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Sialidase activity in aerobic vaginitis is equal to levels during bacterial vaginosis

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ABSTRACT

Objective: To evaluate levels of proinflammatory cytokines and sialidase activity in aerobic vaginitis (AV) in relation to normal vaginal flora and bacterial vaginosis (BV).
Study design: In this cross-sectional study, a total of 682 consecutive non-pregnant women attending the gynecology service were assessed and 408 women were included. Vaginal rinsing samples were collected from 223 women with microscopic finding of BV ($n = 98$), aerobic vaginitis ($n = 25$) and normal flora ($n = 100$). Samples were tested for interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and sialidase activity.
Results: Compared to women with normal flora, vaginal levels of IL-1β were highly increased in both BV and AV ($p < 0.0001$). Significantly higher vaginal IL-6 was detected in AV ($p < 0.0001$) but not in BV, in relation to normal flora. Women with AV also presented increased IL-8 levels ($p < 0.001$), while those with BV presented levels similar to normal flora. Sialidase was increased in BV and AV compared with the normal group ($p < 0.0001$) but no difference in sialidase activity was observed between BV and AV.
Conclusion: A more intense inflammatory host response occurs for AV than for BV when compared with normal flora. Furthermore, the increased sialidase activity in AV and BV indicates that both abnormal vaginal flora types can be harmful to the maintenance of a healthy vaginal environment.

1. Introduction

Abnormal vaginal flora have been linked to many severe gynecological and obstetric complications, such as pelvic inflammatory disease, increased risk of sexually transmitted diseases (STD) and preterm birth [1–6]. Changes in vaginal flora activate the local immunity as reflected in the locally elevated cytokine levels [7–9] produced by the resident monocytes/macrophages and lymphocytes [10].

Changes in vaginal cytokine levels occur even in response even to minor shits in the composition of the vaginal microbiota [11]. Vaginal epithelial cells express on their surfaces important receptors for microbial recognition, such as toll-like receptors (TLR). These receptors recognize molecular patterns present on the bacterial surface and induce production of proinflammatory cytokines through nuclear factor kappa B activation [10]. According to a previous study, Interleukin (IL)-1β is increased in bacterial vaginosis (BV) and aerobic vaginitis (AV) [8]. In the same study, however, the levels of IL-6 were elevated only in AV [8]. Data regarding the neutrophil chemotactic IL-8 in BV are still conflicting [9,12] and it has not been evaluated in AV.

Several studies suggest that the diversity in microbial composition of the vaginal flora, and differences in the composition of bacterial products present, is a probable cause of diversity in the inflammatory response. As recently demonstrated, short chain fatty acids produced by anaerobes in bacterial vaginosis (BV), can interfere with the TLR-mediated inflammatory response [13]. Additionally, hydrolytic bacterial enzymes known as sialidases are produced by BV-associated bacteria, such as *Gardnerella vaginalis* and *Prevotella bivia* [14]. High sialidase levels have been considered a risk factor for preterm birth [15,16]. Sialidase plays a role in downregulating the innate immune system in BV, since it degrades host defence molecules such as Immunoglobulin-A (IgA) against *G. vaginalis* hemolysin [17], which can be harmful to the maintenance of a healthy vaginal environment. Recent work demonstrated that removal of sialic acid from secretory IgA is the mechanism by which sialidase leads to IgA proteolysis and consequent lowered local immune response [18]. Confirming the effect of sialidase in the vaginal flora, Santiago et al. [19] reported that *G. vaginalis* sialidase-producer strains have higher adherence capacity to

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epithelial cells and biofilm formation, reinforcing the importance of this enzyme in the pathogenesis of BV and its likelihood of recurrence.

Considering the variability in the innate immune response to the different types of abnormal vaginal flora and the importance of sialidase in the pathogenesis of these conditions, we aimed to determine the sialidase profile in AV, as well as to measure the associated levels of proinflammatory cytokines in relation to normal flora and BV.

2. Materials and methods

In this cross-sectional study, a total of 682 consecutive, premenopausal, non-pregnant women attending one unit of primary medical care in Botucatu, Brazil, from May 2010 to April 2011, for routine Pap smear screening were candidates for enrollment in the study. Before the standard pelvic examination, all women were informed about the aims of the study and signed a consent term. This study was approved by the Ethics Committee Board at the Botucatu Medical School (Protocol 2936/2008). Women were not sampled if they presented one of the following conditions: vaginal bleeding, use of systemic antibiotic or vaginal medication during the last 30 days, or having had sexual intercourse or any vaginal procedure in the preceding 72 h.

After inserting a non-moisturized speculum, two smears from the mid-lateral vaginal wall were taken. Vaginal pH was measured using pH strips (range 4.0–7.0, Merck, Germany), by pressing against the vaginal wall. The whiff test was performed by adding 10% KOH on the sampled swab and results were categorized as positive, negative or doubtful. Vaginal rinsing with 3 mL of sterile 0.9% NaCl solution was performed with a sterile plastic pipette allowing contact of the solution with the vaginal wall. The professionals in charge of the sampling procedures were trained on how to perform the rinsing and instructed to recover exactly 3 mL. Samples containing volumes with less than 3 mL or with blood were discarded. The vaginal rinse samples were centrifuged ($800 \times g$, 10 min) and pellet and supernatant were stored separately at $-20\,^{\circ}C$

Cervical samples were taken using a cytobrush to test for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection by polymerase chain reaction (PCR) [20,21]. For detection of *Trichomonas vaginalis*, vaginal vault samples were taken with an Ayre's spatula, spread on a microscope slide and after adding a drop of saline immediately examined under 400× magnification with a light microscope (Olympus CX31, Tokyo, Japan).

Microscope slides were prepared by adding a drop of 0.9% NaCl and a cover slip to each swab smear for observation at 400× magnification in a phase-contrast microscope (Olympus BX41, Tokyo, Japan). The protocol for classification of the vaginal flora was performed according to Donders et al. [8]. Lactobacillary grades (LBG) were classified as LBG I, IIa, IIb or III depending on the balance between quantity of *Lactobacillus* morphotypes and other bacteria found in the smears. The LBG I category reflected the predominance of *Lactobacillus* sp. morphotypes and was considered as normal flora. In LBG IIa, smears presented a normal lactobacillary pattern but a with significant quantity of non-*Lactobacillus* sp. morphotypes, while in LBG IIb the non-lactobacillary flora exceeded the quantity of *Lactobacillus* sp although they were still seen. Finally the smears showing total replacement of lactobacilli by other microorganisms were classified as LBG III and further diagnosed as BV, AV or even mixed infections. Bacterial vaginosis was diagnosed if its typical granular anaerobe microflora were found on the smear, while AV was defined as the presence of small bacilli and/or cocci in pairs or chains, taking into account the lactobacillary grade, ratio of leukocytes to epithelial cells, presence of toxic leukocytes, and parabasal cells. Candidiasis was identified when unequivocal pseudohyphae and/or blastophores were detected on the smears.

Interleukin-1β, IL-6, IL-8 and TNF-α concentrations in the supernatants were quantified by ELISA with Duo Set Kits (R&D Systems, Minneapolis, MN). All the samples were tested in duplicate, and if the concentration was beyond the standard curve range, the sample was diluted (1:5 and 1:10) and tested again. Intra-assay variability was <10.0% for IL-1β, IL-6, and IL-8 and 21.4% for TNF-α. Inter-assay variability was 10.0% for IL-1β, 0.15% for IL-6, 4.5% for IL-8 and 23.0% for TNF-α. The minimum detectable levels for IL-1β, IL-6, IL-8 and TNF-α assays were, respectively, 0.4 pg/mL, 4.7 pg/mL, 15.6 pg/mL and 0.23 pg/mL.

Sialidase activity in the rinse supernatants was assayed quantitatively using the fluorogenic substrate 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN; Sigma–Aldrich, St. Louis, MO), as previously described [22]. In a 96-well plate (OptiPlate-96F, PerkinElmer, Waltham MA), 50 μL of each supernatant was added to 50 μL of 0.35% MUAN (wt/vol) in 3 mM sodium acetate (pH 4.5) and incubated for 30 min at 37 °C. The fluorescence was measured at 450 nm, using 365 nm excitation and 420 nm cutoff filters in an Epoch spectrofluorometer with Gene5 software (Biotek, Winooski VT). The positive controls were dilutions from 1000.0 ng/mL to 1.0 ng/mL of purified *Clostridium perfringens* neuraminidase (Sigma–Aldrich, St. Louis, MO), from which the unknown values were interpolated. A negative control consisting of a sample previously heated at 95 °C for 20 min, and a sialidase-positive control sample with visible fluorescence on UV transilluminator, were run in every assay. All samples were tested in duplicate.

Cytokine and sialidase levels among the groups were compared with Kruskal–Wallis analysis of variance by ranks, followed by the Dunn's multiple comparison post-test when group effects were significant. All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA) and $P < 0.05$ was considered as significant.

3. Results

Among the 682 candidates for inclusion in the study, we excluded 166 women that tested PCR-positive for *C. trachomatis* ($n = 161$, 23.5%) and/or *N. gonorrhoeae* ($n = 8$, 1.2%). From the group that tested negative for both pathogens ($n = 516$), those with microscopic finding of candidosis ($n = 44$, 8.6%), LBGIIa or IIb ($n = 50$, 9.7%), cytolitic vaginosis ($n = 2$, 0.4%), mixed AV and BV ($n = 5$, 1.0%) or whose smears were inadequate for microscopic analysis ($n = 7$, 1.3%) were also excluded from the study. We included the remaining 408 women, of whom 285 had normal flora, 98 had microscopic finding of full BV, and 25 had moderate or severe AV. Demographic and gynecological characteristics from the included women are summarized in Table 1. The population was homogeneous as none of those variables differed among the groups, except that years at school approval was significantly lower in the BV group than the normal flora group ($P = 0.01$). Vaginal pH was significantly increased in abnormal vaginal flora when compared to the control group.

The cytokine and sialidase levels were measured in all women with BV and AV included in the study. From the normal flora group, we evaluated samples from the first consecutive 100 women, in order to equal the sample size of the biggest group presenting abnormal vaginal flora (BV, $n = 98$). The levels of IL-1β, IL-6, IL-8 and TNF-α are shown in Fig. 1. Interleukin 1β was detectable in 77.0%, 96.0%, and 92.9% women with normal flora, AV and BV, respectively. Interleukin-1β concentrations were significantly higher in women with AV (median 178.8 pg/mL; range 0.0–656.3) and BV (median 71.2 pg/mL; range 0.0–1950.0) when compared to women with normal flora (median 5.0 pg/mL; range

Table 1
Demographic, behavioral and gynecological characteristics from women included in the study.

Subjects characteristics	Normal flora ($n = 285$)	Aerobic vaginitis ($n = 25$)	Bacterial vaginosis ($n = 98$)	P
Age, median (range), years	29 (18–52)	32 (18–50)	33 (18–53)	0.08[†]
Ethnicity (self-defined), n (%)				
White	197/272 (72.4)	18/23 (78.3)	54/85 (63.5)	
Nonwhite	75/272 (27.6)	5/23 (21.7)	31/85 (36.5)	0.20[‡]
Education, years, median (range)	11 (0–18)[b]	8 (4–16)[ab]	8 (0–16)[a]	*0.01*[†]
Marital status, n (%)				
Single	76/275 (27.6)	7/23 (30.4)	32/96 (33.3)	
Married	199/275 (72.4)	16/23 (69.6)	64/96 (66.7)	0.57[‡]
Sexually active, n (%)	267/275 (97.1)	21/24 (87.5)	93/96 (96.9)	0.06[‡]
Steady partner (among sexually active only), n (%)	241/267 (90.3)	16/21 (76.2)	85/93 (91.4)	0.10[‡]
Smoking habit, n (%)	58/275 (19.8)	6/24 (25.0)	26/95 (32.6)	0.44[‡]
Contraceptive use, n (%)	108/247 (43.7)	12/22 (54.5)	39/89 (43.8)	0.61[‡]
Type of contraception, n (%)				
Oral	62/108 (57.4)	3/12 (25.0)	21/39 (53.8)	
Injection	4/108 (3.7)	2/12 (16.7)	3/39 (7.7)	
Condom	18/108 (16.7)	5/12 (41.7)	10/39 (25.6)	
Intrauterine device	3/108 (2.8)	2/12 (16.7)	4/39 (10.3)	NC[*]
Vaginal pH, median (range)	4.4 (4.0–4.7)[a]	5.0 (4.4–5.8)[b]	5.0 (4.0–7.0)[b]	*<0.0001*[†]

Total of women may vary among the categories, as some of the data were unavailable or due to patient's refusal to answer. Different letters in superscript following values indicate statistical significance.
[*] Not calculated as women may had reported use of more than one method of contraception.
[†] Kruskal–Wallis with Dunn's post-test.
[‡] Chi-squared test.

0.0–250.8) ($P < 0.001$). Levels of IL-6 were below the detection limit in most of normal (82.0%) and BV samples (69.4%) but only in 28.0% of AV group. Women with AV had significantly increased vaginal IL-6 (median 14.0 pg/mL; range 0.0–735.1) compared to normal (median 0.0 pg/mL; range 0.0–156.1) and BV (median 0.0 pg/mL; range 0.0–73.3). Interleukin-8 was detected in 92 women with normal flora, 24 with AV and 87 with BV, with levels increased in AV (median 517.9 pg/mL; range 0.0–2744.0) as compared to the normal (median 184.3 pg/mL; range 0.0–1985.0) and BV (median 195.6 pg/mL; range 0.0–2200.0). As shown in Fig. 1, only a few samples had detectable levels of TNF-α: 20.0%, 12.0% and 11.2% from normal, AV and BV, respectively, with no differences among the groups ($P > 0.05$).

Sialidase activity was detected in 16 women from the AV group (64.0%) and 72 (73.5%) from the BV group. Only two of 100 women with normal flora had detectable sialidase activity. As shown in Fig. 2, both groups with abnormal vaginal flora had increased sialidase activity when compared to normal flora.

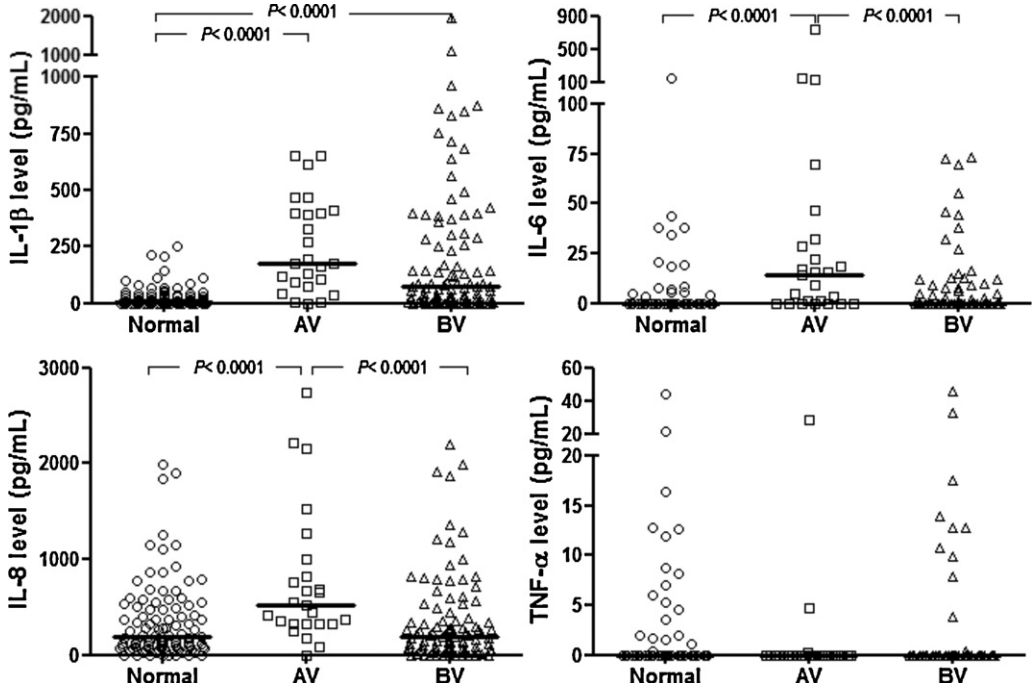

Fig. 1. Cytokine levels in vaginal wash samples from women with normal ($n = 100$), AV ($n = 25$) and BV ($n = 98$) flora. Horizontal bars represent median values.

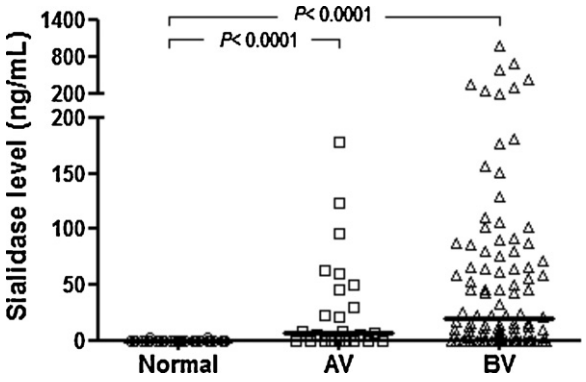

Fig. 2. Sialidase levels in vaginal wash samples from women with normal ($n = 100$), AV ($n = 25$) and BV ($n = 98$) flora. Horizontal bars represent median values.

4. Comment

There is an increasing interest in establishing the microbiological and immunological profiles of abnormal vaginal flora, since it has been widely associated with several adverse gynecological and obstetric conditions [1,4,5]. More recently, several authors have pointed out that abnormal vaginal flora should no longer continue to be seen as a single, homogeneous entity, as a great diversity in the bacterial core and inflammatory response is encountered in the vaginal environment [8,23]. Many characteristics of each type of flora abnormality and their repercussions on women's health still remain to be elucidated, however. In this study, although education level was significantly lower in women with BV, which is a known factor linked to this type of abnormal vaginal flora [24], we did not observe any further association of demographic data with vaginal flora pattern. Several authors have shown that factors such as smoking habit, ethnicity, and multiple sex partners are associated with BV, but those studies evaluated a much larger population than ours [24–26]. In relation to the cytokine and sialidase findings, we demonstrated that besides the classical anaerobic BV, AV can also be considered as a likely candidate to provoke intense local immunologic changes, as well as an associated increase in the bacterial sialidase activity, a recognized risk factor for preterm birth [16,27].

Although BV is not defined as an inflammatory condition, IL-1β is found in particularly high levels in the vagina of women with BV when compared to pregnant [7,9] and non-pregnant women with normal flora [8,12], strongly agreeing with the current findings. Regarding IL-1β in AV, it has been reported [8] that women with AV have significantly higher vaginal levels than women with BV, but we found a significant increase only when compared to the normal flora group. When pregnant women were excluded from the prior analysis [8], however, IL-1β increased equally in both BV and AV conditions when compared to normal flora.

The current report shows that AV is a condition associated with the full activation of the inflammatory cascade. Unchanged IL-6 concentration in cervical samples [7] and vaginal rinses [8,9] has been reported in women with BV in relation to normal controls, while IL-6 showed highly increased levels in AV compared to both BV and normal flora [8]. Our data are in full agreement with those findings regarding IL-6, reinforcing the idea that abnormal vaginal flora do not behave as a homogeneous group in terms of modifying the host's immune response. No previous studies have evaluated IL-8 in AV, but our data show strongly increased IL-8 concentrations in AV. This should not come as a surprise, given the influx of leucocytes observed microscopically in cases with moderate and severe AV [8]. We also found that IL-8 levels remained unchanged in BV. As IL-8 is a potent neutrophil chemotactic factor, and BV is not associated with increases in neutrophil counts, higher IL-8 levels are not to be expected in cases of BV [12], but the reason for this typical non-inflammatory response in the presence of so many potential pathogens still has not been elucidated. TNF-α is normally found in low, even undetectable, levels in most of cervicovaginal samples with or without BV [7,28], as supported by the present study.

We detected sialidase activity in almost 75% of women with BV, which is similar to previous studies [14,17] even though different methodologies for sialidase detection were used. To our knowledge, this is the first study to evaluate the level of sialidase in a condition like AV. The finding of elevated sialidase activity in 64% of AV cases, a prevalence similar to that in cases of BV, confirms our understanding that AV should also be considered a potentially serious condition in pregnancy, because former studies [16,27] showed that the presence and amount of this bacterial enzyme is predictive of poor gestational outcome. Because AV cultures yield aerobes more frequently than BV, it has been proposed that such species occupy a larger portion of the local flora differently from BV, which is mostly composed of anaerobes [8]. Additionally, the sialidase-producer *Gardnerella vaginalis* can be recovered in only 20% of AV samples [8], and therefore an alternative source of this enzyme is very likely in AV. One study that focused on the correlation between the sialidase and cytokine levels in BV showed a positive correlation of sialidase with IL-1β, but not with IL-8 levels [29]. Those investigators suggested a possible participation of this enzyme in attenuating the downstream production of cytokines in BV after IL-1β release, leading to unchanged IL-8 levels and low neutrophil counts, based on the finding of inverse correlation of sialidase levels with the IL-8/IL-1β ratio. This seems unlikely, however, considering that BV itself is associated with high sialidase and IL-1β levels, but with unchanged IL-8. Indeed, the combination of high IL-8 levels and sialidase activity in AV opposes the idea that microbial sialidases can interfere with the cytokine cascade leading to diminished IL-8 levels. Thus, the reason for unchanged IL-8 levels in BV, despite the increase in IL-1β, remains undetermined. Recent work [18] showing that sialidase promotes proteolysis of IgA illustrates how this bacterial product may be linked to persistence of abnormal vaginal flora and its consequences.

We suggest that the great diversity in microbial composition in abnormal vaginal flora is more important than sialidase alone in modulating the host's inflammatory response. Taking into account the importance of bacterial synergism in this environment, further studies are essential to establish what role alterations in the vaginal microbiota play in the activation of the immune response and how they are related to gynecological and obstetric complications.

Conflict of interest

The authors do not have any conflict of interest to declare.

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