Ma, James G, Gordon S, Gilbert GL. Molecular genotyping of human *Ureaplasma* species based on multiple-banded antigen (MBA) gene sequences. *Int J Syst Evol Microbiol* 2000;50:1921–1929.
- Cordova CMM, Cunha RAF. Relevant prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum* serogroups in HIV-1 infected men without urethritis symptoms. *Rev Inst Med Trop S Paulo* 2000;42:185–188.
- Cordova CMM, Cunha RAF. Importância da pesquisa de micoplasmas no trato urogenital. *Laes Haes* 2000;121:110–124.
- Teng L-J, Zheng X, Glass JI, Watson HL, Tsai J, Cassel GH. *Ureaplasma urealyticum* biovar specificity and diversity are encoded in multiple-banded antigen gene. *J Clin Microbiol* 1994;32:1464–1469.
- Zheng X, Teng L-J, Watson HL, Glass JI, Blanchard A, Cassel GH. Small repeating units within the *Ureaplasma urealyticum* MB antigen gene encode serovar specificity and are associated with antigen size variation. *Infect Immunol* 1995;63:891–898.
- Zheng X, Lau K, Frazier M, Cassel GH, Watson HL. Epitope mapping of the variable repetitive region within the MB antigen of *Ureaplasma urealyticum*. *Clin Diagn Lab Immunol* 1996;3:774–778.
- Kong F, James G, Zhenfang Ma, Gordon S, Bin W, Gilbert GL. Phylogenetic analysis of *Ureaplasma urealyticum*—support for the establishment of a new species, *Ureaplasma parvum*. *Int J Syst Bacteriol* 1999;49:1879–1889.
- Grattard F, Pozzetto B, Barbeyrac B, Renaudin H, Clerc M, Gaudin OG, Bébérar C. Arbitrarily-primed PCR confirms the differentiation of strains of *Ureaplasma urealyticum* into two biovars. *Mol Cel Probes* 1995;9:383–389.
- Nelson S, Matlow A, Johnson G, Th'ng C, Dunn M, Quinn P. Detection of *Ureaplasma urealyticum* in endotracheal tube aspirates from neonates by PCR. *J Clin Microbiol* 1998;36:1236–1239.
- Kong F, Zhu X, Wang W, Zhou X, Gordon S, Gilbert GL. Comparative analysis and serovar-specific identification of multiple-banded antigen genes of *Ureaplasma urealyticum* biovar 1. *J Clin Microbiol* 1999;37:538–543.
- Ruifu Y, Minli Z, Guo Z, Wang X. Biovar diversity is reflected by variation of genes encoding urease of *Ureaplasma urealyticum*. *Microbiol Immunol* 1997;41:625–627.
- Watson HL, Blalock DK, Cassell GH. Variable antigens of *Ureaplasma urealyticum* containing both serovar-specific and serovar-cross-reactive epitopes. *Infect Immunol* 1990;58:3679–3688.
- Zheng X, Watson HL, Waites KB, Cassel GH. Serotype diversity and antigen variation among invasive isolates of *Ureaplasma urealyticum* from neonates. *Infect Immunol* 1992;60:3472–3474.
- Cassell GH, Waites KB, Watson HL, Crouse AT, Harasawa R. *Ureaplasma urealyticum* intrauterine infection: role in prematurity and disease in newborns. *Clin Microbiol Rev* 1993;6:69–87.
- Blanchard A, Hentschel J, Duffy L, Baldus K, Cassel GH. Detection of *Ureaplasma urealyticum* by polymerase chain reaction in the urogenital tract of adults, in amniotic fluid, and in the respiratory tract of newborns. *Clin Infect Dis* 1993;17(Suppl I):S148–S153.
- Waites KB, Bébérar CM, Robertson JA, Talkington DF, Kenny GE. Cumitech 34. In: Nolte FS, editor. Laboratory diagnosis of mycoplasma infections. Washington, D.C.: American Society for Microbiology; 2001. p 1–129.
- Clegg A, Passey M, Yoannes M, Michael A. High rates of genital myco-

- plasma infection in the highlands of Papua New Guinea determined both by culture and by a commercial detection kit. *J Clin Microbiol* 1997;35:197–200.
25. Clerc MT, Renaudin H, Barbeyrac B. PCR, a useful tool for biovar determination in *Ureaplasma urealyticum*. *IOM Lett* 1994;3:326–327.
26. Povlsen K, Thorsen P, Lind I. Relationship of *Ureaplasma urealyticum* biovars to the presence or absence of bacterial vaginosis in pregnant women and to the time of delivery. *Eur J Clin Microbiol Infect Dis* 2001;20:65–67.
27. Povlsen K, Jensen JS, Lind I. Detection of *Ureaplasma urealyticum* biovars by PCR and biovar determination by liquid hybridization. *J Clin Microbiol* 1998;36:3211–3216.
28. Neyrolles O, Ferris S, Behbahani N, Montagnier L, Blanchard A. Organization of *Ureaplasma urealyticum* urease gene cluster and expression in suppressor strain of *Escherichia coli*. *J Bacteriol* 1996;178:647–655.
29. Matlow A, Th'ng C, Kovach D, Quinn P, Dunn M, Wang E. Susceptibilities of neonatal respiratory isolates of *Ureaplasma urealyticum* to antimicrobial agents. *Antimicrob Agents Chemother* 1998;42:1290–1292.
30. Koch A, Bilina A, Teodorowicz L, Stry A. *Mycoplasma hominis* and *Ureaplasma urealyticum* in patients with sexually transmitted diseases. *Wien Klin Wochenschr* 1997;109:584–589.
31. Cedillo-Ramirez L, Gil C, Zago I, Yanez A, Giono S. Association of *Mycoplasma hominis* and *Ureaplasma urealyticum* with some indicators of non-specific vaginitis. *Rev Latinoam Microbiol* 2000;42:1–6.