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High Prevalence of Genital Mycoplasmas among Sexually Active Young Adults with Urethritis or Cervicitis Symptoms in La Crosse, Wisconsin

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Sexually active young adults in the small college town of La Crosse, Wisconsin, were evaluated for conventional sexually transmitted pathogens and tested for infections with mycoplasmas. The prevalence in 65 symptomatic men or women and 137 healthy volunteers (67 men and 70 women) was compared. Urine specimens from both cohorts were tested by ligase chain reaction for *Chlamydia trachomatis* or *Neisseria gonorrhoeae*. In addition, the urethral or cervical swabs from the symptomatic subjects were tested by PCR for *Mycoplasma genitalium* and cultured for *Mycoplasma hominis* and the ureaplasmas. The results confirmed a relatively low prevalence of gonorrhea among symptomatic men (12%) and chlamydia among symptomatic men (15%) and normal women (3%). In contrast, infections with mycoplasmas, especially the ureaplasmas (57%), were common and the organisms were the only potential sexually transmitted pathogen detected in 40 (62%) symptomatic subjects. Because of the high prevalence, we also evaluated urethral swabs from an additional 25 normal female volunteers and recovered ureaplasmas from 4 (16%) subjects. Additionally, the participants rarely used protection during sexual intercourse and some symptomatic subjects apparently acquired their infections despite using condoms regularly. The findings demonstrate a strong association between abnormal urogenital findings and detection of myoplasmas, particularly ureaplasmas, and suggest the infections will remain common.

Genitourinary infections, including sexually transmitted diseases (STDs), are caused by a large number of diverse microbial agents that cause considerable morbidity and mortality worldwide. While "classical" sexually transmitted pathogens such as *Neisseria gonorrhoeae* and *Treponema pallidum* continue to produce serious illnesses among certain (primarily urban) populations in the United States (6, 7), less urbanized communities have a proportionally higher incidence of urethritis and cervicitis caused by *Chlamydia trachomatis*. In addition, mycoplasmas commonly colonize the genital tracts of men and women (34), and the ability of some species to cause nongonococcal urethritis has been well established. However, the prevalence and complete pathogenic potential of this bacterial group is still being elucidated (2, 4, 34, 35).

La Crosse, Wisconsin, a small city with a relatively large number of college students, has a low incidence of gonorrhea and syphilis. In addition, the incidence of *C. trachomatis* infections declined until 1997, though it has increased in recent years. This may be due in part to the introduction of more sensitive DNA-based assays. The countywide annual incidence of *Chlamydia* was 247/100,000 in 2001 (La Crosse County Public Health data). More significantly, a large proportion of urethritis and cervicitis cases remain nongonococcal and nonchlamydial, and we hypothesized a significant contribution from genital mycoplasmas. Accordingly, we evaluated a cohort of sexually active young adults with clinical symptoms suggestive of STD (i.e., urethritis or cervicitis) for pathognomonic signs from infections with herpes simplex virus, papillomaviruses, or *Haemophilus ducreyi*, and we performed microscopic examinations for *Trichomonas vaginalis*. In addition, we cultured for ureaplasmas and *Mycoplasma hominis* and tested for *Mycoplasma genitalium* by PCR. The results confirmed a relatively low incidence of well-characterized STDs and a high prevalence of symptomatic subjects positive for mycoplasmas, particularly *Ureaplasma urealyticum* and/or *Ureaplasma parvum* (30), collectively referred to as ureaplasmas in this study.

MATERIALS AND METHODS

Patients and controls. Young adults presenting to Options in Reproductive Care (La Crosse, Wisc.) or the Student Health Center at the University of Wisconsin—La Crosse (UW-L) from April 1999 to March 2000 with urethral or cervical discharges, burning or pain during urination, testicular or pelvic pain or inflammation, or cervical bleeding were solicited for participation. Subjects who had urinated within the previous hour or received antibiotics within the previous 2 weeks were excluded. Control urine samples were obtained from asymptomatic sexually active students attending the UW-L. The symptomatic subjects and normal volunteers also completed a standardized questionnaire and normal subjects with other illnesses or previous histories of STD were excluded. After the study was begun, an additional 25 normal female subjects attending Options in Reproductive Care for routine Pap smears were asked to provide cervical swab samples to culture for ureaplasmas or *M. hominis*. The additional participants were not required to complete the study questionnaire. All participants signed a standard informed consent agreement, and the procedures were preapproved by the Institutional Review Boards and Biosafety Committees at Gundersen Lutheran Medical Center and the UW-L.

Clinical evaluation and testing. Symptomatic patients were initially questioned regarding a previous history of syphilis, chancroid, or genital herpes and then examined for lesions suggestive of these infections. In addition, the patients were examined for *Trichomonas vaginalis* trophozoites by a standard wet prep

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microscopic evaluation. Approximately 20 ml of clean-catch urine was then collected in a sterile plastic container, followed by sampling with two Dacron-tipped swabs (LCx Probe System; Abbott Laboratories, Chicago, Ill.) that were inserted approximately 2 cm into the cervix or urethra and rotated several times. To maximize the detection of *M. genitalium* DNA, the first swab collected was broken off into a screw top test tube containing 1 ml of SP-4 broth (38) and the second swab was placed in a screw cap test tube containing 5 ml of pleuropneumoniae-like organism medium (12). Both swabs and the urine sample were stored at 4°C until transport to the laboratory.

Detection of *C. trachomatis* and *N. gonorrhoeae*. A portion of the urine samples were tested for *C. trachomatis* or *N. gonorrhoeae* within 24 h of collection by ligase chain reaction (LCR). DNA from both organisms was amplified according to the manufacturer's instructions for the LCx probe system (Abbott Laboratories).

Detection of ureaplasmas and *M. hominis*. After transport to the laboratory, the swab in the pleuropneumoniae-like organism medium was removed and placed in a test tube containing 5 ml of Boston broth (18). After incubation overnight at 37°C, 3 drops of the Boston broth was subcultured to an A-7 agar plate (Remel, Lenexa, Kans.), and the plate was incubated at 37°C under microaerophilic conditions using a GasPak (CampyPak; Becton Dickinson). The plates were then examined periodically for several weeks for brown mulberry-shaped colonies characteristic of ureaplasmas or "fried-egg" colonies characteristic of other mycoplasmas. As a control to confirm the efficacy of the A-7 agar, a separate plate was inoculated with Boston broth spiked with *U. urealyticum* (ATCC 700970) and a *M. genitalium* (ATCC 33530) acclimated to growth on culture medium.

Colony morphology was considered sufficient to identify the ureaplasmas, and *M. hominis* was identified using a modification of a previously described PCR technique (1). Briefly, a colony was resuspended in 300 μl of proteinase K buffer. After incubation for 5 min at 56°C and 10 min at 90°C, a 2-μl amount was combined with primers M1 (5′-CAATGGCTAATGCCGGATACGC-3′) and M2 (5′-GGTACCGTCAGTCTGCAAT-3′) and a 334-bp product of the 16S rRNA was amplified during 40 cycles, each consisting of a 20-s denaturation step at 95°C, a 60-s annealing step at 62°C, and a 60-s elongation step at 72°C in a volume of 50 μl and 1 U of Amplitaq DNA polymerase (Applied Biosystems, Foster City, Calif.). A final extension for 7 min at 72°C was used to fully extend any truncated strands. A 15-μl amount of the reaction was then electrophoresed at 100 V in a 3% agarose gel and detected by staining with a 1:10,000 dilution of SYBR Green I (Molecular Probes, Eugene, Oreg.) in 1× Tris-acetate-EDTA buffer.

Detection of *M. genitalium*. *M. genitalium* is difficult to recover on laboratory culture medium (15, 19), so PCR was used to detect the organisms in the remaining urine and the urogenital swab sample that had been placed into the SP-4 broth. A 10-ml amount of urine was transferred to a sterile 50-ml tube and centrifuged at 10,000 × *g* for 20 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was resuspended in 1 ml of phosphate-buffered saline (pH 7.2). In addition, the swab was removed from the SP-4 broth after vortexing for 15 s and transferred to a sterile 1.5-ml microcentrifuge tube where it was centrifuged at 14,000 × *g* for 5 min at room temperature. The supernatant was then discarded and the pelleted cells were lysed by adding 20 μl of proteinase K buffer prior to centrifuging as above. A 20-μl volume was then transferred to a sterile 1.5-ml microcentrifuge tubes, and 5 μl of StrataClean resin (Stratagene, La Jolla, Calif.) was added. After incubation for 5 min at room temperature, the sample was centrifuged at 14,000 × *g* for 1 min, and the supernatant was transferred to an additional sterile 1.5-ml microcentrifuge tube. PCR was then performed on a 2-μl portion of the sample using the 5′ primer MgPa-1 (5′-AGTTGATGAAACCTTAACCCCTTGG-3′) and 3′ primer MgPa-3 (5′-CCGTTGAGGGGTTTTCCATTTTTGC-3′) (Sigma-Genosys, The Woodlands, Tex.) to amplify a 281-bp region of the *M. genitalium* adhesin gene (16). Final concentrations of reagents in the PCR buffer were 2.5 μM each primer, 2.5 mM $MgCl_2$, 400 μM each dNTP, 1.25 U of Amplitaq DNA polymerase (Applied Biosystems), 1× Amplitaq buffer, and 1.4 μl of TaqStart antibody (Clontech, Palo Alto, Calif.). The DNA was then amplified by an initial 5-min denaturation at 95°C followed by 35 cycles of a 30-s denaturation at 95°C, a 30-s annealing at 60°C, and a 30-s extension at 72°C. A final extension for 7 min at 72°C was used to fully extend any truncated strands. A 15-μl amount of the reaction was then electrophoresed in a 3% agarose gel and detected by staining with a 1:10,000 dilution of SYBR Green I in 1× Tris-acetate-EDTA buffer.

Prior to beginning the study, the specificity of the PCR was confirmed by attempting to amplify DNA from other organisms. No products were detected using DNA from *M. hominis*, *U. urealyticum*, *Corynebacterium diphtheriae*, *Streptococcus agalactiae*, *Moraxella catarrhalis*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Proteus vulgaris*, human papillomavirus, cytomegalovirus, herpes simplex virus types I and II, *Gardnerella vaginalis*, *Escherichia coli*, *Lactobacillus* spp., *Saccharomyces cerevisiae*, *Candida albicans*, *N. gonorrhoeae*, or *C. trachomatis*. Protective measures to prevent false-positive results from cross-contamination included using separate rooms to mix reagents, amplify DNA, and analyze amplified products; use of aerosol-resistant tips (Molecular Bio-Products Inc., San Diego, Calif.); and thorough cleaning of counter surfaces and equipment with DNA-Erase (ICN Biomedics Inc., Aurora, Ohio). In addition, three negative controls containing buffers only were included with each run.

To detect false-negative reactions, we constructed a plasmid (pKLS25) that coded for a truncated *M. genitalium* adhesin gene using a 5′ primer JRS-RF (5′-TCATCTCGAGGTATCTCAATGCTGTTGAG-3′) and a 3′ primer JRS-RR (5′-TCATCTCGAGCTAATCCAAATCATCCTGG-3′). The primers were combined with 0.02 U of Elongase (Gibco BRL, Rockville, Md.)/μl, 1× Elongase buffer, 1.5 mM $MgCl_2$, and 400 μM each dNTP and amplified by an initial 5-min denaturation at 95°C followed by 35 cycles of a 1-min denaturation at 95°C, a 1-min annealing at 60°C, and a 3-min extension at 72°C. The resultant approximately 3.2-kb DNA fragment was then purified (Wizard PCR Preps DNA purification system; Promega, Madison, Wis.), digested with XhoI (Gibco BRL), ligated, and transformed into *E. coli* JM109. One hundred molecules of pKLS25 were then added to the recovered DNA from each urine or urogenital swab sample prior to performing the PCR. When the *M. genitalium* DNA from the plasmid was not detected, the sample was considered invalid unless *M. genitalium* DNA was detected despite the inhibition of the control. In a preliminary experiment, the sensitivity of the PCR was evaluated by spiking normal urine with decreasing concentrations of the laboratory *M. genitalium* isolate and pKLS25. The procedure detected approximately 80 *M. genitalium* organisms when inhibiting factors were not present.

Statistical analyses. Statistical significance was determined using χ^2 and Fisher's exact tests. The *P* value was set at <0.05 prior to beginning the study.

RESULTS

A total of 26 symptomatic men and 39 symptomatic women represented the study group. Urine samples and urogenital swabs were obtained from all participants. Controls consisted of urine specimens from 137 healthy volunteers (67 men and 70 women). The demographics of the study and control groups were similar (data not shown).

Lesions associated with Herpes simplex virus, papillomavirus, or *H. ducreyi* were not seen. However, two symptomatic subjects reported previous diagnoses of genital warts (i.e., human papillomavirus infection). *Trichomonas vaginalis* trophozoites were also not detected. In addition, infections with *C. trachomatis* or *N. gonorrhoeae* were detected only rarely and there were significant differences among the cohorts. *C. trachomatis* was detected in four symptomatic but no asymptomatic men ($P = 0.0051$) and in two asymptomatic but no symptomatic women (Table 1). In addition, *N. gonorrhoeae* was recovered from three symptomatic men ($P = 0.02$) and one symptomatic woman. One of the above men was infected with both organisms.

In contrast, recovery of mycoplasmas was common, especially from the symptomatic subjects. Mycoplasmas were recovered from five of the seven symptomatic subjects with chlamydia or gonorrhea. More significantly, mycoplasmas were recovered from an additional 19 (73%) symptomatic men and 21 (54%) symptomatic women from whom pathognomonic symptoms or direct evidence of other STD pathogens were not detected. In these subjects, urethral or vaginal discharges and burning or pain during urination were common complaints (Table 2).

The least prevalent organism, *M. genitalium*, was detected from the urine or urogenital swabs from eight (12%) symptomatic subjects and the urine from five (5%) asymptomatic subjects. In addition, the prevalence of *M. genitalium* was likely underestimated significantly because the PCR procedure was less than optimal. The negative results from 19 (38%) and 27

TABLE 1. Detection of microbial agents among sexually active adults

Diagnostic method	Symptomatic			Asymptomatic[a]			*P* value
	Men (n = 26)	Women (n = 39)	Total (n = 65)	Men	Women	Total	
Culture for ureaplasmas	16 (62)[b]	21 (54)	37 (57)	NT[c]	4/25 (16)	4/25 (16)	0.0036
Culture for *M. hominis*	3 (12)	10 (26)	13 (20)	NT	0/25	0/25	0.0047
PCR for *M. genitalium*[e]	3 (12)	5 (13)	8 (12)	1/46 (2)	4/50 (8)	5/96 (5)	NS[d]
LCR for *C. trachomatis*	4 (15)	0	4 (6)	0/67	2/70 (3)	2/137 (1)	0.0051
LCR for *N. gonorrhoeae*	3 (12)	1 (3)	4 (6)	0/67	0/70	0/137	0.02

[a] Denominator equals number tested. The denominators used to calculate percentages vary, because data were not available for all subjects for all variables.
[b] Percentage of positive tests is in parentheses.
[c] NT, not tested.
[d] NS, not significant.
[e] Results from symptomatic subjects are combined findings from urine and swab samples. Results from asymptomatic subjects are from urine samples. Samples contaminated with inhibitory factors that prevented amplification of the internal control DNA were not included.

(22%) urine samples from symptomatic and asymptomatic subjects, respectively, were not included because the truncated control was not amplified. In addition, the PCR results from the swab and urine samples diverged frequently. *M. genitalium* DNA was detected in both the urine and swab samples from five symptomatic subjects, only the urine sample from two subjects, and only the swab sample from one subject.

In contrast, ureaplasmas were recovered from 16 (62%) and 21 (54%) symptomatic men and women, respectively. In addition, *M. hominis* was recovered from 3 (12%) symptomatic men and 10 (26%) symptomatic women. A comparison of the prevalence of *M. hominis* in asymptomatic men was not possible because urethral swab samples were not collected from the cohort. We did, however, culture cervical swabs collected from an additional 25 normal women to determine the prevalence in this cohort. *M. hominis* was not detected ($P = 0.0047$), but ureaplasmas were recovered from four (16%) of the normal women ($P = 0.0036$).

We then compared the responses from the questionnaires of the symptomatic and asymptomatic subjects from whom chlamydia, gonococci, and/or mycoplasmas were recovered (n = 53) to those obtained from the normal subjects (n = 131) from whom sexually transmitted pathogens were not suggested. Both cohorts averaged 21 years of age and were almost entirely heterosexual (99%) and Caucasian (Table 3). In addition, the participants were sexually active for at least the previous 4 years and were with similar numbers of partners. The majority was also sexually active during the previous 3 months. However, abnormal Pap smears ($P = 0.0001$) and past diagnoses of STD ($P = 0.0377$) were significantly more common among subjects infected with chlamydia, gonococci, or mycoplasmas. In addition, barrier methods to prevent STD, e.g., condoms, were not used consistently by either group, though significantly ($P = 0.0024$) more symptomatic subjects reported occasional use. More notably, mycoplasmas were recovered from six (29%) and five (16%) symptomatic men and women, respectively, despite their claims that condoms were always used.

DISCUSSION

At least 13 species of mycoplasmas (41) and two species of ureaplasmas (30) are common commensals of the human urogenital tract, and these diminutive microbes are transmitted efficiently by sexual contact. Most colonized individuals remain asymptomatic, but there is considerable evidence, albeit largely circumstantial, that *M. genitalium*, *M. hominis*, and the ureaplasmas also cause disease. Despite occasional conflicting results (25), an overwhelming number of studies (9, 13, 14, 20–23, 26, 29, 32, 37) have demonstrated significantly greater isolation rates of *M. genitalium* and the ureaplasmas from patients with nongonococcal urethritis or cervicitis. More compelling, intraurethral inoculation of chimpanzees with *M. genitalium* resulted in urethritis and significantly increased antibody responses (39). In addition, real-time PCR correlated the elimination of *M. genitalium* after treatment with the resolution of symptoms in humans (8). *M. hominis* has also been implicated as causal factors of bacterial vaginosis (31), pelvic inflammatory disease (23, 24), infertility (27), miscarriage (11), preterm labor (5, 17), respiratory distress in newborns (36), and neonatal meningitis (40), and the ureaplasmas are associated with preterm labor (10), intrauterine lung disease (28), and neonatal pneumonia (3).

With these findings in mind, we sought to determine the prevalence of possible causative agents of STDs, including *M. genitalium*, *M. hominis*, and the ureaplasmas, among symptomatic sexually active young adults in La Crosse, Wisconsin. We found no indirect pathognomonic evidence that the clinical abnormalities were caused by infections with herpes simplex virus, papillomaviruses, or *H. ducreyi* and failed to detect *Trichomonas vaginalis*. In addition, *N. gonorrhoeae* and *C. trachomatis* were detected in only 4 (6%) of the 65 symptomatic subjects and asymptomatic infections with *C. trachomatis* were rare in normal men (0%) or women (3%). The findings therefore echoed a nationwide trend of decreasing prevalence of these well-defined sexually transmitted pathogens in this relatively homogeneous Midwestern population of European de-

TABLE 2. Clinical symptoms of subjects testing positive for mycoplasmas only

Symptom	No. (%) of subjects with symptom		
	Men (n = 19)	Women (n = 21)	Total (n = 40)
Urethral or vaginal discharge	11 (58)	18 (86)	29 (73)
Burning or pain with urination	12 (63)	9 (43)	21 (53)
Testicular or pelvic pain	2 (11)	9 (43)	11 (28)
Spotting between periods	NA[a]	5 (24)	5 (24)
Cervical inflammation	NA	4 (19)	4 (19)

[a] NA, not applicable.

TABLE 3. Demographic, gynecologic, and behavioral characteristics of men or women with or without STD

Demographic characteristic	With STD			Without STD			*P* value
	Men (n = 21)[a]	Women (n = 32)	Total (n = 53)	Men (n = 66)	Women (n = 65)	Total (n = 131)	
Mean age (range)	21 (19–28)	20 (18–26)	21 (18–28)	21 (16–26)	20 (18–35)	21 (16–35)	NS[b]
Heterosexual	21 (100)	32 (100)	47 (100)	66 (100)	64 (98)	130 (99)	NS
Previous STD	7 (33)	5 (16)	12 (23)	0	1 (2)	1 (1)	0.0001
Abnormal Pap smear	NA[c]	8 (25)	NA	NA	6 (9)	NA	0.0377
Oral contraceptives	NA	18 (56)	NA	NA	45 (69)	NA	NS
Depo-provera	NA	2 (6)	NA	NA	2 (3)	NA	NS
Mean age at first intercourse (range)	17 (11–19)	17 (13–22)	17 (11–22)	17 (11–21)	17 (14–21)	17 (11–21)	NS
Mean no. of partners, lifetime (range)	8 (3–20)	5 (1–20)	5 (1–20)	6 (1–40)	3 (1–10)	4 (1–40)	NS
Mean no. of partners in last 3 mo (range)	2 (1–12)	1 (0–5)	2 (0–12)	1 (0–3)	1 (0–3)	1 (0–3)	NS
Condom use							
Always	6 (29)	5 (16)	11 (21)	23 (35)	17 (26)	40 (31)	NS
Sometimes	11 (52)	11 (34)	22 (40)	14 (21)	12 (18)	26 (20)	0.0024
Never	4 (20)	7 (22)	11 (21)	29 (44)	18 (28)	47 (36)	NS

[a] Data are number infected out of the total number tested (%), unless otherwise indicated.
[b] NS, not significant.
[c] NA, not applicable.

scent. *C. trachomatis* infections were responsible for 35 to 50% of nongonococcal urethritis cases during the 1970s and 1980s, but incidences have decreased significantly, presumably because of effective screening and treatment programs (33).

However, clinical abnormalities that are the likely result of a sexually transmitted infection remain relatively common in young adults from this region. Our findings provide strong evidence that the mycoplasmas are responsible for most cases because infections with more typical sexually transmitted pathogens could explain the symptoms in only a few patients. In contrast, mycoplasmas, especially the ureaplasmas, were recovered from 40 (85%) of the 47 symptomatic subjects whose symptoms, including most typically urethritis or cervicitis, pain, spotting between periods, and frank inflammation, appeared most likely to be attributable to a sexually transmitted organism.

In addition, we likely underestimated the prevalence of *M. genitalium* because the PCR method was hindered by inhibiting factors that confounded the results in a significant number of urine samples. In hindsight, we should have determined whether diluting the inhibiting factors to undetectable levels would have allowed evaluation without significant loss of sensitivity. We also did not evaluate the mycoplasma carriage rates in normal subjects adequately because we did not test the normal males and only a small number of normal females were evaluated. Despite the shortcomings, however, the findings also provided strong evidence that mycoplasma infections will remain common unless appropriate educational and prevention programs are implemented. Ureaplasmas and *M. genitalium* were detected in 4 (16%) of the 25 normal women and 5 (5%) of the 96 normal men and women tested, respectively. In addition, a significant number of asymptomatic volunteers ignored barrier methods that might have prevented transmission and, most disconcerting, some symptomatic subjects apparently became infected with mycoplasmas despite using condoms regularly.

In conclusion, we demonstrate a strong association between abnormal urogenital findings in sexually active young adults and the ability to detect mycoplasmas, particularly the ureaplasmas. Additional studies to more completely assess the significance of these findings are ongoing.

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