Vaginal yeast colonisation, prevalence of vaginitis, and associated local immunity in adolescents

M M Barousse, B J Van Der Pol, D Fortenberry, D Orr, P L Fidel Jr

Objectives: To evaluate point prevalence vaginal yeast colonisation and symptomatic vaginitis in middle adolescents and to identify relation of these yeast conditions with reproductive hormones, sexual activity, sexual behaviours, and associated local immunity.

Methods: Middle adolescent females (n = 153) were evaluated for sexually transmitted infections (STIs), asymptomatic yeast colonisation, and symptomatic vulvovaginal candidiasis (VVC) by standard criteria. Also evaluated were local parameters, including vaginal associated cytokines, chemokines, and antibodies, vaginal epithelial cell antifungal activity, and Candida specific peripheral blood lymphocyte responses. Correlations between yeast colonisation/vaginitis and local immunomodulators, reproductive hormones, douching, sexual activity, condom use, and STIs were identified.

Results: Rates of point prevalence asymptomatic yeast colonisation (22%) were similar to adults and similarly dominated by Candida albicans, but with uncharacteristically high vaginal yeast burden. In contrast with the high rate of STIs (18%), incidence of symptomatic VVC was low (< 2%). Immunological properties included high rates of Candida specific systemic immune sensitisation, a Th2 type vaginal cytokine profile, total and Candida specific vaginal antibodies dominated by IgA, and moderate vaginal epithelial cell anti-Candida activity. Endogenous reproductive hormones were in low concentration. Sexual activity positively correlated with vaginal yeast colonisation, whereas vaginal cytokines (Th1, Th2, proinflammatory), chemokines, antibodies, contraception, douching, or condom use did not.

Conclusion: Asymptomatic vaginal yeast colonisation in adolescents is distinct in some ways with adults, and positively correlates with sexual activity, but not with local immunomodulators or sexual behaviours. Despite several factors predictive for VVC, symptomatic VVC was low compared to STIs.

Vulvovaginal candidiasis (VVC), caused by Candida species, is an opportunistic fungal infection that is common in women of childbearing age. Although VVC in pre-menarchal and post-menopausal women is rare, there are several exogenous factors that predispose menarchal women to acute VVC, including several hormonal modulations associated with pregnancy, luteal phase of the menstrual cycle, oral contraceptive use, hormone replacement therapy (HRT), and non-hormonal factors such as antibiotic use and uncontrolled diabetes mellitus. There is also a population of women (5–10%) that suffer from recurrent VVC (RVVC) with no known predisposing factors, hormonal or otherwise. Cell mediated immunity (CMI) by T helper (Th) 1 type responses is generally considered to be associated with resistance to mucosal candidiasis, whereas Th2 type responses are associated with susceptibility to infection. Adolescents represent a menarchal population that is newly influenced by reproductive hormones and is also extremely susceptible to sexually transmitted infections (STIs). In fact, an estimated three million adolescents acquire an STI annually in the United States. The new influence of reproductive hormones in adolescents that causes vast changes in tissues may also increase the susceptibility to VVC. Accordingly, the purpose of the present study was to characterise symptomatic and asymptomatic yeast colonisation, reproductive hormones, Candida specific systemic immunity, and vaginal immunity in adolescent females, and to identify correlations between these parameters and vaginal yeast carriage.

MATERIALS AND METHODS
Participants/specimen collection
Enrolment was based on attendance at an adolescent health clinic for multiple reasons (oral contraception, symptoms, pregnancy testing, attendance with a friend, etc) and participants were enrolled into a study conducted through the Mid-America Adolescent Sexually Transmitted Disease Clinical Research Center between May 1999 and September 2001. Having an STI or symptoms was not a prerequisite. Informed consent was obtained from all participants as well as permission from the accompanying parent/guardian for entry into the study. All procedures were followed in the conduct of clinical research in accordance with the institutional review boards at Louisiana State University Health Sciences Center (LSUHSC), New Orleans, LA, and Indiana University Medical Center, Indianapolis, IN. Participants were enrolled and the specimens were collected at Indiana University Medical Center. Specimens were subsequently
Specimens collected from enrolled subjects included a vaginal lavage, vaginal swab, endocervical swab, vaginal smears, and blood (30 ml) by venepuncture into Vacutainer CPT cell preparation tubes or red top tubes (without EDTA) (Becton Dickinson, Sparks, MD, USA). All specimens were collected at scheduled visits by a provider. The vaginal lavage was collected after a 30–40 second aspiration with 5 ml of non- pyrogenic sterile saline. An endocervical brush was used in the vagina before lavage and lateral swab to facilitate the release of sloughing vaginal epithelial cells. The lavage fluid was processed as previously described and stored at −70°C until use. The lavage cell pellet was stored at −70°C in cryopreserved medium (50% fetal bovine serum (FBS), 30% RPMI 1640 medium, and 20% DMSO; Gibco, Grand Island, NY, USA) until use. The vaginal swab was plated on Candida Chromagar (ChroMagar, Paris, France) and incubated at 37°C for 48 hours. Green colony forming units (cfu) were further evaluated for germ tube formation (evidence for C. albicans) in FBS for 2–3 hours in a 37°C water bath. Non-germ tube forming colonies were speckled using API 20 AUX (bioMerieux, Hazelwood, MO, USA). Subjects were considered to have VVC if fungi were observed in the KOH smear, the subject was positive for signs and symptoms of vaginitis (assessed by a provider), and had a positive vaginal smear culture. One vaginal smear was spray fixed and stained by the Papanicolaou technique. The second vaginal smear was Gram stained. Blood in CPT tubes were centrifuged (1000 × g) before being shipped to LSUHSC. Upon arrival, peripheral blood mononuclear cells (PBMCs) were washed twice with Hanks balanced salt solution (HBSS) (Gibco, Grand Island, NY, USA) and enumerated using trypan blue dye exclusion. Serum was collected from clotted blood following centrifugation and stored at −70°C until use.

Subjects were asked to complete a detailed questionnaire including information on sexual behaviours, contraceptive use, and demographics. Enrolled middle adolescent females (n = 153) had a mean age of 15.4 (SD 0.9) (ages ranged from 14–17; 14 years of age (n = 29), 15 years of age (n = 53), 16 years of age (n = 52), 17 years of age (n = 19)) and were predominantly African-American (85%) (14% white, 1% Hispanic). Hormonal contraceptive methods used by the adolescent cohort were surveyed. Of those enrolled, 39 (29%) subjects were using progesterone contraception (Depo-Provera), 29 (21%) were using an oestrogen form of oral contraception (Depo-Provera), and 29 (21%) were using a vaginal form of estriol or estradiol. The participants were asked to report on sexual activity (vaginal intercourse) and the interval of time from the last sexual intercourse. Results indicated that 84% of the cohort was sexually active at the time of testing. Data on condom use were available for 82 subjects and stratified into three groups: having used condoms for every sexual intercourse (always; n = 38), having used condoms for some incidences of sexual intercourse (sometimes; n = 21), or having never used condoms for any sexual intercourse (never; n = 23).

Data on douching were available for 146 subjects and 53 reported having douched ever. Bacterial vaginosis (BV), identified by Nugent score from vaginal Gram stain, was detected in 33% (36/108) of available subjects. C. trachomatis, T. vaginalis, and N. gonorrhoeae were identified by polymerase chain reaction (PCR) using the Amplicor and the Cobas Amplicor tests from endocervical swabs. In all, 104 subjects tested negative for any STI, six tested positive for T. vaginalis, four tested positive for N. gonorrhoeae, and 10 tested positive for C. trachomatis. Some tested positive for more than one STI (one with N. gonorrhoeae and T. vaginalis, two with C. trachomatis and T. vaginalis, and four with C. trachomatis and N. gonorrhoeae). Total rate of STIs was 18%, which is consistent with published reports.

**Identification of stage of menstrual cycle**

Stage of menstrual cycle, excluding those on Depo-Provera, was verified by oestradiol and progesterone concentrations in sera by radioimmunoassay (RIA) at the clinical endocrinology laboratory in the Division of Reproductive Endocrinology at the Detroit Medical Center, Detroit, MI. Local cellular maturation indices from a vaginal smear stained by the Papanicolaou technique were also evaluated for epithelial cell morphology. The epithelial cells were identified as basal, intermediate, or superficial by viewing 10 fields/slide. Results showed that 34% of vaginal smears exhibited superficial epithelial cells. Normal ranges in the menstrual cycle for superficial epithelial cells are follicular phase (20%), ovulatory phase (70%), and luteal phase (60%). The mean oestrogen level was 80.4 pg/ml (range 5–269.9 pg/ml) and the mean progesterone level was 2.4 ng/ml (range 0.21–21.7 ng/ml). Normal ranges in the menstrual cycle for oestrogen are: follicular phase (50–250 pg/ml), ovulatory phase (>350 pg/ml), and luteal phase (200–350 pg/ml). Normal ranges in the menstrual cycle for progesterone are follicular phase (1–3 ng/ml), ovulatory phase (2–3 ng/ml), and luteal phase (>8 ng/ml).

**Systemic immune responses**

PBMC proliferation was conducted as previously described. PBMCs were cultured in serum free AIM V lymphocyte media (Gibco, Grand Island, NY, USA) supplemented with glutamine and either C. albicans heat killed blastospores (HKB), C. albicans soluble cytoplasmic substances (SCS) (kind gift of Judith Domer, Appalachian State University, Boone, NC, USA), or phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 6 hours at 37°C, 5% CO₂, the cells were harvested onto glass fibre filter paper and the incorporated radioactivity was measured by liquid scintillation (Beckman Instruments, Fullerton, CA, USA). Data were expressed as proliferation indices (mean counts per minute (cpm) for stimulated cells/mean cpm for non-stimulated cells).

**Cytokines and chemokines**

Cytokines and chemokines in vaginal lavages were quantified by capture ELISA (BD Pharmingen, San Diego, CA, USA) according to manufacturer’s instructions and as previously described. Absorbance was read at 450 nm using an automated plate reader (Bio-Tek Instruments, Winooski, VT, USA). Cytokine and chemokine concentrations were expressed as pg/ml. The total protein content was determined for each vaginal lavage sample using the BCA protein assay kit (Pierce Chemical, Rockford, IL, USA) and bovine serum albumin (BSA) as the standard (Sigma-Aldrich, St Louis, MO, USA). Concentrations for each sample were extrapolated from the standard curve and expressed as mg/ml. All cytokines and chemokines were ultimately normalised to total protein in the sample and expressed as pg/mg protein.

**Total antibodies**

Total IgA and IgG immunoglobulin concentrations in vaginal lavages were quantified for each sample by ELISA as previously described. Total antibody concentrations were expressed as ng/mg protein.

**Candida specific antibodies**

The procedure for Candida specific antibody detection was similar to that for cytokines and total antibodies, except the
plates were coated with *Candida* soluble cytoplasmic substances (SCS). Because commercial *Candida* specific antibodies were not available to use as the standard, data were expressed as optical density (OD) and normalised to the OD for total immunoglobulin of that isotype for each sample.

**Epithelial cell growth inhibition of C albicans**

A $^3$H glucose incorporation assay was employed. Enriched epithelial cells from all patients with sufficient numbers of cells were tested independently. *C albicans* blastosporidia and epithelial enriched cells at various effector to target (E:T) ratios (40:1 to 1:25:1), along with *Candida* and epithelial cells cultured alone (controls), were incubated for 9 hours at 37°C, 5% CO$_2$ in the presence of 1 μCi [${}^3$H] glucose. Following incubation, cultures were harvested and incorporated $^3$H glucose was measured by liquid scintillation. Percentage growth inhibition was calculated as follows: 1 [(mean cpm experimental well – mean cpm effector cell wells)/mean cpm *C albicans* wells] x 100.

**Statistics**

Contingency table analysis including point and interval estimates and odds ratios investigating the association of potential risk factors with the presence of colonisation was performed using the $\chi^2$ test. The unpaired Student's $t$ test was used to analyse the immunological parameters. Significant differences were defined at a $p$ value of $<0.05$. Not all subjects had sufficient specimens for testing or the samples became limiting for some tests. The sample size for each parameter, however, represented all available specimens.

**RESULTS**

**Vaginal yeast colonisation**

Asymptomatic vaginal yeast colonisation was detected in 22% (34/153) of participating adolescents. Of these 34 subjects, 31 (91%) were colonised with *C albicans*, two (6%) were colonised with *C glabrata*, and one (3%) was colonised with *C tropicalis*. Two subjects were colonised with both *C albicans* and *C glabrata*. Semiquantitative vaginal yeast burden showed that 53% had low level colonisation (<200 cfu/swab culture), 18% had intermediate (200–1000 cfu/swab culture), and 29% had high level colonisation (>1000 cfu/swab culture) (table 1). In those sexually active (n = 118), vaginal yeast colonisation was detected in 24%, compared to only 4% of those not sexually active (n = 24) (table 2). The number of sexual partners did not affect the percentage of those colonised (data not shown). Of those sexually active, colonisation was not affected by condom use, douching, or hormonal contraception (table 2). There was no association between the presence of colonisation and bacterial vaginosis. Finally, 22% of those with an STI (n = 7/32) exhibited yeast colonisation, which was equally distributed between low and high levels of fungal burden. Two individuals (1.3%) had symptomatic vaginitis, both of which were sexually active with one taking progesterone contraception. Neither subject had BV.

**Candida immune sensitisation**

Subjects that complied with blood withdrawal (n = 63) were tested for *Candida* specific systemic responsiveness by PBMC proliferation in response to *Candida* HKB and SCS. Results showed that 95% of the adolescents tested responded to one or both *Candida* antigens with a proliferation index of at least 3.0. The mean proliferation index (PI) for HKB was 52.5 (8.2) with 95% of enrollees with positive responses and for SCS was 28.9 (4.8) with 84% of enrollees with positive responses. Responses to PHA, used as a quality control for the culture conditions, showed 100% of enrollees had positive responses (data not shown).

**Vaginal epithelial cell antifungal activity**

Anti-*Candida* activity by vaginal epithelial cells was examined for 70 non-colonised subjects with sufficient cellular recovery. Results showed a dose response with a mean of $\sim$26% growth inhibition at a 40:1 effector to target (E:T) ratio, $\sim$20% at a 20:1 E:T ratio, $\sim$14% at a 10:1 E:T ratio, and $\sim$9% at E:T ratios of 5:1 and lower.

**Vaginal associated immunomodulators**

**Antibodies**

The vast majority of available vaginal samples (~98%) had detectable concentrations of both total lavage and *Candida* specific antibodies. Total and *Candida* specific IgA antibodies were in higher concentrations than IgG antibodies (fig 1). Total and *Candida* specific IgA and IgG concentrations for those with and without yeast colonisation was not significantly different.

**Cytokines**

Th1 (IL-2, IL-12, IFN-γ) and Th2 (IL-4, IL-10, TGF-β) type cytokines were detectable (above the sensitivity of the assay) in $\sim$79% of available samples with Th1 type cytokines dominated by IL-2 and Th2 type cytokines dominated by TGF-β (fig 2). Th1 type cytokines were generally in lower concentrations and varied less than Th2 type cytokines. Proinflammatory cytokines (IL-6, IL-1α, TNF-α) were present in all of the samples tested and were dominated by IL-1. Chemokines (IL-8, RANTES, MCP-1) (fig 3) were present in $\sim$75% of the available samples and were dominated by IL-8. Concentrations of Th1 and Th2 type cytokines and chemokines in those with or without vaginal yeast colonisation were not significantly different (figs 2 and 3).

**DISCUSSION**

This study was conducted to gather information on asymptomatic and symptomatic vaginal yeast colonisation and vaginal immunity in female adolescents who are newly influenced by reproductive hormones; and to identify a relation between the presence of vaginal yeast carriage and sexual behaviours, reproductive hormones, and local immunomodulators. In the first part of the study, a microbiological survey showed that adolescents were colonised asymptotically with yeast at a rate of 22%. Compared to adults, this represents the high end of the normal range reported as 15–25%, and is considerably higher than the 17% reported in a recent adult study from this laboratory and 11% from a recent European study. Adolescents, like adults, are colonised most frequently with *C albicans* (85–90%) followed by *C glabrata*. An interesting observation in the adolescent population was the relatively high vaginal fungal burden in

<table>
<thead>
<tr>
<th>Table 1 Asymptomatic vaginal yeast colonisation/speciation in adolescents</th>
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<tr>
<td><strong>Species (% subjects)</strong></td>
</tr>
<tr>
<td>No</td>
</tr>
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those asymptotically colonised (~50% with >500 cfu/swab culture). This is considerably higher than adults in which >70% had <100 cfu/swab with no more than 20% with >500 cfu/swab.16 Despite the high asymptomatic fungal burden in adolescents, point prevalence of symptomatic vaginitis was extremely low (<2%). Longitudinal analysis of the adolescents for >3 years showed consistent yeast colonisation and confirmed low rates of VVC (<4%) (Fidel, in preparation). A recent point prevalence study in adults showed a 6.3% rate of symptomatic VVC.16 Interestingly, there was no association between oestrogen contraception and VVC, which although controversial, is generally considered to be a predisposing factor for VVC in adults.20 There was also no association between reduced vaginal yeast colonisation or VVC and progestogen contraception as reported previously.21 22 In fact, one of the two subjects with VVC was on Depo-Provera.

The high levels of asymptomatic vaginal fungal burden in adolescents may be attributed to sexual activity, as sexually active adolescents (84% of the cohort) were more likely to have vaginal yeast colonisation than those not sexually active. Although a report showed a similar relation in adults, this is the first of its kind for adolescents.23 Several exogenous factors were also examined for a correlative relation to yeast colonisation including presence of an STI, BV, douching, condom use, and contraceptive use. None showed any association with the prevalence of detectable vaginal yeast colonisation. Other parameters evaluated were Candida immune sensitisation and vaginal epithelial cell anti-Candida activity that we recently reported in mice,24 macaques,25 and adult women.26 Candida immune sensitisation in adolescents which resulted in a 95% rate of positive PBMC responses to Candida antigen is virtually identical to what is seen for adults.27 Similarly, the presence of Candida specific IgG and IgA in vaginal secretions from nearly all subjects tested is consistent with adults.28 In contrast, vaginal epithelial cell anti-Candida activity which exhibited a ~26% growth inhibition rate at a 40:1 E:T ratio in non-colonised adolescents is at the low end of normal for healthy adults at each stage of the menstrual cycle.29 Levels of activity may be higher in those colonised, but the assay at present can only be tested on those not detectably colonised. We postulate that this activity may represent an innate host defence mechanism to control yeast commensalism and infection. If so, it may be expected that adolescents would be at higher risk for VVC, and that this relatively low anti-Candida activity might contribute to the high vaginal yeast burden.

With regard to immunomodulators in vaginal secretions, Th1 and Th2 type cytokines measured in CVL showed a predominant Th2 type cytokine profile unlike the Th0/Th1 type profile reported for adults.9 The level of TGF-β, a potent downregulatory cytokine,25 is higher than that observed in adults at the follicular stage of the menstrual cycle, but comparable to that observed under higher influence of

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Condition (% colonisation)</th>
<th>Odds ratio (CI)*</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>Sexual activity</td>
<td>Yes (24)</td>
<td>7.2 (2.3 to 22.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>No (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condom use</td>
<td>Always (32)</td>
<td>1.143 (0.696 to 1.878)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Sometimes (23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Never (39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Douching</td>
<td>Yes (26)</td>
<td>1.398 (0.633 to 3.086)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>No (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraceptive use</td>
<td>Oestrogen (15)</td>
<td>0.541 (0.18 to 1.626)</td>
<td>NS</td>
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<tr>
<td></td>
<td>Progestogen (26)</td>
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CI = confidence interval; NS = not significant.
Vaginal-associated chemokines in cervicovaginal lavage fluid. Lavage fluids from adolescents (n=64) were evaluated for IL-8, RANTES, and MCP-1 concentrations by ELISA and stratified by those with or without detectable vaginal yeast colonisation. The figure shows mean concentrations (SEM) normalised to total protein (pg/mg).

**Key messages**

- VVC is low in adolescents compared with STIs or BV
- Adolescents can have high vaginal fungal burden in the absence of symptoms of vaginitis
- There is no association between yeast colonisation and local vaginal immunity in adolescents
- Sexual activity is positively correlated with vaginal yeast colonisation in adolescents

Oestrogen and progesterone (ovulatory and luteal stages of the menstrual cycle). Proinflammatory cytokines in adolescents are predominated by IL-1β and IL-6 and chemokines are predominated by IL-8, which is similar to adults. A final distinction to adults was total immunoglobulins in vaginal secretions where levels of IgA were higher than IgG, which is contrary to several reports for adults. Vaginal yeast colonisation did not appear to have any effect or influence on vaginal cytokines, chemokines, or antibodies.

Taken together, the presence of systemic and local immune sensitisation to Candida would suggest some level of protection against Candida infection. This is consistent with the low incidence of candida vaginitis. However, this would not have been predicted based on the Th2 type vaginal cytokines (associated with susceptibility to Candida infections), the high vaginal fungal burden in a large number of those tested, and the relatively low epithelial cell anti-Candida activity. In fact, high vaginal fungal burden has been directly associated with symptoms in adults. Thus, adolescents appear to possess strong protective mechanisms that withstand high vaginal yeast burden without triggering symptomatic infection. However, at present, it is unclear what contributes to the protection. Cell mediated immunity is possible although the majority of studies to date suggest little if any role for T cells against Candida at the vaginal mucosa. Other possibilities include high concentrations of putative "protective" antibodies in adolescent vaginal secretions, high levels of proinflammatory cytokines that may override the effects of Th2 type cytokines, and/or low levels of endogenous reproductive hormones.

Overall, the present study provides much needed vaginal microenvironment and immunological information on a middle adolescent population fairly naive to reproductive hormones. While there were several similarities to adults, several distinctions were also observed. Among these distinctions were an extremely low incidence of VVC in the presence of high levels of asymptomatic vaginal colonisation suggesting a strong resistance to vaginal Candida infections between the ages of 14 and 17. While it remains unclear as to what factors are most critical in protection against Candida infections, when better understood, perhaps the factors involved in resistance to VVC in adolescents could be duplicated in adults by immunotherapy in an effort to reduce the incidence of adult VVC or RVVC.

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**CONTRIBUTORS**

This study was initiated and conducted by PF, DF, and DO; MB was responsible for the collection and registration of data, statistical analysis, all laboratory work, and for the reporting of the results including writing this manuscript; BVDP was responsible for organising the recruitment of patients, sample collection, and shipping of specimens to LSUHSC.

**Authors’ affiliations**

M M Barousse, P L Fidel, Department of Microbiology, Immunology, and Parasitology, Louisiana State University, Health Sciences Center, New Orleans, LA, USA
B J Van Der Pol, D For tenberry, D Orr, Department of Medicine, Division of Infectious Diseases, Indiana University School of Medicine, Indianapolis, IN, USA

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