Original Study

Longitudinal Changes in Vaginal Microbiota Composition Assessed by Gram Stain Among Never Sexually Active Pre- and Postmenarcheal Adolescents in Rakai, Uganda

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A B S T R A C T

Study Objective: To describe changes in vaginal microbiota and pH over time among never sexually active adolescents at different menarcheal stages.
Design: A cohort of 49 sexually inexperienced Ugandan adolescents provided weekly self-collected vaginal swabs and behavioral/health information for up to two years. Menarcheal stage was classified as: not experiencing menarche during follow-up (premenarcheal, n = 9), achieving menarche during follow-up (perimenarcheal, n = 20), and being postmenarcheal (n = 20) at enrollment. Vaginal microbiota were characterized as morphotypes of large gram-positive rods, small gram-negative or variable rods, and curved gram-negative rods based on Nugent Gram-stain criteria. Baseline measures were compared using nonparametric tests. Mean changes (β) in morphotypes and pH over time were estimated using longitudinal mixed-effects models.
Results: The baseline median (IQR: interquartile range) Nugent score was 8 (7–8) in premenarcheal, 4.5 (1–8) in perimenarcheal, and 1 (0–3) in postmenarcheal girls (P = 0.001). For each respective menarcheal stage, the median (IQR) counts of gram-positive rods were 0 (0–0), 10 (0–30), and 30 (18–30) (P = 0.002) and gram-negative or variable rods were 30 (30–30), 16 (0.5–30), and 0.5 (0–2.5) (P = 0.002) at enrollment. Counts of gram-positive rods increased (β = 0.259, 95% CI: 0.156, 0.362) and gram-negative or variable rods decreased (β = -0.201, 95% CI:-0.298,-0.103) significantly over time in premenarcheal girls, but not in other groups. Vaginal pH declined significantly in peri- and postmenarcheal girls only.
Conclusion: Vaginal microbiota composition varied by menarcheal stage at enrollment. Over time, significant changes in vaginal morphotypes occurred in premenarcheal girls, suggesting this may be an important period of transition.
Key Words: Vaginal microbiota, pH, Menarche, Lactobacilli, Bacterial vaginosis, Gram stain, Nugent score

Introduction

The vaginal microbial environment is a complex and dynamic system and varies with different stages of biologic maturation. In postmenarcheal women, bacteria from the genus Lactobacillus are usually the predominant vaginal micro-organisms and have been associated with a healthy vaginal ecosystem.[1] Lactobacilli are large gram-positive rods that metabolize glycogen from the vaginal epithelium into lactic acid under estrogenic stimulation, which is required to maintain an acidic vaginal pH (4.0–4.5) and considered to be a primary mechanism for inhibiting the growth of pathogenic organisms.[2–4] Bacterial vaginosis (BV) is characterized by the replacement of lactobacilli by an overgrowth of facultative and obligate anaerobic gram-negative bacteria and genital mycoplasmas. Moreover, epidemiologic studies have suggested an increased risk of HIV and sexually transmitted infections among women with BV and/or those who lack lactobacilli.[5–8]

In prepubescent girls, the vaginal pH is alkaline and the vaginal microbiota is comprised of both anaerobic and aerobic rods and cocci with low frequencies of lactobacilli, *Gardnerella vaginalis (G. vaginalis),* and Mobiluncus.[9–11] During puberty, estrogen levels rise to reach concentrations found in mature females.[12] Given that endocrine changes begin prior to onset of first menses and estrogen stimulates glycogen deposition in the vaginal epithelium, shifts in the composition of vaginal microbiota may precede first menses. One study among sexually active, postmenarcheal adolescents found a reduction in BV prevalence with increasing time since first menses (gynecologic age) and increasing breast and pubic hair development.[13] However, since all participants were sexually active, the study could not disaggregate the effects of vaginal microbiota changes due to early sexual activity from the effects of pubertal maturation. In addition, we know of no studies that have investigated changes in vaginal microbiota over time in girls at or around the time of menarche, which may

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be an important transitional period for determining a woman's predominant vaginal microbiota in adulthood.[14] In this paper, we present data on differences in vaginal microbiota assessed by Gram stain using Nugent morphotype scoring criteria and vaginal pH by menarcheal status among girls aged 13 to 18 who had not initiated sexual activity.

Materials and Methods

Study Design and Data Collection

Between 2001 and 2003, the Rakai Health Science Program (RHSP) conducted a 2-year cohort study of 312 consenting females aged 13–39 years in rural Rakai District, Uganda, to assess weekly changes in vaginal microbiota and factors associated with BV progression and resolution. Women were included regardless of HIV status, current or prior pregnancy, or history of sexual experience. Girls between 13 and 19 years were oversampled compared to women in the older age groups in order to assess vaginal microbiota at younger ages. For this publication, a secondary analysis was conducted on 49 adolescents from this cohort who reported never having had sexual intercourse at enrollment (age range: 13–18 years). In these 49 females, the period of observation was truncated at the time of sexual debut.

For all women enrolled in the cohort, data collection was conducted in participants' homes every week for 2 years. At each weekly visit, self-collected vaginal swabs and pH samples were collected and a short questionnaire was administered on sexual activity, menstrual history, and vaginal symptoms and treatment. At baseline and every 6 months, detailed data were collected on demographic characteristics, household environment, menstrual history, sexual behaviors, vaginal hygiene, and health status. Data on household dwelling characteristics and assets were collected at annual census visits.

Menarcheal stage was classified as: never experiencing menarche during follow-up (premenarcheal), achieved menarche during follow-up (perimenarcheal), and being postmenarcheal at enrollment. Gynecological age was estimated by subtracting the reported age at first menstruation from the age at enrollment (in years) in postmenarcheal girls and time to menarche was calculated as weeks from enrollment to reported first menses in perimenarcheal girls. To assess potential confounding of socioeconomic status, which has been associated with vaginal flora and age at menarche in the literature,[15,16] we generated a relative household wealth index based on a sum of possession of modern objects (radio, bicycle, vehicle) and dwelling characteristics (materials used for roof, walls, and floor, and availability of electricity and latrine) weighted by the inverse of the prevalence within the population. This weight has been shown to have good agreement between other weighting methods for constructing wealth indices using binary variables.[17] Vaginal hygiene behaviors included genital washing frequency, insertion of substances into the vagina, materials used for bathing, and water sources for bathing classified as protected (protected well, tap, or borehole), partially protected (unprotected well), and unprotected (rainwater or pond/lake) and based on the least protected source reported by the respondent out of three possible responses.

Vaginal swabs were rolled onto slides and air dried, Gram stained, and assessed for vaginal microbiota under oil immersion using the Nugent quantitative morphologic classification, which characterizes vaginal microbiota by the presence of morphotypes of large gram-positive rods, small gram-negative to variable rods, and curved gram-variable rods.[18] In postmenarcheal women, these morphotypes most often correspond to Lactobacillus, *G. vaginalis* or *Bacterioides*, and *Mobiluncus* spp., respectively; however, premenarcheal girls may differ in vaginal bacterial composition as described previously.[9–11] The frequency of morphotypes were quantified as none (no organisms), less than 1, 1 to 4, 5 to 30, or more than 30 organisms per high power field and a morphotype score from 0 to 4 was assigned to large gram-positive rods (weighted such that the absence yielded the highest score) and small gram-negative to variable rods and a score from 0 to 2 for curved gram-negative rods. The morphotype scores were summed to obtain an overall Nugent score ranging from 0 to 10. A Nugent score of 7–10, which is typically used to diagnose BV in postmenarcheal women, was not used to diagnose BV in premenarcheal girls. In addition, we assessed large gram-positive rods and small gram-negative to variable rod morphotype scores separately. For each respective group, we assigned a count value of 0, 0.5, 2.5, 17.5, and 30 using the midpoints of the intervals corresponding to the original counts of the average number of organisms per field. Slides were read in batches within 2 weeks of collection by 2 independent laboratory technicians and discrepancies resolved by a third senior technician. A 10% random sample was sent to Dr. Sharon Hillier's lab at the Magee-Women's Research Institute, University of Pittsburgh, for external quality control.Vaginal pH was determined by BAKER-pHIX pH papers (pH 4.0–9.0, Phillipsburg, NJ) affixed to a spatula. Vaginal pH samples were not assessed during menstruation in the study and vaginal microbiota readings collected during menstruation were excluded from analyses.

This study was reviewed and approved by institutional review boards (IRBs) in Uganda (the Science and Ethics Committee of the Uganda Virus Research Institute) and the United States (the Johns Hopkins Bloomberg School of Public Health IRB and the Columbia University Medical Center IRB).

Statistical Methods

Baseline measures were compared by menarcheal status using Fisher exact tests for categorical variables and Kruskal-Wallis tests for continuous variables. Longitudinal linear mixed-effects models using a normally distributed random intercept (mean = 0) were used to estimate mean changes (β) in vaginal microbiota and pH over weekly follow-up. Confidence intervals were based on bootstrap estimations of the standard error with 500 replications.[19] The interaction between menarcheal groups and time was assessed by pairwise comparisons of the beta coefficients