# Periurethral Anaerobic Microflora of Healthy Girls

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The periurethral anaerobic and aerobic microfloras were investigated in 18 healthy premenarcheal girls, 5 to 14 years of age, by using a quantitative sampling method. Colonization of the female periurethral area with enterobacteria seems to be an important step in the development of urinary tract infections, and the present study was undertaken as a stage in elucidating factors that might control the establishment of urinary tract pathogens periurethrally. The study showed that obligate anaerobic bacteria constituted 95.0% (standard error, ±5.8%) of the total colony-forming units per square centimeter of periurethral area. An average of 7.0 different anaerobic and 2.7 different aerobic strains per specimen was obtained. The flora was dominated by anaerobic gram-positive cocci and gram-positive rods, whereas anaerobic gram-negative rods comprised a minor part. The most commonly encountered anaerobic isolates were peptococci and peptostreptococci, propionibacteria, bifidobacteria, eubacteria, and bacteroides in decreasing order of frequency. The aerobic flora consisted most commonly of nonhemolytic streptococci and diphtheroids. The findings suggest that the periurethral microenvironment is a distinctive ecological niche, separate from the fecal and skin biotas, although it has some characteristics in common with the vaginal flora.

The periurethral region of healthy girls probably forms a barrier against urinary tract infections (UTI). A previous study of ours on the periurethral aerobic bacterial flora revealed no or very small numbers of gram-negative bacteria and enterococci in healthy girls, whereas UTI-prone individuals were colonized with urinary tract pathogens even during infection-free periods (2, 3). This was in accordance with studies on the introital aerobic flora of adult women (8, 24).

Our knowledge is fragmentary about the mechanisms creating a microenvironment devoid of enterobacteria in the normal individual but failing to do so in UTI-prone persons. Properties of the periurethral mucosal cells seem to be one important factor, cells in UTI-prone girls having a significantly higher capacity for adherence of bacteria than those in healthy girls (12).

With other human microbiotas, it has been shown that the indigenous flora might interfere with the establishment of potential pathogens; e.g., alpha-streptococci in the pharynx interfere with the beta-hemolytic group A streptococci (4), cervical lactobacilli interact correspondingly with *Neisseria gonorrhoeae* (20), and interfer-

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ence between strains of *Staphylococcus aureus* has been useful in curtailing staphylococcal disease among newborn infants (22). Thus, it is conceivable that the indigenous periurethral microflora may also play a role in controlling the establishment of urinary tract pathogens in healthy individuals and that this balance for some reason may be altered in UTI-prone ones.

To study possible interactions between organisms in the periurethral area, it is necessary to have a clear picture of the normal flora. For that reason, the periurethral indigenous bacterial flora was studied in healthy girls. A sampling method was used that permitted a quantitative evaluation of the microflora. The main purpose was to characterize the anaerobic bacterial flora since the aerobic flora has been analyzed earlier (2). Aerobic cultivation was, however, also performed to allow comparison of the relative frequencies of aerobic vis-à-vis anaerobic bacteria.

## MATERIALS AND METHODS

**Subjects.** Eighteen girls attending either a child health care center or a pediatric outpatient clinic for minor surgical complaints were studied. None of them had any history of UTI and urine cultures were negative. None had any infectious disease at the time of examination, and none had taken any antibiotics during the 2 months before sampling. Ten girls were 5 to 10 years old and eight girls were 11 to 14 years old.

**Sampling technique.** A quantitative method for periurethral sampling, previously described in detail (2), was used to determine the anaerobic and aerobic periurethral floras. A 5-ml plastic syringe was filled with sterile 12% (wt/vol) gelatin (Difco Laboratories, Detroit, Mich.), kept anaerobically at 4°C to obtain a firm consistency, and then kept at room temperature for 10 min before use. The needle end of the syringe was cut off, and the gelatin, with a cross-sectional area of approximately 1 $cm^2$, was pressed against the urethral orifice.

A 2-mm slice (0.2 ml of gelatin) with the bacteria-bearing surface was cut off and put into a sterile tube, which had been gassed out with carbon dioxide. The tube was transported to the laboratory in an anaerobic jar (GasPak system, BBL Microbiology Systems, Cockeysville, Md.).

**Cultivation techniques.** The further handling of the specimens was performed within 2 h of collection. A 2.3-ml portion of Virginia Polytechnic Institute dilution salts solution (11) was added to the gelatin sample under a flow of oxygen-free $CO_2$. The gelatin was dissolved at 37°C for 10 min and homogenized by agitation in a Vortex mixer. Portions (0.1 ml; i.e., 1/25 of the dissolved gelatin sample) were spread onto freshly prepared blood agar plates (10% [vol/vol] horse blood in agar base; Oxoid, London, England) for anaerobic and aerobic incubation and onto different selective culture media (Table 1). Serial dilutions (1/10, 1/100, 1/1,000) of the dissolved gelatin sample were also made in the Virginia Polytechnic Institute dilution salts solution, and 0.1-ml portions were inoculated onto blood agar plates for anaerobic and aerobic incubation. Selective media (Table 1) were also inoculated with some of these dilutions.

Anaerobic plates were incubated at 37°C by using the GasPak system and examined after 10 days. Aerobic media were incubated in air or 5% $CO_2$ at 37°C and examined after 48 h and once again after 10 days. Total counts and counts of different organisms based on colony morphology were determined and recorded as colony-forming units (CFU) per square centimeter of periurethral area. In general, the anaerobically incubated blood agar plates inoculated with low dilutions of the specimens showed heavy growth, so that colony counting and isolation of different organisms was possible only on blood agar plates bearing higher dilutions of the specimens (1/100, 1/1,000).

Colony types were enumerated on plates containing 30 to 300 colonies. The selective media revealed some additional strains in low numbers, but in some cases organisms present in small numbers were probably missed. Each different type of colony was isolated. Organisms isolated from anaerobic media were incubated anaerobically and aerobically to determine air tolerance.

**Identification of microorganisms.** All isolates were Gram stained, and the slides were evaluated by two persons independently of each other. Gram-negative rods belonging to *Enterobacteriaceae* were identified to species by the API 20E test kit system (Analytab, Plainview, N.Y.) (17). Biochemical identification of streptococci was performed according to Facklam et al. (7). The following tests were performed: growth on agar containing 6.5% (wt/vol) NaCl (Difco), growth in Trypticase soy broth (BBL) containing 40% (wt/vol) bile (Difco), growth on hematin agar (Oxoid) with 0.04% (wt/vol) potassium tellurite, hippurate hydrolysis in peptone broth (Difco) containing 1% (wt/vol) sodium hippurate, hydrolysis of esculin in Enterococcosel agar (BBL), and acid production from arabinose and raffinose. Identification of *Streptococcus agalactiae* was verified by a precipitation test with group-specific antiserum (14). The following biochemical tests were used for the identification of staphylococci (*S. aureus* and *S. epidermidis*): oxidation-fermentation test with glucose and tests for production of coagulase and deoxyribonuclease. Free coagulase was detected in a tube assay using citrated rabbit plasma. Production of deoxyribonuclease was detected

TABLE 1. *Media used for identification of anaerobic and aerobic microorganisms in the periurethral area according to the Wadsworth Anaerobic Bacteriology Manual (25)*

| Medium | Incubation | Dilutions | Purpose |
|---|---|---|---|
| Blood agar (10% horse blood; Oxoid) | Aerobic | $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$ | Total aerobic counts, predominant flora |
| Cystine–lactose–electrolyte-deficient agar (Oxoid) | Aerobic | $10^0$ | *Enterobacteriaceae* |
| Enterococcosel agar (BBL) | Aerobic | $10^0$ | Enterococci |
| Mitis salivarius agar (Difco) | Aerobic | $10^0$ | α-Streptococci |
| Hematin agar (Oxoid) | 5% $CO_2$ | $10^0$, $10^{-1}$ | *Haemophilus, Neisseria* |
| Blood agar (10% horse blood) | Anaerobic | $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$ | Total anaerobic counts, predominant flora |
| Kanamycin-vancomycin blood agar (Oxoid) | Anaerobic | $10^0$, $10^{-1}$ | Bacteroides |
| Neomycin-vancomycin blood agar (Oxoid) | Anaerobic | $10^0$, $10^{-1}$ | Fusobacteria |
| Phenylethyl alcohol agar (Difco) | Anaerobic | $10^0$, $10^{-1}$, $10^{-2}$ | Peptococci, peptostreptococci |
| Egg yolk-neomycin agar (Oxoid) | Anaerobic | $10^0$, $10^{-1}$ | Clostridia |
| Rogosa SL agar (Difco) | Anaerobic | $10^0$, $10^{-1}$ | Lactobacilli |
| Veillonella agar (Difco) | Anaerobic | $10^0$ | Veillonella |

on deoxyribonuclease agar (Difco) (5). When staphylococcal colony counts exceeded eight colonies per plate (>200 CFU/cm²; three strains), isolates were identified to species according to Kloos and Schleifer (13) for identification of, e.g., *Staphylococcus saprophyticus*. The following tests were performed: hemolysis of blood agar (5% [vol/vol] bovine blood in agar base), nitrate reduction in peptone broth (Difco) containing 0.2% potassium nitrate, and aerobic acid production from various carbohydrates in a broth medium (Oxoid). The following carbohydrates were tested: fructose, arabinose, ribose, maltose, lactose, sucrose, trehalose, xylitol, xylose, and mannitol. Diphtheroids were identified by colony appearance, typical Gram staining, and a positive catalase test. Catalase-negative, gram-positive aerobic rods producing lactic acid from glucose fermentation (identified by gas-liquid chromatography) were characterized as facultative lactobacilli. Yeasts were identified according to standard laboratory procedures (23).

Anaerobic bacteria were identified to genus level by their morphological appearance in Gram staining and by analysis of end products of glucose metabolism by gas-liquid chromatography according to Holdeman and Moore (11).

## RESULTS

**Total viable counts and relative frequencies of anaerobic versus aerobic microorganisms.** Total viable counts (including anaerobic and aerobic bacteria and yeast cells) per square centimeter of periurethral area showed great variations between individual specimens. The mean total count was $1.4 \times 10^6$ CFU/cm², the median count was $4.8 \times 10^5$ CFU/cm², and the range was $6.6 \times 10^3$ to $6.3 \times 10^6$ CFU/cm².

Aerobic and anaerobic microorganisms were recovered from 17 samples. The ratio of aerobic/anaerobic bacteria was fairly constant in all specimens. Thus, anaerobic microorganisms constituted 95.0% (standard error, ±5.8%) of the total CFU per square centimeter. The remaining specimen yielded a pure culture of facultative lactobacilli.

**Anaerobic microflora.** An average of 5.5 (range, 0 to 11) different anaerobic strains (i.e., anaerobic isolates identified to genus level) per specimen was obtained from the anaerobically incubated blood agar plates. Some additional types of microorganisms present in small numbers could be found by the use of selective media, and with these strains included, an average of 7.0 (range, 0 to 14) different strains per specimen was found. In the following presentation, the mean numbers of strains per specimen take account of findings from both blood agar plates and selective media.

Table 2 lists the counts of different anaerobic bacteria grouped according to Gram staining properties. The proportions of different anaerobic bacteria expressed as a percentage of total

CFU per square centimeter are shown in Fig. 1.

Gram-positive cocci were frequently isolated, with an average of 2.9 different strains per sample. Specimens with a preponderance of gram-positive cocci (>50% of total CFU per square centimeter) originated in six instances out of seven from girls of less than 11 years of age. Anaerobic nonsporulating gram-positive rods also often dominated the periurethral flora. Five of six samples with a preponderance of gram-positive rods were obtained from girls in the older age group. The mean number of different types of bacteria identified per sample was 2.2. Figure 2 shows the distribution of the different genera of gram-positive rods in terms of percentage of the total CFU per square centimeter. In contrast, clostridia were only isolated in one instance, from a 10-year-old girl ($7.5 \times 10^2$ CFU/cm²).

Anaerobic gram-negative rods were isolated from several specimens, but were never predominant. An average of 1.7 different strains per specimen was obtained, but the majority of these

TABLE 2. *Types and concentration of organisms present in periurethral samples from 18 healthy girls*

| Morphological group | No. of specimens at (CFU/cm² of periurethral area): | | |
|---|---|---|---|
| | ≤10³ | >10³–10⁵ | >10⁵ |
| Anaerobic microorganisms | | | |
| Gram-positive cocci | 4 | 5 | 9 |
| Gram-positive rods | 2 | 4 | 12 |
| Gram-negative cocci | 17 | 1 | 0 |
| Gram-negative rods | 7 | 9 | 2 |
| Clostridia | 18 | 0 | 0 |
| Aerobic microorganisms | | | |
| Gram-positive cocci | 9 | 8 | 1[a] |
| Gram-positive rods | 10 | 7 | 1[b] |
| Gram-negative cocci | 18 | 0 | 0 |
| Gram-negative rods | 18 | 0 | 0 |
| Yeasts | 16 | 2 | 0 |

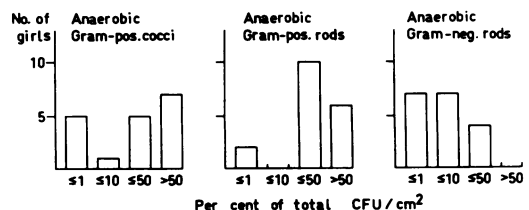[a] *S. agalactiae.*
[b] *Lactobacillus* species.



FIG. 1. *Presence of anaerobic gram-positive cocci, gram-positive rods, and gram-negative rods, expressed in percentage of total CFU per square centimeter in the periurethral samples from 18 healthy girls.*
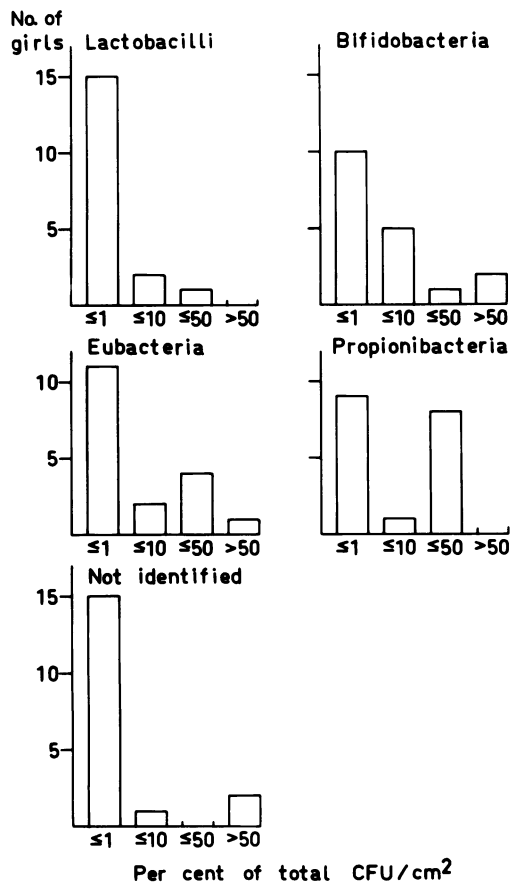
No. of
girls


FIG. 2. *Distributions of different anaerobic gram-positive rods, expressed in percentage of total CFU per square centimeter in the periurethral samples from 18 healthy girls.*

most frequent aerobic bacteria (Table 2). One 5-year-old girl was heavily colonized with *S. aga-lactiae* ($1.3 \times 10^6$ CFU/cm$^2$), and one 11-year-old girl carried a homogeneous lactobacillus flora ($6.3 \times 10^6$ CFU/cm$^2$).

Of potential pathogens of the urinary tract, gram-negative rods belonging to the *Enterobacteriaceae* and enterococci were isolated from only one and two samples, respectively, and in low counts. *S. epidermidis* was isolated from several specimens, but in small numbers (Fig. 4). Three strains of coagulase- and deoxyribonuclease-negative straphylococci were identified to
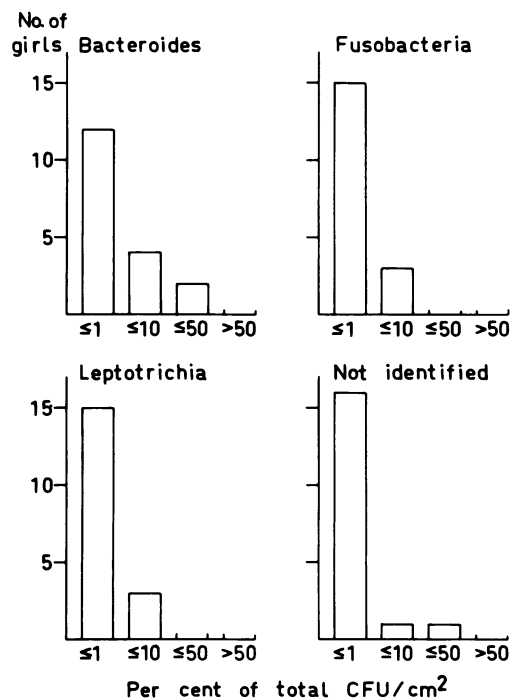


FIG. 3. *Distributions of different anaerobic gram-negative rods, expressed in percentage of total CFU per square centimeter in the periurethral samples from 18 healthy girls.*
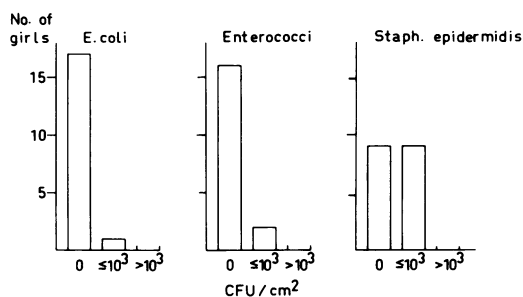
strains were recovered only from selective media and in very small numbers. They represented less than 1% of total CFU per square centimeter (Fig. 1). The quantitative distributions of different genera of gram-negative rods are given in Fig. 3 as a percentage of the total CFU per square centimeter. All strains of *Bacteroides* species, which constituted 1% or more of the total CFU per square centimeter (Fig. 3), were isolated from the blood agar plates, whereas the selective medium (kanamycin-vancomycin blood agar [Oxoid]) yielded no bacteroides or very few colonies.

Anaerobic gram-negative cocci (*Veillonella*) were isolated in one instance, from a 12-year-old girl ($2.3 \times 10^3$ CFU/cm$^2$).

**Aerobic microorganisms.** The mean number of various aerobic strains isolated per specimen was 2.7 (range, 1 to 4). Gram-positive cocci (mainly nonhemolytic streptococci) and gram-positive rods (mainly diphtheroids) were the



FIG. 4. *Viable counts of Escherichia coli, enterococci, and S. epidermidis in the periurethral samples from 18 healthy girls.*

species. None turned out to be *S. saprophyticus.*

No strains of *S. aureus* or neisseriae were isolated. *Haemophilus vaginale* was not encountered, which may either reflect the real absence of this organism or be due to lack of the use of a selective agar. *Candida albicans* was recovered from three girls, 11, 10, and 7 years old ($1.0 \times 10^3$, $1.3 \times 10^3$, and $5.0 \times 10^2$ CFU/cm², respectively), and *Saccharomyces cerevisiae* was recovered from one 14-year-old girl ($2.5 \times 10^4$ CFU/cm²).

## DISCUSSION

This investigation of healthy premenarcheal girls shows an indigenous periurethral flora that is multibacterial and consists mainly of anaerobes, the most common being gram-positive cocci and rods. There were great variations among individuals with respect to the total viable counts per square centimeter of periurethral area and to the numbers of bacterial genera represented by the isolates. However, the ratio of aerobes to anaerobes was remarkably constant.

Herein, a quantitative method has been used to describe the periurethral microflora. Most other studies of the urogenital region concern only qualitative aspects, and certain bacteria may have been overemphasized with a disregard for others. In some previous studies, the specimens were precultured in broth before being inoculated onto solid media for isolation of different bacteria, a method that might favor rapidly growing species.

By using qualitative methods, certain bacteria, e.g., *Bacteroides* species, may receive undue emphasis, especially when present in low numbers, as a result of the choice of particular selective media. In this respect, quantitative methods are potentially advantageous since it may be more obvious when certain bacteria constitute a minor part of the flora. The risk of overestimating transient contaminants in small numbers from adjacent microbiotas is accordingly reduced.

The findings of this study suggest that the periurethral region is a bacteriological niche, with a flora different from those of feces and skin. The aerobic fecal flora comprises mainly enterobacteria and enterococci, whereas these bacteria are absent or very scanty in the periurethral region of healthy girls (2). The present study shows a similar dissociation between these areas concerning the anaerobic flora. Thus, although the anaerobic fecal flora is dominated by *Bacteroides* species (6), these bacteria were absent or found in comparatively small numbers in the periurethral area. In addition, all bacteroides strains constituting 1% or more of total CFU

were isolated from the blood agar plates but did not grow on the selective medium (kanamycin-vancomycin blood agar) used for isolation of bacteroides of fecal origin. This phenomenon may be indicative of distinct properties of the bacteroides strains peculiar to the periurethral region.

With regard to the skin surrounding the urogenital area, the findings are more complex. The majority of the bacteria isolated from the periurethral area, e.g., peptococci, peptostreptococci, bifidobacteria, and eubacteria, have not been reported in the skin flora, indicating that these two microenvironments may be distinct from each other (16). Nevertheless, such organisms as *S. epidermidis,* diphtheroids, and propionibacteria, which are typical constituents of the skin flora (16), were often isolated also from the periurethral area. However, the regular periurethral findings of *S. epidermidis* and diphtheroids make it probable that these bacteria are true constituents of the flora of the periurethral area, just as they are considered to be of the flora of the vagina (1). Although propionibacteria were found in fairly high counts in several periurethral samples, contamination seems unlikely since the samples did not show correspondingly high counts of the other species typical of the skin flora.

A more complicated matter is whether the periurethral niche is distinct from the adjacent urethral and vaginal microenvironments. Similarities could well be expected as embryologically this whole area derives from the urogenital sinus and is covered with squamous epithelium, which in adults shows cyclic variations due to hormonal influence (26). Other factors such as different secretions may, however, create differences in the microenvironment.

Existing data suggest that the aerobic microflora is similar throughout the distal urogenital area, i.e., constituted of *S. epidermidis,* diphtheroids, nonhemolytic and α-streptococci, and, in adult women, lactobacilli (8, 10, 19), indicating that this whole area might reasonably be looked upon as an entity. The whole area is also characterized by scanty findings of potential uropathogens such as enterobacteria and enterococci (2, 10, 19). *S. saprophyticus,* another common cause of UTI in young adult women (18), was not registered with the method used in this study. More extensive studies concerning the urethral flora (21) and the periurethral flora of adult women (Bollgren, unpublished data) suggest that *S. saprophyticus* rarely is a constituent of the normal flora in the distal urogenital area.

Comparisons with studies concerning the anaerobic microflora of these different parts of the distal urogenital area are more complex. Re-

cently, a study on the vaginal anaerobic microflora of girls was published (9). This study, in accordance with ours, revealed a multibacterial anaerobic flora, with a mean of 5.3 anaerobic species per vaginal sample, compared to 7.0 different strains per specimen in the present periurethral investigation. The results of the vaginal flora study, however, diverged from ours in other respects, e.g., high isolation rates of clostridia and *Bacteroides* species. Apart from the methodological differences, more important may be the fact that the two investigations studied different populations. Hammerschlag et al. (9) thus reported that *Bacteroides* species were more frequently isolated from girls under 3 years of age than from girls 3 to 15 years of age. These findings of anaerobic bacteria of probable fecal origin in the vagina correspond well with our previous studies showing high numbers of aerobic fecal bacteria in the periurethral area of young girls (2).

Most other studies on the vaginal and urethral anaerobic microfloras concern adult women, where the high glycogen content of the mucosal cells due to hormonal influences creates a micromilieu quite different from that in premenarcheal girls. In spite of this, our findings in many respects agree well with some quantitative studies on the anaerobic vaginal flora in adult women (1, 15), which showed that anaerobic bacteria often constituted about 90% of the adult vaginal flora, with a preponderance of gram-positive cocci and rods.

From a functional point of view, the introital area in adult women seems, at least, to have characteristics in common with the periurethral region in girls, since both areas may act as barriers against UTI.

## ACKNOWLEDGMENTS

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