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Cervicovaginal cytokines, sialidase activity and bacterial load in reproductive-aged women with intermediate vaginal flora

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ABSTRACT

Studies have shown that not only bacterial vaginosis, but also intermediate vaginal flora has deleterious effects for women's reproductive health. However, literature still lacks information about microbiological and immunological aspects of intermediate flora. Objective: To characterize intermediate flora regarding levels of Interleukin (IL)–1beta, IL-6, IL-8, tumor necrosis factor-alpha, interleukin 1 receptor antagonist (IL-1ra), IL-10, sialidase; loads of *Gardnerella vaginalis*, total bacteria and to verify whether it is closer related to normal flora or bacterial vaginosis. This cross-sectional study enrolled 526 non-pregnant reproductive-aged women distributed in 3 groups according to pattern of vaginal flora using Nugent's system in normal, intermediate and bacterial vaginosis. Cervicovaginal levels of cytokines, sialidases, loads of *G. vaginalis* and total bacteria were assessed by ELISA, conversion of MUAN and quantitative real-time PCR, respectively. A principal component analysis(PCA) using all measured parameters was performed to compare the three different types of flora. Results showed that intermediate flora is associated with increased cervicovaginal IL-1beta in relation to normal flora($P < 0.0001$). When compared to bacterial vaginosis, intermediate flora has higher IL-8 and IL-10 levels($P < 0.01$). Sialidases were in significantly lower levels in normal and intermediate flora than bacterial vaginosis($P < 0.0001$). Loads of *G. vaginalis* and total bacterial differed among all groups($P < 0.0001$), being highest in bacterial vaginosis. PCA showed that normal and intermediate flora were closely scattered, while bacterial vaginosis were grouped separately. Conclusion: Although intermediate flora shows some differences in cytokines, sialidases and bacterial loads in relation to normal flora and bacterial vaginosis, when taken together, general microbiological and immunological pattern pattern of intermediate flora resembles the normal flora.

1. Introduction

Normally, *Lactobacillus* species are the most frequent components of the vaginal microbiota (Ravel et al., 2011; Sobel, 2000). Maintenance of a healthy vaginal flora is crucial for protecting women against several sexually transmitted infections (STI), such as those caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* and human immunodeficiency virus (HIV) (Brotman et al., 2012; Martin et al., 1999; Sewankambo et al., 1997; Weiesenfeld et al., 2003). The most common type of abnormal vaginal flora is a polymicrobial entity called bacterial vaginosis in which the lactobacilli-dominated flora is replaced by an overgrowth of anaerobic bacteria (Spiegel et al., 1980). Mechanisms by which bacterial vaginosis increases the risk for STI acquisition and transmission remain poorly understood (Schwebke, 2001; Sturm-Ramirez et al., 2000), but it was already shown that the high levels of proinflammatory cytokines and bacterial sialidases found in this condition contributes for increasing the local vulnerability for infections (Cauci et al., 2003a; Cherpes et al., 2003). In fact, vaginal non-lactobacilli load of bacteria is positively correlated with higher levels of interleukin (IL)-1 beta and several strains of the bacterial vaginosis-associated *Gardnerella vaginalis* are recognized as good sialidases-producers (Marconi et al., 2013a; Santiago et al., 2011).

Bacterial vaginosis can be detected in about 30% of reproductive aged women, but only half of them report symptoms such as increased vaginal discharge and unpleasant vaginal odor (Amsel

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et al., 1983; Klebanoff et al., 2004). The high rates of asymptomatic women with bacterial vaginosis challenges its diagnosis, leaving an expressive rate of the population in risk for STI acquisition. Currently, the gold standard for bacterial vaginosis diagnosis is the Nugent's scoring system, which is based in the microscopic semi-quantification of bacterial morphotypes found in Gram-stained vaginal smears (Nugent et al., 1991). Using this method, vaginal flora can be classified as normal (scores 0–3), intermediate (scores 4–6) and bacterial vaginosis (scores 7–10). Although this is considered a good tool for bacterial vaginosis detection, the clinical meaning and importance of intermediate flora for women's reproductive health remains a matter of discussion. Intermediate flora is found in 19.2% to 30.0% of the population and is even less symptomatic than bacterial vaginosis (Cauci et al., 2002a; Guédou et al., 2014). Some studies showed that intermediate flora shares several of bacterial vaginosis risk factors and, more importantly, it is also associated with vulnerability for HIV (Guédou et al., 2012). However, little is known about the local microbiological and immunological aspects of women with intermediate flora and literature data have been controversial whether it should be considered as a transitory type of flora between normal and bacterial vaginosis or a single different entity (Donders, 2007; Srinivasan et al., 2010).

Considering the increasing evidences from the literature around the deleterious effect of intermediate flora for women's health and the few information available regarding the cytokine and sialidase profile of this condition, the aim of this study was to determine the cytokine levels of IL-1beta, IL-1ra, IL-6, IL-8, IL-10 and Tumor Necrosis Factor (TNF)-alpha, as well as sialidase activity, *G. vaginalis* and total bacterial load in intermediate flora. We also aimed to verify whether its overall profile is comparable to normal flora or bacterial vaginosis.

2. Material and methods

From 2010–2013, 783 non pregnant reproductive-aged women attending in one unit of Primary Health Care in Botucatu for routine cervical cancer screening were invited to participate of this cross-sectional study. Women were not eligible if they were menstruating, reported urinary loss, used antibiotics (30 days), had sexual intercourse (48 h), presented vaginal candidosis or cytolytic vaginosis, as well as those who tested positive for *C. trachomatis* and/or *N. gonorrhoeae* endocervicitis. All subjects were informed about the study aims and signed a consent form. This study was approved by the Ethics Committee Board of Botucatu Medical School, São Paulo State University (Protocol #3629-2010). Sociodemographic and behavioral data were acquired by applying a standardized questionnaire.

During the physical exam for routine pap-smear screening, additional vaginal samples were taken to assess the local pH, 10% KOH testing and to detect, by microscopy, the presence of *Candida* sp. pseudo hyphae, *Trichomonas vaginalis* and for classifying the vaginal flora according to the Nugent's scoring system in normal (scores 0–3), intermediate (4–6) and bacterial vaginosis (7–10) (Nugent et al., 1991). Endocervical samples were also taken for assessing of *C. trachomatis* and *N. gonorrhoeae* status by PCR, according to methods previously described (Ho et al., 1992; Marconi et al., 2012). Additionally, cervicovaginal lavages were performed using 3 mL of a sterile 0.9% NaCl solution and, after allowing contact of the solution with the vaginal wall, samples were recovered using a plastic pipette. Cervicovaginal lavages samples were centrifuged at 800 x *g* for 10 min within 4 h after collection, with pellets and supernatants stored separately at −80 °C until analysis. We excluded of this study those women whose vaginal smears were not satisfactory for analysis (n = 24) and those with blood in the cervicovaginal samples (n = 11).

A total of 179 (22.9%) women were tested positive for chlamydial endocervicitis, 14 (1.8%) for gonorrhea and 2 (0.3%) for both infections and therefore were excluded of this study, as well as those who presented vaginal vulvovaginal candidiasis (n = 25, 3.2%) and cytolytic vaginosis (n = 2, 0.3%). Therefore, among the 526 women considered eligible for this study, bacterial vaginosis was detected in 145 (27.6%), while 63 (12.0%) had intermediate flora and 318 (60.5%) had lactobacilli-dominated vaginal flora. All pellets and supernatant samples from cervicovaginal lavages from women with bacterial vaginosis (n = 145, 27.6%), intermediate flora (n = 63, 12.0%) and 145 (27.6%) out of 318 randomly selected women with normal flora were subjected to determination of bacterial load and cytokine and sialidases quantification.

Bacterial DNA was extracted from the pellets of 1 mL of cervicovaginal samples using DNEasy Blood &Tissue Kit (Qiagen, Valencia, CA) with final elution in 100 μL. Loads in number of copies/mL of cervicovaginal samples of *G. vaginalis* and the conserved sequence of 16S rRNA were determined by quantitative real-time PCR, using previously described primers (Smits et al., 2004; Zariffard et al., 2002). Reactions were performed individually in a final volume of 13 μL using Maxima SYBR Green/ROX (Fermentas, St. Leon-Rot, Germany) in a LineGeneK equipment (Bioer, China), according to methods described elsewhere (Marconi et al., 2013a).

Interleukin-1beta, IL1-ra, IL-6, IL-8, IL-10 and TNF-alpha levels were measured in the supernatants by ELISA using Duo Set Kits (R&D Systems, Minneapolis, MN) of cervicovaginal lavages, according to manufacturer's instructions. All samples were tested in duplicate and those with values set above the standard curve range were diluted (1:5 and 1:10) and retested. The minimum detectable levels in the IL-1beta, IL-1ra IL-6, IL-8 and TNF-alpha assays were, respectively, 0.1 pg/mL, 775.0 ng/mL, 1.3 pg/mL, 7.8 pg/mL, 0.4 pg/mL. Intra and inter-assay variability remained <10.0% for all cytokines.

Measurement of sialidase activity in the supernatants was performed using the fluorogenic substrate 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN; Sigma-Aldrich, St. Louis, MO), according to protocol previously described (Marconi et al., 2013a,b). Comparison of discrete and continuous quantitative variables among the women with normal vaginal flora, intermediate and bacterial vaginosis was performed, respectively, by Chi-squared and non-parametric Kruskal Wallis test, followed by Dunn's multiple comparison post-test, when group effects were significant. Bacterial load, cytokine and sialidase levels among the groups were also compared with Kruskal-Wallis, followed by Dunn's test. Analyses were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA) and $P < 0.05$ was considered as significant. Principal component analysis (PCA) is a tool by which complex data are converted in simple values (principal components), allowing the evaluation of the samples based on the different variables that they express. We performed this analysis to compare the general profile of normal flora, intermediate and bacterial vaginosis samples based on the combination of their cytokine levels, sialidase activity, loads of *G. vaginalis* and total bacterial loads. An auto-scaled PCA analysis was performed with $\log_{10}$ transformed data using MVSP software, version 3.13a (Kovach Computing Services, Pentraeth, UK). Post-hoc analysis showed a power test superior to 95%, considering the difference of IL-1beta among the groups with normal, intermediate flora and bacterial vaginosis.

3. Results

Sociodemographic, behavioral and gynecologic characteristics of the women enrolled are shown in Table 1, distributed according their pattern of vaginal flora. The three study groups did not