Anaerobic and Aerobic Urethral Flora in Healthy Females

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We characterized the aerobic and anaerobic urethral flora of five healthy females by performing urethral and midstream urine cultures once weekly for 8 weeks. Aerobic cultures were performed monthly for an additional 3 months. *Lactobacillus* spp. were isolated from 52 of 57 samples, *Staphylococcus epidermidis* from 42 of 57, *Corynebacterium* spp. from 26 of 57, and alpha-hemolytic streptococci from 14 of 57. Two subjects had *E. coli* serogroup O6 and group B streptococci isolated on five occasions, respectively. Anaerobes were isolated from 32 of 35 urethral urines (91%). *Bacteroides melaninogenicus* accounted for 46% of these isolates. The anaerobic urethral flora varied slightly from week to week, and a similar anaerobic flora was isolated from the introitus, fourchette, and cutaneous perineum. In addition, anaerobes were isolated from 16 of 18 healthy females who had a urethral urine sample cultured once only, and *B. melaninogenicus* was the most frequent isolate. Of the 21 *B. melaninogenicus* isolates identified to subspecies, 14 were subsp. *intermedius*.

Urinary infections in nonpregnant women are common, varying with both the age of the patient and the population under study (15). It is estimated that about 10 to 20% of women experience a urinary infection in their lifetime (13). Some of these women will have recurrent urinary tract infections. The aerobic introital and urethral flora of patients with recurrent urinary tract infections have been shown to be different from the aerobic flora of healthy volunteers who do not have urinary tract infections in that large numbers of pathogenic bacteria persist on the mucosal surfaces of the introitus and urethra of those women with recurrent urinary tract infections (16). The reasons for this difference remain to be delineated. The aerobic bacterial flora of the urethra in healthy females has been studied infrequently (14, 16), and the anaerobic flora has not been characterized.

As part of our efforts to define the role of the "normal" urethral flora as a host defense mechanism, we have delineated the urethral flora of healthy females with no history of urinary tract infection.

MATERIALS AND METHODS

Five healthy females aged 18 to 20 years were followed for 5 months. A history regarding symptoms of urinary tract infection was taken at each visit. For the first 8 weeks they were seen weekly, and once monthly for an additional 3 months.

Specimen collection. At each of the initial four visits, the urethral flora was cultured by introducing

a sterile cotton swab moistened in brain heart infusion broth into the urethra a distance of one centimeter and then swabbing one half the circumference with a circular motion. A second swab was used to sample the remaining half. A midstream urine specimen was then collected.

At each of the subsequent four weekly visits, two urine specimens were collected. The first consisted of the initial 5 to 10 ml of voided urine ("urethral urine"), the second a midstream urine. In addition, on the eighth visit, the introitus, fourchette, cutaneous perineum, and 1 cm² of the right groin were sampled using moistened cotton swabs. At each of the three subsequent monthly visits, urethral and midstream urines were collected. One subject had two additional visits. Eighteen healthy females aged 18 to 40 years had urethral urine samples cultured once only.

Inoculation and incubation. One of the urethral swabs was immediately inoculated onto a Brucella blood agar plate containing 10 µg of vitamin K₁ per ml and 5% sheep blood (BAK) and onto a kanamycin-vancomycin-laked-blood agar plate containing Brucella agar base, 5% laked sheep blood, 10 µg of vitamin K₁ per ml, 100 µg of kanamycin per ml, and 7.5 µg of vancomycin per ml (LKV). Both these plates were prepared in our laboratory and were held under anaerobic conditions prior to use. These plates were then incubated anaerobically in GasPak jars for 48 h. Disposable hydrogen-carbon dioxide generators (GasPak, BBL, Cockeysville, Md.) were used in the jars to supply a gas mixture containing hydrogen and carbon dioxide. The swab was then placed into liquid thioglycolate medium and, after 24 h of incubation, was subcultured to BAK and LKV plates. The other swab was placed into a tube containing 1 ml of BHI broth and mixed in a Vortex mixer. Samples were streaked onto blood agar (Trypticase soy agar containing 5% sheep blood), MacConkey agar, and chocolate

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agar (GC agar base [BBL], 2% hemoglobin [Difco, Detroit, Mich.], and IsoVitalX [BBL] at 10 ml/liter, using 0.01- and 0.001-ml quantitative loops. The blood and MacConkey agar plates were incubated aerobically for 18 h, and the chocolate plate was incubated in an atmosphere of 5 to 10% CO₂ for 36 h. The midstream and aerobic urethral urine specimens were inoculated onto blood, MacConkey, and chocolate agar using 0.01- and 0.001-ml quantitative loops and incubated as for the aerobic urethral swab. The urethral urine specimen (0.01 ml) was inoculated onto BAK and LKV plates and incubated anaerobically. The introitus, fourchette, cutaneous perineum, and groin swabs were cultured aerobically and anaerobically as outlined above.

Isolation and identification. (i) Aerobes. All aerobic isolates were identified by conventional techniques for gram-positive cocci and *Enterobacteriaceae* (1, 5, 6, 12). *Escherichia coli* isolates were serogrouped using antisera obtained from Difco Laboratories.

(ii) Anaerobes. All colonies growing on the BAK and LKV plates were examined for fluorescence under UV light (365 nm). Plates that exhibited brick-red fluorescence were held for 3 weeks to detect pigment production. All colonial types were Gram stained and inoculated to plates incubated anaerobically to obtain pure cultures and to plates incubated aerobically in an atmosphere of 5 to 10% CO₂ to identify facultative and microaerophilic organisms. Organisms that grew only anaerobically were inoculated into 9 ml of fluid thioglycolate medium with dextrose and without indicator (BBL 135C) containing hemin (5 µg/ml), vitamin K₁

(0.1 µg/ml), sodium bicarbonate (1 mg/ml), and Fildes enrichment (BBL) to a final concentration of 5% (vol/vol). All organisms isolated were identified according to methods outlined in the Virginia Polytechnic Institute Anaerobe Laboratory Manual (11). Only the anaerobic isolates from weeks 4, 6, 7, and 8 were fully identified to species. The prereduced, anaerobically sterilized media were obtained from Carr-Scarborough Microbiologicals, Stone Mountain, Ga.

RESULTS

The five subjects who were followed for 5 months (11 visits) had 57 urethral and 57 midstream urine specimens cultured aerobically. (One subject, L.H.R., made 13 visits.) Twenty urethral swab samples and 15 urethral urine samples were cultured anaerobically. During this 5-month period only one subject, L.H.R., had symptoms of dysuria, frequency, and urgency. Two separate midstream samples obtained when she was symptomatic yielded $>10^5$ colony-forming units of *Staphylococcus epidermidis* per ml of urine.

Aerobic flora. The results of culture of the urethral samples (20 obtained by swabs and 35 by culturing the first few milliliters of urine) are shown in Fig. 1. Since no obvious differences were noted when the results of the urethral flora obtained by swab samples were compared with

FIG. 1. Species and quantity of aerobic urethral microorganisms isolated from five healthy females over a 5-month period, 57 specimens (20 urethral swab samples and 37 urethral urine samples).

those obtained by urethral urine samples, the data are combined in Fig. 1. A mean of 2.5 organisms was isolated per specimen. *Lactobacillus* spp. was the most common isolate and was present in the highest counts. Of the 52 samples that grew *Lactobacillus* spp., 49 had counts of 10^5 per ml. *S. epidermidis* was the other main constituent of the aerobic urethral flora. The counts were lower than those of *Lactobacillus* spp., and ranged from 10^2 to 10^4 colony-forming units per ml. *Corynebacterium* spp. was isolated from about one-quarter of the samples (Fig. 1). All five *E. coli* isolates were from one subject, and all group B streptococcal isolates were from another. The five *E. coli* isolates were serogroup O6. There was one isolate each of *Staphylococcus aureus* and *Micrococcus* spp.

The isolates from the midstream urines were similar to those isolated from the urethra, but they were generally lower in number, and 42% of the specimens had $>10^2$ organisms per ml (Fig. 2). *S. epidermidis* was the predominant isolate, and the *E. coli* and group B streptococci were from the same two patients, who had these organisms from their urethras.

Anaerobic flora. Thirty-two of the 35 urethral cultures (91%) grew anaerobes, with a mean of 1.4 organisms being isolated per specimen. (All week 5 anaerobic samples were lost in a laboratory accident.) Organisms of the *Bacteroides melaninogenicus* group accounted for 46% of the anaerobes, and those of the *Bacteroides fragilis* group were the next most common isolate (Table 1). Four of the latter group

were *B. distasonis*, two were *B. vulgatus*, and there was one each of *B. ovatus* and *B. fragilis*. *B. melaninogenicus* was isolated repeatedly from all five subjects. In two subjects, it was isolated from each of the seven samples. The anaerobic urethral flora varied from week to week, as shown in Table 2 for weeks 6, 7, and 8.

Quantitation of the urethral anaerobes was carried out during weeks 6 to 8 when the urethral urine sample was cultured anaerobically. As shown in Fig. 3, the counts were low, 10^2 to 10^4 /ml. The anaerobic flora of the urethra, introitus, fourchette, and cutaneous perineum was similar (Tables 2 and 3). In all five subjects, anaerobes were recovered from the fourchette and the cutaneous perineum. No anaerobes were isolated from the introitus of subject L.H.A. *B. melaninogenicus* was recovered from the groin of one of the five. Noteworthy was the isolation

TABLE 1. Anaerobic microorganisms isolated from the urethras of five healthy females over a period of 7 weeks^a

Organism	No. of positive cultures
<i>B. melaninogenicus</i> group	23
<i>B. fragilis</i> group	9
<i>Bacteroides</i> spp.	5
<i>Peptococcus</i>	6
<i>Peptostreptococcus</i>	2
<i>Eubacterium</i>	3
<i>Clostridium</i> spp.	2

^a 20 urethral swab samples and 15 urethral urine samples.

FIG. 2. Species and quantity of aerobic microorganisms isolated from midstream urine specimens obtained from five healthy females over a 5-month period, 57 specimens.

TABLE 2. Anaerobic urethral flora from week to week in five healthy females (urethral urine samples)

Week	Subject				
	N.T.	L.H.R.	C.A.	L.B.	L.H.A.
6	<i>B. melaninogenicus</i> subsp. <i>intermedius</i>	<i>B. melaninogenicus</i> subsp. <i>intermedius</i> <i>Peptococcus</i> spp.	<i>B. melaninogenicus</i> subsp. <i>melanino-</i> <i>genicus</i> <i>Peptococcus</i> spp.	<i>B. melaninogenicus</i> subsp. <i>intermedius</i> <i>B. vulgatus</i>	<i>B. distasonis</i>
7	<i>B. melaninogenicus</i> subsp. <i>intermedius</i> <i>B. distasonis</i> <i>B. ovatus</i>	<i>B. melaninogenicus</i> subsp. <i>intermedius</i> <i>B. asaccharolyticus</i>	<i>B. melaninogenicus</i> subsp. <i>melanino-</i> <i>genicus</i>	<i>B. melaninogenicus</i> subsp. <i>intermedius</i> <i>Bacteroides</i> spp.	<i>B. distasonis</i> <i>B. vulgatus</i> <i>B. melaninogenicus</i> subsp. <i>intermedius</i>
8	<i>Bacteroides</i> spp.	<i>B. melaninogenicus</i> subsp. <i>intermedius</i>	<i>B. melaninogenicus</i> subsp. <i>melanino-</i> <i>genicus</i>	<i>B. melaninogenicus</i> subsp. <i>intermedius</i> <i>B. fragilis</i>	<i>B. distasonis</i> <i>B. melaninogenicus</i> subsp. <i>intermedius</i>

FIG. 3. Species and quantity of anaerobic urethral microorganisms isolated from five healthy females over a 3-week period by culturing the first 5 to 10 ml of urine (urethral urine) anaerobically, 15 specimens.

of only two anaerobic gram-positive organisms (*Peptococcus* 2) from the 15 urethral urine samples, whereas 11 such organisms (*Peptococcus* 4, *Peptostreptococcus* 2, *Eubacterium* 3, *Clostridium* spp. 2) were recovered from 20 urethral swab samples.

Anaerobes were recovered from the urethras of 16 of the 18 (90%) females who had urethral urine cultured on one occasion only. *B. melaninogenicus* was isolated from 12 and *Bacteroides* spp. from 6. Two subjects had two subspecies of *B. melaninogenicus* recovered from the same

specimen. No anaerobic gram-positive bacteria were recovered.

Of the 41 isolates of *B. melaninogenicus* recovered from the urethras of both groups of females, 21 were identified to subspecies. Fourteen of the 21 isolates were subspecies *intermedius*. Four of the remainder were subspecies *melaninogenicus*, and three were subspecies *Asaccharolyticus*, now designated as *B. asaccharolyticus* (8). None of the subsp. *intermedius* isolates turned black even when held for 30 days. They were all tan in color. We had some of these non-black-pigmented strains confirmed as *B. melaninogenicus* subsp. *intermedius* by Vera Sutter, Wadsworth Anaerobic Bacteriology Laboratory, Wadsworth V.A. Hospital, Los Angeles, Calif.

DISCUSSION

The aerobic urethral flora of healthy females has been delineated in two other studies (14, 16). Our results are similar to those obtained in these studies. *Lactobacillus* spp. constituted the bulk of the urethral flora and was usually present in counts of $>10^5$. *S. epidermidis* was the next most common isolate. *Corynebacterium* spp. and alpha-hemolytic streptococci were isolated less frequently, from approximately one-half and one-quarter of the specimens, respectively. *E. coli* was isolated from one subject repeatedly, and all isolates were serogroup O6. At no time did she have pyuria or symptoms of urinary tract infection. Over the 8 months of observation, only one subject had dysuria, frequency, and urgency. This was associated with $>10^5$ colony-forming units of *S. epidermidis* per ml in midstream urine cultures. The symptoms lasted for 4 days, then subsided spontaneously. All of her midstream urine cultures prior to and subsequent to this episode grew *S. epidermidis* $<10^4$ /ml. Almost half the midstream urines were sterile ($<10^2$ organisms per ml). Urethral aerobes were recovered from the remaining midstream urines, always in lower counts than those ob-

TABLE 3. *Anaerobic microorganisms isolated from various sites in five healthy females—week 8*

Site sampled	Subject				
	N.T.	L.H.R.	C.A.	L.B.	L.H.A.
Introitus	<i>Bacteroides</i> spp.	<i>B. melaninogenicus</i>	<i>B. melaninogenicus</i>	<i>B. melaninogenicus</i>	0 ^a
Fourchette	<i>Bacteroides</i> spp.	<i>B. melaninogenicus</i>	<i>B. melaninogenicus</i>	<i>B. melaninogenicus</i>	<i>B. melaninogenicus</i>
		<i>B. fragilis</i>	<i>Peptococcus</i> spp.		<i>B. fragilis</i>
Cutaneous perineum ^b	<i>Bacteroides</i> spp.	<i>B. melaninogenicus</i>	<i>B. melaninogenicus</i>	<i>Bacteroides</i> spp.	<i>B. melaninogenicus</i> <i>Bacteroides</i> spp.
Groin	0	<i>B. melaninogenicus</i>	0	0	0

^a 0, No anaerobic microorganisms isolated.^b The area between the posterior fourchette and the anus.

tained from the first few milliliters of voided urine collected immediately before the mid-stream urine. This represents a washout effect, as demonstrated by Henning and Tornvall (10). These investigators collected voided urine in fractions by placing containers on a movable circular dish, the speed of which could be varied. Rotation of the dish was started before the beginning of micturition and was stopped after completion. They showed decreasing bacterial counts towards the end of the sampling. Fourteen percent of the fractions were sterile.

None of the study subjects carried *Streptococcus faecalis*. This organism is infrequently isolated from the urethras of healthy females (10, 16) despite the observation that it inhibits the growth of lactobacilli in vitro (15).

Anaerobes were isolated from the urethras of 90% of the females studied. In a study carried out by Finegold et al. (8), anaerobes were recovered from 14 of 100 random urines, some of which had been held at room temperature for several hours. They were able to isolate anaerobes from 19 of 29 specimens (65%) of urethral urine (the first 10 to 20 ml voided). Stamey et al. isolated *Bacteroides* spp. from 17 of 67 (25%) of urethral urine specimens from healthy volunteers (16). It would seem from our data that most healthy females have anaerobes as part of their endogenous urethral flora. This anaerobic flora is also present on the introitus, posterior fourchette, and the cutaneous perineum. The anaerobes isolated from the urethral urine did not result from contamination of this fraction of urine by the introital and fourchette anaerobes because we were able to isolate anaerobes from the urethra by using a swab to collect the specimen so that there was no chance of contamination. Furthermore, the urethral flora is different from the vaginal flora. Bartlett et al. found that vaginal anaerobes outnumbered aerobes (2). *Peptococci* were the most frequently

recovered anaerobes. *B. melaninogenicus* was isolated from 8 of the 22 women studied (2). All five of our subjects had *B. melaninogenicus* isolated repeatedly from their urethras. We were also able to isolate it from 66% of the 18 additional subjects who had urethral urine cultured once only. *B. melaninogenicus* subsp. *intermedius* was most common. Strains of this subspecies have been isolated from sites above and below the diaphragm (9). Burdon isolated *B. melaninogenicus* from an area about the clitoris in all 35 patients cultured (3). Whether there is selective colonization of the urethra and clitoral area by *B. melaninogenicus* deserves further study.

The species *B. melaninogenicus* contains all strictly anaerobic, nonmotile, nonsporing, gram-negative bacilli that produce black- or brown-pigmented colonies when grown on media containing blood. The pigment is an intracellular or cell-associated derivative of hemoglobin assimilated from the medium (4). The characteristic pigmentation remains the basic criterion for differentiation from other *Bacteroides* species (11). However, not all isolates of *B. melaninogenicus* produce a black pigment when grown on blood-containing media. Harding and co-workers (9) characterized 58 human isolates of *B. melaninogenicus*. Three isolates (two *B. melaninogenicus* subsp. *melaninogenicus* and one subsp. *intermedius*) did not produce a black pigment. All our isolates of *B. melaninogenicus* subsp. *intermedius* failed to produce a black pigment while growing on blood or laked-blood agar in spite of prolonged incubation. However, all isolates fluoresced brick red under UV light.

The role of the urethral flora as a host defense mechanism remains to be determined. The resident flora of the urethra may prevent colonization with aerobic gram-negative rods by the production of inhibitors or by prevention of adherence of these organisms to the epithelial cells,

thereby enabling them to be washed out in the urine before they can ascend to the bladder.

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