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Aluna: Heloise Ranucci Luchiari

Orientadora: Dra. Camila Marconi

Co-orientadora: Prof. Dra. Márcia Guimarães da Silva

Supervisora: Prof. Dra. Maria de Lourdes Ribeiro de Souza da Cunha

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Total bacterial counting and the response to treatment of bacterial vaginosis with metronidazole[*]

Heloise R. Luchiari [1], Márjorie A. Golim[2], Márcia G. da Silva[1], Camila Marconi[1].

[1]Department of Pathology, Botucatu Medical School, UNESP – Univ Estadual Paulista, Brazil

[2]Blood Center, Botucatu Medical School, UNESP - Univ Estadual Paulista, Brazil

Reprint requests and corresponding author:

Camila Marconi, e-mail: marconi.cml@gmail.com

Department of Pathology, Botucatu Medical School, UNESP – Univ Estadual Paulista Botucatu, São Paulo, Brazil. Zip code: 18618-970. Phone: +55 14 3880-1655; Fax: +55 14 38152348.

Key words: Bacterial vaginosis, metronidazole, flow cytometry, total bacterial count

Abstract

Objective: To evaluate if the total bacterial count of vaginal samples with bacterial vaginosis assessed by flow cytometry influences the response to treatment with metronidazol. **Methods:** In this cross-sectional study, 273 low-risk reproductive aged women were enrolled. Vaginal samples were taken to evaluate the pattern of vaginal flora according to Nugent's criteria, as well as the presence of trichomoniasis and candidosis. Cases identified of bacterial vaginosis were treated with metronidazole and controlled after 45 days. Cervical infection by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were also assessed. Flow cytometry for total bacterial counting was performed in propidium iodide stained cervicovaginal samples, using fluorescent beads at a known concentration. Non-parametric Mann-Whitney test was used to compare total bacterial count between groups of interest, at $p < 0.05$. **Results:** From the total of 273 women enrolled, 50 were excluded as they presented at least one of the infections investigated. Bacterial vaginosis was detected in 79 women (35.4%), of which 33 (41.8%) returned for re-evaluation after treatment, being 21 cases successfully treated and 12 with persistent abnormal vaginal flora. Flow cytometric data showed that total bacterial counting does not differ between normal flora and bacterial vaginosis samples ($p=0.14$). Also, no difference was found between the cases of treated and persistent bacterial vaginosis ($p=0.48$). **Conclusion:** Total bacterial counting does not influence the response to metronidazole treatment of bacterial vaginosis.

Introduction

Vaginal flora is typically defined as normal when *Lactobacillus* sp. predominates over the numerous other bacterial species that can be found in this environment [1]. Bacterial vaginosis is the most common type of abnormal vaginal flora (AVF) and is characterized by the total or partial replacement of the local lactobacilli by other bacteria, anaerobes mostly [2]. In reproductive aged-women, this condition is particularly important as it is associated with poor pregnancy outcomes and increased risk for acquisition and transmission of sexually transmitted infections [3-5].

Although bacterial vaginosis has deleterious effects on women's reproductive health, the treatment of this condition remain a challenge for clinical practice. Current recommended treatment for this AVF consists in a 7-day regimen with metronidazole [6], with treatment failure rate of 36.0% one month after treatment cessation [7]. Although the treatment failure is frequent and the recurrence rate after six months of treatment is more than 50% [8] the microbiological aspects involved in this difficulty on flora restoration remain to be defined.

It is well established that women with bacterial vaginosis present a significantly increased diversity of bacterial species in their vaginal flora [9,10]. In addition to flora disruption, some studies have shown that bacterial vaginosis is accompanied by an increase in the number of vaginal bacteria [11,12]. Corroborating with this idea, bacterial biofilms are more frequently found in vaginal samples from women with bacterial vaginosis when compared to those with normal flora, which contribute for increases in bacterial number [13,14]. Considering that most vaginal bacterial species are fastidious or non-cultivable, the determination of the total number of vaginal bacteria should be performed using culture-independent

methods.[10] Recently, the use of flow cytometry for total bacterial counting on vaginal fluids was successfully demonstrated [15].

Therefore, considering that the microbiologic aspects of bacterial vaginosis linked to therapeutic failure or resistance remain under investigation, the aim of the current study was to evaluate if the total bacterial count in vaginal samples influences the response to treatment of bacterial vaginosis with metronidazole.

Methods

Study population

From September 2012 to October 2013, a total of 273 reproductive-aged women attending one unity of primary medical care in Botucatu-SP for routine pap-test were invited to participate of this cross-sectional study. Women that reported vaginal bleeding, urinary loss, recent sexual intercourse (<72h), puerperium (<3 months after delivery), use of intrauterine device (IUD), antibiotics (<30 days) and confirmed/probable pregnancy were not included. Demographic, behavioral and clinical data were obtained by interview using a standard questionnaire. Study aims and procedures were explained and all women enrolled provided written informed consent. Study was reviewed and approved by the Ethics Board of Botucatu Medical School (Protocol 306.547).

Sampling procedures

During physical examination, using sterile speculum, vaginal pH was assessed by pressing the commercial pH strips (4.0-7.0, Merck, Darmstadt, Germany) for approximately 1 minute against the vaginal wall and then comparing the result with the standard provided by the manufacturer. Whiff test was performed

by adding 10% KOH solution to the vaginal swabs and the results were interpreted as positive, doubtful or negative by the practitioner.

For microscopic evaluation of the vaginal flora, samples were taken using sterile cotton swabs from mid-lateral vaginal wall, rolled on microscope slides and Gram stained for classification according Nugent et al. (1991) [16] in normal, intermediate flora or bacterial vaginosis. Another vaginal smear was prepared for wet mount evaluation with addition of 0,9%NaCl solution and examined under light microscope at 400 magnification (Olympus CX31, Tokyo, Japan) to detect the presence of *Trichomonas vaginalis* and *Candida* sp. morphotypes. Additionally, cervical samples were taken using cervical brush for assessment of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection by PCR.

Finally, cervicovaginal rinsings were performed by allowing the contact of 3mL of sterile 0.9% NaCl with the lateral vaginal wall and ectocervix and recovering the total liquid volume with sterile pipettes. Rinsing samples were stored at -80°C until analysis and discarded if volume recovered was inferior to 3mL or in the presence of blood.

Detection of endocervicitis

Cervical samples were submitted to DNA extraction using the commercial Tissue & cells genomicPrep Mini Spin kit (GE Healthcare, Buckinghamshire, UK), following the manufacturer's protocol for Extraction of genomic DNA from animal tissue. Efficiency of the extraction was confirmed by amplification of the constitutive gene of β-globin using the primers PCO4 (5'-CAACTTCATCCACGTTCACC-3') and GH20 (5'-GAAGAGCCAAGGACAGGTAC-3') for a 268bp product [17]. Reaction was performed following parameters previously established in our laboratory consisting of initial denaturation at 95°C for 9 min, followed by 35 cycles of 95°C for 1 min,

annealing at 52°C for 1 min and extension at 72°C for 1 min, and final extension at 72°C for 5 min. The products of the reactions were observed after electrophoresis in agarose gel 1.5% stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA). Once the amplification of the β-globin sequence was confirmed, samples were tested for *C. trachomatis* and *N. gonorroheae*.

Detection of *C. trachomatis* was performed by multiplex end-point PCR using GoTaq Green Master Mix, 2X (Promega, Madison, WI) and primers CTP1 (5'-TAGTAACTGCCACTTCATCA-3'), CTP2 (5'-TTCCCCTTGTAATTCGTTGC-3'), PL61 (5'-AGAGTACATCGGTCAACGA-3'), PL62 (5'-TCACAGCGGTTGCTCGAAGCA-3') [18], that resulted in products of 201bp and 130bp, respectively. Cycling protocol used was denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C at 1 min, with a final extension at 72°C for 5 min. For all reactions, negative (DNAse-free water) and positive (extracted DNA from McCoy cells infected by *C. trachomatis*) controls were used. Presence of *C. trachomatis* was confirmed when observing both PCR products on 1.5% agarose gel after electrophoresis.

Neisseria gonorroheae was detected by real time PCR using Maxima™ SYBR Green/ROX PCR Master Mix 2X (Fermentas®, Thermo Scientific Inc, Waltham, MA) and primers OH1 (5'-GCTACGCATACCCGCGTTGC-3') and OH3 (5'-CGAAGACCTTCGAGCAGACA-3') [19] resulting in a 390bp product. Melting temperature of 83°C was used to determine the presence of *N. gonorrhoeae* amplicons. For all reactions, negative (DNAse-free water) and positive controls (DNA obtained by pure culture of *N. gonorrhoeae* ATCC® 19424) were used. Reactions were performed on Line-gene K Real-time PCR Detection System (Bioer Technology, China) and Line-gene K software (Bioer Technology, China) was used for data analysis. Cycling parameters were 50°C for 2 min, 95°C for 10 min, followed

by 45 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Finally, fragments were melted from 65 to 95°C at a rate of 0.5 °C/s.

Treatment of bacterial vaginosis and control visit

Treatment for bacterial vaginosis consisted in two-daily doses of metronidazole 500mg for 7 days as recommended by the Center of Disease Control and Prevention.[6] Women were scheduled for a control visit 45 days after the end of treatment. At return, vaginal swabs of mid-lateral vagina wall were taken to a new evaluation of vaginal flora as previously described. It allowed identifying cases of success or failure of the treatment for bacterial vaginosis.

Total bacterial counting by flow cytometry

Cervicovaginal samples were thawed at -80°C and processed 24h before cytometric analyses. Aliquots of 400µL were taken from all samples and fixed with 200µL of paraphormaldehyde 5% overnight at 4°C. To the total volume of 600µL of the fixed samples, 8µL of 0,002% propidium iodide (BD, San Jose, CA) were added and incubated at 37°C for 15 minutes. Samples were briefly centrifuged at 800rpm to remove the largest residues and 500µL of the supernatants were transferred to 5mL cytometry tubes. Prior to analysis, 5µL of fluorescent beads (Bacteria Counting Kit, Invitrogen, Carlsbad, CA) were added to each sample to reach the density of 1.0×10^6 beads/mL.

Flow cytometric analyses were performed at FACScalibur (BD, San Jose, CA). Data were acquired and processed using CellQuest Pro (Version 5.2, San Jose, CA) with double threshold set on 280 and forward scatter (FSC) as primary parameter and side scatter (SSC) as second parameter, both in logarithmic scale. According to methodology previously described by Schellenberg et al. (2008) [15], a total of two cell populations could be identified on the dot plots of cervicovaginal

samples (Figure 1A). The upper population consists of host's shed epithelial cells while the lower represents the total of bacterial cells in the sample. The position of this lowest population consisting of bacterial cells on the dot plot was confirmed by evaluating an overnight culture of *Staphylococcus aureus* (ATCC 19095) in filtered tripticase soy broth, as observed in Figure 1B.

For bacterial counting, total of 10000 events at the beads were recorded for each sample. As observed in Figure 2, gates were created for both bacterial cells population and beads (R5). Considering the initial density of the fluorescent beads, a 10000 beads count is equivalent to 10μL sample volume, which allows determining the total of bacterial cell units per μL of sample based on the events observed on gate R6.

Statistical Analysis

Comparison of discrete variables between the groups of women with normal and AVF was performed by Chi-squared test, while the continuous variables were compared by Mann-Whitney test. For data on bacterial counting, comparison was performed by Mann-Whitney test, between the groups with normal and bacterial vaginosis at baseline, and between those women that presented effective and treatment failure. All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA) and $P<0.05$ was considered as significant.

Results

From the total of 273 women initially included in the study, we excluded those cases of *C. trachomatis* (n=21, 9.4%), *N. gonorroheae* (n=2, 0.9%), concomitant *C. trachomatis* and *N. gonorrhoeae* (n=1, 0.5%), *Trichomonas vaginalis* (n=2, 0.9%), vaginal candidosis (n=24, 10.8%). The demographic, behavioral and

gynecological characteristics of the 223 women finally included in the study are shown in Table 1, according to the vaginal flora classification using Nugent's scoring system in normal (scores 0-3) and AVF (scores 4-10). Married women were less likely to present flora alterations than single women (p=0.02). Self-reported previous episode of bacterial vaginosis, but not sexually transmitted infections, showed association with current AVF (p=0.02). Considering the gynecological data, women with AVF were more likely to present higher vaginal pH (p<0.0001) and positive or doubtful whiff test (p<0.0001).

Flora classification of the total of 223 women included showed that 132 (59.2%) had normal vaginal flora, while 91 (40.8%) presented AVF of which 12 (5.4%) were classified as intermediate flora and 79 (35.4%) as bacterial vaginosis (BV). From the 79 women with bacterial vaginosis, 33 (41.8%) opt to treat BV and returned to visit for control 45 days after conclusion of treatment. At return visit, evaluation of the vaginal flora showed that in 21 (63.6%) women the treatment was effective, as they returned with normal vaginal flora, while 12 (36.4%) women still presented AVF.

Bacterial counting by flow cytometry was performed in 32 samples of women with normal flora and 33 cases of BV, of which 21 were successfully treated after metronidazole therapy and 12 failed to restore the lactobacilli-predominant flora, as shown in Figure 3. Total bacterial counting, expressed in bacterial cell units per µL of cervicovaginal samples, does not differ significantly between women who had normal flora (median: 366.5, range: 46.0-10530.0) and women with bacterial vaginosis (median: 277.0, range: 7.0-1232.0), (p=0.14). When comparing the results on bacterial counting in first visit samples from women who had flora restoration after treatment (median: 304.0, range: 7.0-1232.0) with those in which treatment was not

effective (median: 236.5, range: 21.0-762.0), no statistical difference was observed (p= 0.48).

Discussion

The evaluation of the demographic and behavioral data from the population enrolled showed that both groups of study, normal and AVF, were similar for most of the variables investigated. Although AVF is commonly associated with smoking habit, number of sex partners and ethnicity [20,21], this study failed to demonstrate such associations. This might be explained by the study design, as its main goal was not to evaluate the characteristics associated with vaginal flora patterns. However, in agreement with findings from Koumans et al., (2007) [21], the current data show that being married is a protector factor for AVF, although the number of sex partners in the last 12 months is not associated with this condition. The report of previous episode of bacterial vaginosis was associated with AVF, which is in agreement with the literature that shows that women with bacterial vaginosis have increased risk to develop more episodes of this condition during their lifetime [8]. The association of clinical findings as pH and positive or doubtful whiff test with an abnormal pattern vaginal flora was well established [22] and could be confirmed by our results.

Bacterial counting by flow cytometry was already described and proposed as a fast tool to evaluate microbiological changes in vaginal flora over the time [15]. Although in this previous study by Schellenberg et al. [15] it was showed that total bacterial counting is increased in normal flora when compared with bacterial vaginosis, our results failed to confirm these findings, as no difference was observed between the two groups in the current study. Of noteworthy, Schellenberg et al. [15] evaluated samples from mid-vagina, while the current study was performed using cervicovaginal lavages, which may explain the inconsistency on the results of both

studies. Nevertheless, these findings have been a matter of discussion, since data from cultures of vaginal swabs show the opposite results, as the number of colony forming units (CFU) correlate positively with Nugent score [11]. This finding is supported by the presence of bacterial biofilms that are frequently detected in cases of bacterial vaginosis [13] and contribute positively for bacterial growth.

Regarding the treatment of bacterial vaginosis, no difference in total bacterial counting was found at baseline samples from women that returned with normal flora against those women who had AVF in the second visit. This finding suggests that total bacterial counting should not be a determining feature on treatment resistance of bacterial vaginosis. Therefore other microbiological features might be linked to treatment failures, such as the presence of metronidazole resistant *Atopobium vaginae* strains [23]. Moreover, in relation to recurrence, women with the concomitant detection of *A. vaginae* and *G. vaginalis* have higher rates of recurrence when compared to those with the detection of *G. vaginalis* alone [24].

Conclusion

Present data show that total bacterial counting assessed by flow cytometry does not influence the response to metronidazole treatment of bacterial vaginosis. It suggests that therapeutic failures might be related to other microbiological aspects of the vaginal flora, such as the bacterial composition and pathogenic potential of the strains.

Conflict of interest: None of the authors have conflicts of interest to mention.

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Table 1. Demographic, behavioral and clinical characteristics of the 223 women included in the study, in relation to the classification of the vaginal flora in normal (scores 0-3) and abnormal vaginal flora (AVF, scores 4-10), according to Nugent's system [16].

Characteristics	Normal (n=132)	AVF (n=91)	p
Age[*]	34 [18-50]	33 [18-49]	0.92
Ethnicity[**]			
White	82 (62.1%)	47 (51.7%)	0.12
Non-White	50 (37.9%)	44 (48.3%)	
Marital status[**]			
Single	34 (25.8%)	37 (40.7%)	0.02[¥]
Married/living together	98 (74.2%)	54 (59.3%)	
Years at school[*]	9 [0-16]	8 [0-15]	0.10
Remunerated Activity (n/total)[**]	71 (53.8%)	49 (53.8%)	0.99
Smoking Habit (n/total)[**]	21 (15.9%)	20 (22.0%)	0.25
Number of sex partner (1 year)[**]			
0 or 1	120 (90.9%)	77 (84.6%)	0.15
2 or more	12 (9.1%)	14 (15.4%)	
Number of vaginal intercourse/week[*]	2 [0-7]	2 [0-7]	0.70
Previous BV[**]	55 (41.7%)	52 (57.1%)	0.02[¥]
Previous STD[**]	11 (8.3%)	10 (11.0%)	0.50
Consistent Condom Use[**]	22 (16.7%)	23 (25.3%)	0.11
Hormonal contraceptive use[**]	64 (48.5%)	33 (36.3%)	0.07
Vaginal pH[*]	4.4 [4.0-5.0]	4.7 [4.0-7.0]	<0.0001[¥]
Cervical ectopy[**]	50 (38.0%)	38 (41.8%)	0.56
Whiff test[**]			
Positive or doubtful	71 (53.8%)	77 (84.6%)	<0.0001[¥]
Negative	61 (46.2%)	14 (15.4%)	

AVF: Abnormal vaginal flora, BV: Bacterial vaginosis; STD: Sexually transmitted infection.
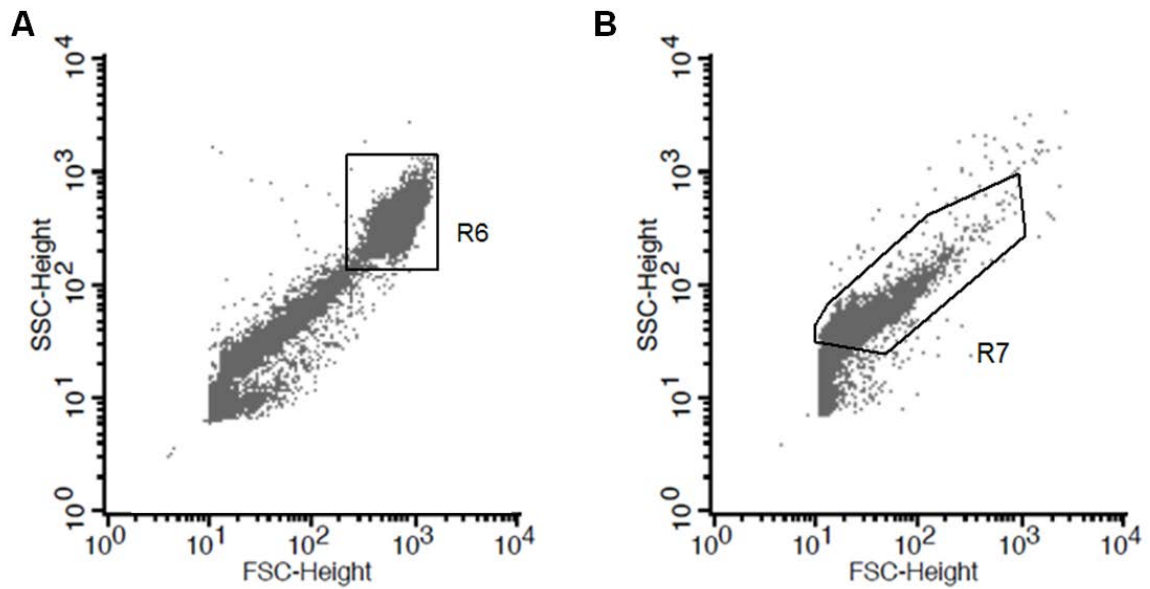[*]Mann-Whitney test; [**]Chi-square test. [¥]p<0,05

Figure 1. A. Scattering profile of a vaginal sample resembling the findings of Schellenberg et al. (2008) [15] with two distinct populations observed. Upper population (R6) consisted of host's epithelial or inflammatory cells and lower of bacterial cells. **B.** Distribution of the bacterial cells on the dot-plots was confirmed using a pure culture of *Staphylococcus aureus* (ATCC 19095) (R7).
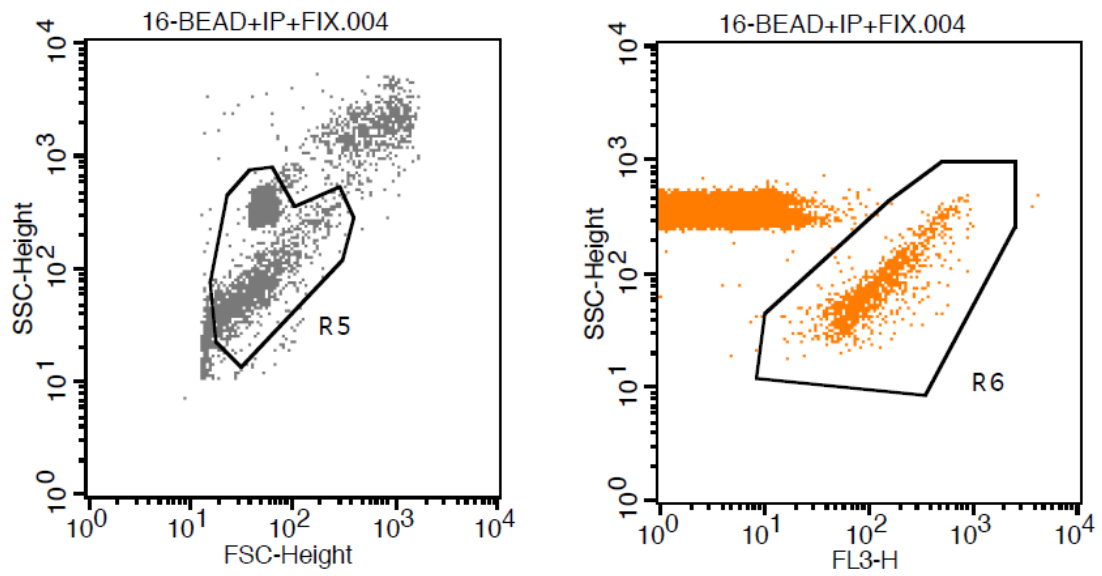
Figure 2. Scattering profiles at FSC/SSC and FL3/SSC showing gates that were created for both bacterial cells population and beads (R5) and bacterial cells (R6) exclusively. Bacterial cells units were determined based on the events observed on gate R6 when 10000 bead-events were recorded.
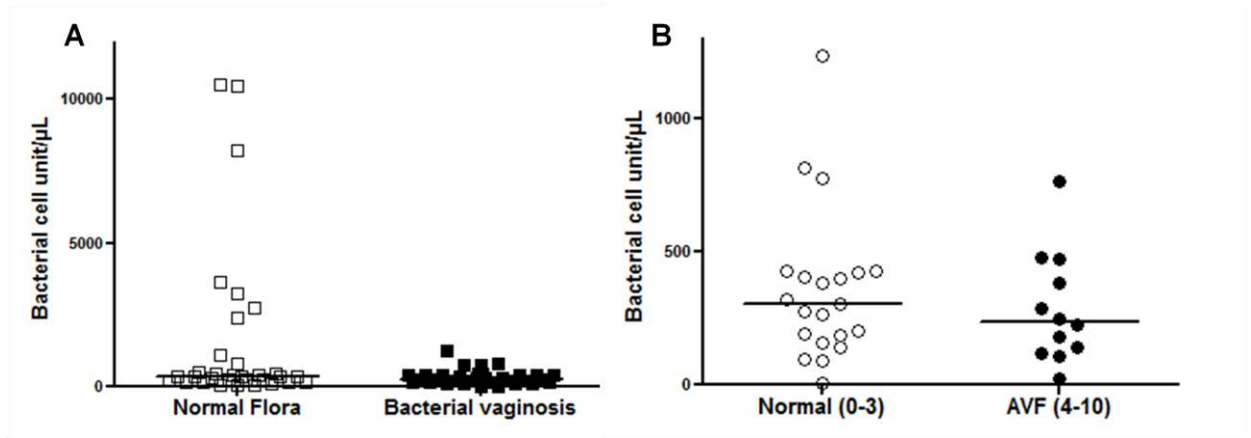
Figure 3. A. Total bacterial counting in cervicovaginal lavages from women with normal flora (n=32) and bacterial vaginosis (n=33) and **B.** from women with bacterial vaginosis successfully treated after metronidazole therapy (n=21) and those with persistence of abnormal vaginal flora (n=12). Horizontal bars represent median values.