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Original Article

Antimicrobial susceptibility and vaginolysin in *Gardnerella vaginalis* from healthy and bacterial vaginosis diagnosed women

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Abstract

Introduction: Bacterial vaginosis (BV) is a syndrome related to *Gardnerella vaginalis* and is characterized by an imbalance in the vaginal microbiota. This work focused on the evaluation of antimicrobial susceptibility patterns and the occurrence of the vaginolysin (*vly*) gene in *G. vaginalis* isolated from BV and non-BV patients.

Methodology: The vaginal secretions were collected randomly and processed for *G. vaginalis* isolation. The isolates were presumptively identified by β-hemolysis and oxidase and catalase tests. Polymerase chain reaction (PCR) was performed to confirm bacterial identity and to detect the *vly* gene. Antimicrobial susceptibility patterns were determined.

Results: Of 89 patients, *G. vaginalis* was isolated from 42 (37 BV and 5 non-BV), and 204 isolates were selected (179 from BV and 25 non-BV). The *vly* gene was detected in all *G. vaginalis* isolated from non-BV women and in 98.3% of the bacteria from BV patients. High resistance was observed for ampicillin (54.4%), metronidazole (59.8%), tinidazole (60.3%) and secnidazole (71.6%).

Conclusions: Further studies are needed to better address the role of *G. vaginalis* and the *vly* gene in BV pathogenesis.

Key words: bacterial vaginosis; *Gardnerella vaginalis*; Nugent score; antimicrobial resistance; clue-cells.

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Introduction

Vaginal microbiota is defined as a diverse group of microorganisms that colonises the vagina without causing disease, considering the host's regular homeostatic conditions [1]. Bacterial vaginosis (BV) is a polymicrobial syndrome mainly related to *Gardnerella vaginalis*, characterized by an imbalance in the healthy vaginal microbiota with an increase in anaerobic bacteria, particularly those producing H_2O_2, which leads to the onset of fetid vaginal discharge [2].

The prevalence of BV is difficult to determine, since a large proportion of infected women are asymptomatic and do not seek medical care, and are not therefore included in the studies. BV is the most common cause of vaginal discharge in women of reproductive age and is more common in black women than white, in those women with intrauterine devices (IUDs), in smokers, in women with multiple sexual partners, and in patients using antibiotics [3].

Anaerobic microorganisms associated with BV mainly include *G. vaginalis*, *Ureaplasma urealyticum*, *M. hominis*, *Mobiluncus* spp., *Bacteroides* spp., and *Prevotella* spp. The microorganisms involved and their products differ significantly between women with BV, and the risk of upper genital tract infections also probably varies between individuals [4]. The isolation of *G. vaginalis* may not be used for BV diagnosis because it is part of the vaginal microbiota of more than 50% of healthy women. A high concentration of *G. vaginalis* is often associated with the presence of BV [1].

As the clinical diagnosis of BV has been shown to be vulnerable, different methods have been proposed, such as those of Amsel and Nugent, which include clinical and laboratory observations [5]. Amsel criteria is used as the main clinical approach to BV diagnosis and requires three of the following four criteria: vaginal pH higher than 4.5; presence of adherent white vaginal discharge; finding of vaginal epithelial cells covered with adherent bacteria, the clue cells; and release of volatile amines following the addition of potassium hydroxide to a small amount of vaginal fluid [5,6]. As

an alternative to the clinical diagnosis based on Amsel criteria, the Nugent score has been proposed to evaluate the vaginal microbiota through examination of vaginal smears under a microscope [7,8].

The Nugent scoring system is built on standardized Gram-stain interpretation-based criteria where vaginal swab smears are graded on a 10-point scale according to presence or absence of *Lactobacillus* morphotypes, Gram-variable or Gram-negative rods, and curved Gram-negative rods [8]. The Nugent score was designed to evaluate the alterations in vaginal microbiota from the healthy to the BV state, and is considered the gold standard in BV diagnosis [2,7,8].

For over half a century, the virulence mechanism of *G. vaginalis* remained poorly understood. Recent reports on the comparative genomic analysis of strains of *G. vaginalis* isolates from BV symptomatic patients and asymptomatic individuals provided information about the potential virulence characteristics of *G. vaginalis*. The difference in cytotoxicity between strains of *G. vaginalis* is attributed to its ability to adhere to vaginal epithelial cells and form biofilm [9].

Although showing low virulence, *G. vaginalis* has some virulence factors that are well established. This microorganism produces a cytolytic toxin that acts as a hemolysin, and it belongs to the family of cholesterol-dependent cytolysins. The toxin, vaginolysin (VLY), is a porin and has selectivity for human red cells and cells of the vaginal epithelium. It has been hypothesized that VLY is involved in the pathogenesis of BV, leading to cell death [10].

The treatment and control of BV is aimed at reducing the population of anaerobic microorganisms, possibly prompting an increase in H_2O_2-lactobacilli-producing species [11]. The anti-anaerobic drugs of choice recommended for BV treatment are metronidazole and clindamycin, by the guidelines for treatment of sexually transmitted diseases from the Centers for Disease Control and Prevention (CDC) and by the Ministry of Health in Brazil [12]. Concern regarding antibiotic therapy is related to the eradication of vaginal lactobacilli from healthy microbiota, which is important for homeostasis [13]. The recommended treatments are still unsatisfactory, and high rates of recurrence and bacterial resistance are frequent, because the pathogenesis process involving *G. vaginalis* is not yet well understood.

In this context, the aim of this work was to evaluate the antimicrobial susceptibility patterns in *G. vaginalis* to drugs that are empirically and regionally prescribed in routine BV treatment, as well as to assess the occurrence of the vaginolysin (*vly*) gene in *G. vaginalis* isolated from both women with BV and women without BV.

Methodology

Patient population sampling

The sampled population comprised 89 women randomly selected from public or private health services between April 2011 and April 2012. This study was approved by the Ethics Committee of the Federal University of Juiz de Fora. The following inclusion criteria were used: patients who had undergone regular Papanicolaou tests; who had not used systemic antibacterial and antifungal drugs in the past 30 days, and had not had sex within the 5 days prior to the examination; who did not use topical vaginal products; and who agreed to participate in the study by signing the instrument of consent. Exclusion criteria were pregnant patients and patients with a clinical and laboratory diagnosis of cervical-vaginal infections from other causes.

Vaginal sample collection and G. vaginalis isolation

Vaginal specimens were collected during the clinical examination. Sterile cotton swabs were saturated with vaginal secretion and placed in a test tube containing 1.0 mL of *Gardnerella* transport medium (proteose peptone #3, 1.35%; glycerin, 10%, pH 6.8) and sent to the Laboratory of Bacterial Physiology and Molecular Genetics on the same day for further experimental procedures. Smears were also prepared on glass slides and Gram stained for visualization of epithelial cells coated with bacteria (clue cells). The quantification of clue cells was considered to determine the Nugent score, based on the established method [8]. Patients with scores of 4–6 were considered to be intermediate, and 7–10 were considered to be BV patients, whereas patients with scores of 0–3 were considered to be non-BV patients.

In the laboratory, selective cultures were performed on Columbia agar (HiMedia, Mumbai, India) supplemented with nalidixic acid (Sigma-Aldrich GmbH, Munich, Germany), gentamicin (Novafarma, Goiás, Brazil), amphotericin B (Sigma-Aldrich GmbH, Munich, Germany), proteose peptone (HiMedia, Mumbai, India), Tween (10%) (Sigma-Aldrich GmbH, Munich, Germany), and blood (5%) (vaginalis modified agar [VMA]) [14]. Culture plates were incubated under an anaerobic atmosphere, at 37°C for 48–72 hours. For presumptive identification, small colonies with β-hemolysis or diffuse hemolysis were Gram stained. Five different Gram-variable coccobacilli suggestive of *G. vaginalis* were picked for each patient and spread in

VMA to obtain pure culture and cell mass. Oxidase and catalase tests were also performed. *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as positive controls in the oxidase or catalase tests. The characterized bacteria were frozen and stored at -20°C.

Confirmation of bacterial identity from culture and vaginal secretions by polymerase chain reaction (PCR) and screening of vly gene

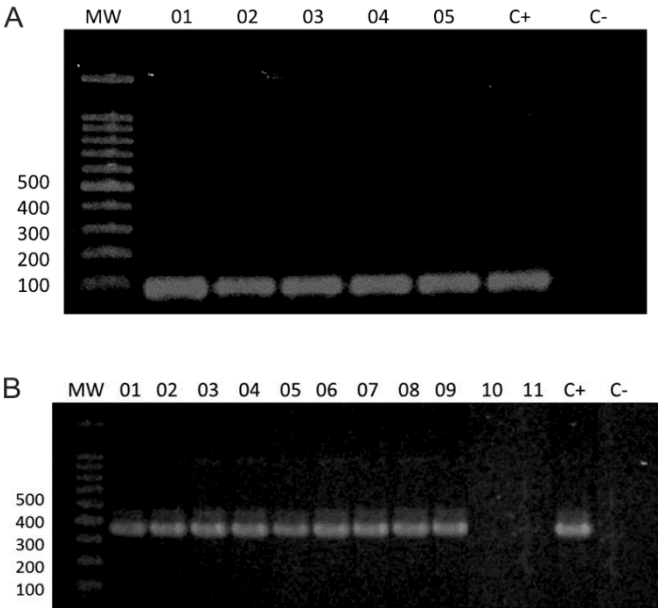
Bacterial genomic DNA from culture and from vaginal secretions was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instructions. For specific molecular identification, the primers *G. vaginalis* R (5' CAG CAA TCT TTT CGC CAA CT 3') and F (5' CGC ATC TGC TAA GGA TGT TG 3') were used according to established procedures [15]. For the *vly* gene screening, the primers R (5' ACA TAA GCT TGG CCA CGG TC 3') and F (5' GCC CTT GAA GAA AGA CAG CC 3') were used. The reactions were performed in a final volume of 25 μL containing 0.5 mL of each primer, 1.0 μL of DNA template, and 12.5 μL of PCR Master Mix (Promega), containing Taq DNA polymerase, dNTPs, $MgCl_2$, and buffer. The conditions for amplification of the vaginolysin (*vly*) gene were initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds, followed by a final extension of 72°C for 5 minutes. PCR reactions were performed in duplicate in an automated thermal cycler (Biometra, Göttingen, Germany). The amplicons in each reaction were visualized on 1.5% agarose gel in 0.5X TBE, after electrophoresis at a constant voltage. The gels were analyzed under transilluminator ultraviolet light after being treated with ethidium bromide (Promega, Madison, USA). As a molecular weight standard, 100 bp DNA ladder (Promega, Madison, USA) was used. As a positive control in PCR reactions, *G. vaginalis* ATCC 14018, ATCC 14019, and ATCC 49145 were included. As a negative control, PCR reactions were performed without template DNA.

As quality control, random sequencing of amplicons was performed, comprising 10% of the total positive PCR reactions (*G. vaginalis* molecular identification and *vly* gene screening). The PCR products were sequenced in an ABI Prism 3730 DNA sequencer (Applied Biosystems, Carlsbad, USA).

Evaluation of antimicrobial drug susceptibility

The minimum inhibitory concentration (MIC) for antimicrobial drugs was determined by the agar dilution method, based on the Clinical and Laboratory Standards Institute (CLSI) guideline [16]. Suspensions of each bacterial isolate were prepared in Columbia agar broth (HiMedia, Mumbai, India), using a 0.5 McFarland standard, for a total concentration of 1 x 10^8 CFU mL^{-1}. All isolates were simultaneously inoculated onto each antibiotic dilution plate with a Steers replicator. Antibiotic stock solutions were added to melted Muller-Hinton (HiMedia, Mumbai, India) agar to obtain final concentrations ranging from 0.06 to 1,024 μg mL^{-1}. The plates were incubated anaerobically for 48–72 hours at 37°C. The antimicrobial drugs were selected on the basis of microbial characteristics and clinical relevance: ampicillin (Novafarma Pharmaceutical Ltd., Goiás, Brazil), ampicillin/sulbactam (Cellofarm Pharmaceutical Ltd., Serra, Brazil), clindamycin, chloramphenicol, metronidazole, secnidazole, and tinidazole (all manufactured by Sigma-Aldrich GmbH, Munich, Germany). The reference strains *G. vaginalis* (ATCC 14018, ATCC 14019, and ATCC 49145) and *Bacteroides fragilis* (ATCC 25285) were included as controls, and all tests were performed in duplicate. The

Figure 1. Representative electropherograms of *Gardnerella vaginalis* PCR identification (A) and *vly* gene screening (B). A: MW: molecular weight standard; lanes 1 to 5: bacteria isolated from vaginal secretions; *G. vaginalis* ATCC 14018, positive control (C+); negative control (C-). Expected amplicon size: 100 bp. B: MW: molecular weight standard; lanes 1 to 9: *vly* positive reaction; lanes 10 and 11: *vly* negative reactions; *G. vaginalis* ATCC 14018, positive control (C+); negative control (C-). Expected amplicon size: 459 bp.



MICs were determined using the breakpoint for metronidazole (≥ 32 μg mL^{-1}).

Statistical analysis

Standard calculations for the measurement of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were used, considering culture and molecular methods.

Results

Presumptive identification, identity confirmation of G. vaginalis and vly gene screening by PCR

Of 89 patients (41 BV symptomatic and 48 asymptomatic) classified according to Nugent score, *G. vaginalis* was isolated from 42 (37 BV and 5 non-BV patients). Whenever possible, 5 representative colonies were selected from each vaginal secretion, totalling 204 isolates (179 from BV and 25 from non-BV). The identity of all presumptively isolated bacteria was confirmed by PCR (Figure 1A). The *vly* gene was detected in all *G. vaginalis* isolated from non-BV women and in 98.3% of the bacteria isolated from BV-symptomatic patients (Figure 1B).

Comparison among isolation of G. vaginalis by culture methods and bacteria detection by PCR

Among the 89 clinical specimens of patients' vaginal secretions, the isolation of *G. vaginalis* by culture methods was not possible in 47 of them. Thus, the total DNA extracted from the vaginal secretions was used as PCR templates in order to compare the sensitivity of the methods. In 55.3% (26/47) of the samples, *G. vaginalis* was detected; 24 were non-BV, 1 was BV, and 1 had an intermediate Nugent score. Comparing molecular and culture methods, the sensitivity, specificity, PPV, and NPV were found to be 61.7%, 100%, 100%, and 44.7%, respectively.

Evaluation of antimicrobial drug susceptibility

As no guidelines or consensus were available for antimicrobial susceptibility patterns of *G. vaginalis*, the antimicrobial breakpoints described for anaerobic bacteria by the CLSI document M11-A7 [16] were used (Table 1). The results of the antimicrobial susceptibility tests are shown in Table 2 and are presented in terms of MIC_{50}, MIC_{90}, and the range of MICs. Chloramphenicol, ampicillin/sulbactam, and

Table 1. Antimicrobial susceptibility patterns determined for control strains, including *Bacteroides fragilis* ATCC 25285, recommended by the Clinical and Laboratory Standards Institute guidelines.

Antimicrobial drugs	Minimum inhibitory concentration (μg mL^{-1})			
	***G. vaginalis* ATCC 14018**	***G. vaginalis* ATCC 14019**	***G. vaginalis* ATCC 49145**	***B. fragilis* ATCC 25285**
Ampicilin	0.5	0.5	0.5	64.0
Ampicilin/sulbactam	0.25	0.25	0.25	1.0
Clindamycin	0.0625	0.0625	0.0625	0.25
Chloramphenicol	4.0	4.0	4.0	2.0
Metronidazole	16.0	16.0	16.0	4.0
Secnidazole*	4.0	4.0	4.0	4.0
Tinidazole*	4.0	4.0	4.0	2.0

*The CLSI guide (2007) does not offer breakpoints for tinidazole and secnidazole. Due to the similarity in structure and activity, values for metronidazole were used.

Table 2. Susceptibility profiles of isolates of *G. vaginalis* to antimicrobial drugs of microbiological and clinical relevance.

Antimicrobial drugs	Minimum inhibitory concentration (μg mL^{-1})			Interpretation criteria according to the CLSI		
	$MIC_{50\%}$	$MIC_{90\%}$	Range	S	I	R
Ampicillin	2.0	8.0	0.0625–64.0	25% (51/204)	20.6% (42/204)	54.4% (111/204)
Ampicillin/sulbactam	1.0	4.0	0.0625–16.0	95.1% (194/204)	4.9% (10/204)	0% (0/204)
Clindamycin	0.0625	0.5	0.0625–1,024	93.1% (190/204)	0% (0/204)	6.9% (14/204)
Chloramphenicol	1.0	2.0	0.125–8.0	100% (204/204)	0% (0/204)	0% (0/204)
Metronidazole	32.0	1,024	2.0–>1,024	26% (53/204)	14.2% (29/204)	59.8% (122/204)
Secnidazole	64.0	>64.0	2.0–>64.0	22% (45/204)	6.4% (13/204)	71.6% (146/204)
Tinidazole	32.0	>64.0	1.0–>64.0	32.8% (67/204)	6.9% (14/204)	60.3% (123/204)

CLSI: Clinical and Laboratory Standards Institute; S: sensitive; I: intermediate; R: resistant.

clindamycin were the most effective drugs, and all isolates were sensitive to chloramphenicol. Considering all 204 strains, resistance was observed against secnidazole (71.5%), tinidazole (60.3%), metronidazole (59.8%), and ampicillin (54.4%).

Discussion

The vaginal microbiota under homeostatic conditions is predominantly composed of *Lactobacillus*, followed by other microbial groups, among which exist some opportunistic pathogens [14,15]. Within those opportunistic pathogens, *G. vaginalis*, *A. vaginae*, *Mobiluncus* spp., and *M. hominis* have been noted [15]. *Lactobacillus* plays an important role in the structure of the vaginal ecosystem and has protective properties against colonization by non-resident bacteria or overgrowth of potentially pathogenic species. It is assumed that a decrease of these *Lactobacillus* may interfere with vaginal microbiota status [15]. Bacterial vaginosis may be a result of vaginal microbiota imbalance, and it affects millions of women annually. It is associated with numerous adverse health outcomes, including pre-term birth and the acquisition of sexually transmitted infections [17].

In our study, all clinical specimens collected from patients were Gram stained for visualization of clue cells and determination of the Nugent score to diagnose BV and non-BV patients. Although clinical observations may provide evidence for BV diagnosis, it is not consensual or highly reliable. In this regard, some criteria were proposed, such as the Amsel criteria and Nugent scoring system [5].

The Nugent score, although less sensitive for the identification of BV, appears to be more reliable because it eliminates subjective aspects found in Amsel's criteria (appearance and odor). BV diagnosis by the Nugent scoring system is currently considered the gold standard [11]. Nugent *et al*. [8] proposed a scoring system that involves the identification and quantification of bacterial morphotypes, such as *Lactobacillus*, *Mobiluncus*, and *G. vaginalis*. A score of 0–3 is considered healthy (non-BV) and is characterized by the predominance of Gram-positive rods. A score of 7–10 confers the diagnosis of BV and is marked by the absence of Gram-positive and presence of high concentrations of *G. vaginalis* or *Mobiluncus* spp. morphotypes. A score of 4–6 is designated intermediary, and the morphotypes are characteristic between the two poles.

Using VMA, we isolated 204 strains of *G. vaginalis* from BV and non-BV patients. The VMA was highly selective and differential, allowing optimal visualization of β-hemolysis or diffuse hemolysis, which is an important parameter for identification. All isolates were presumptively identified as *G. vaginalis*, and this was confirmed by PCR using specific primers for *G. vaginalis*.

Due to *G. vaginalis* being present in almost 100% of women with BV, it is likely that this microorganism plays an important role in the development of this condition. Moreover, *G. vaginalis* has been detected in approximately 50% of the vaginal microbiota by microbiological culture, and up to 70% by molecular methods. *G. vaginalis* cultures are not very specific and are expensive for routine clinical practice. Definitive diagnosis with molecular biology tools has been restricted to research [1,5,17].

Sensitivity is the probability that a test is positive in the presence of the disease; that is, the test assesses the ability to detect disease when it is present. Specificity is the probability of a test to be negative in the absence of disease. PPV is the probability that individuals with a positive screening test truly have the disease. NPV is the probability that individuals with a negative screening test truly do not have disease [18].

In our study, PCR was more efficient than the culture method, showing a sensitivity of 61.7%, specificity of 100%, PPV of 100%, and NPV of 44.7%. These values of specificity and PPV eliminate the possibility of false-positive and false-negative results, because specificity is the fraction of those without disease who will have a negative test and PPV is the proportion of true positive between all positive test individuals [18]. Culture may thus not become the gold standard for diagnosis of BV, as the organisms that are involved in BV are found in vaginal microbiota. Gamal *et al*. [17] evaluated different diagnostic methods of bacterial vaginosis and found highest sensitivity using the qPCR method (96.9%), specificity (97%); culture had the least specificity (88%) and lowest PPV (79%). These findings are in accordance with our results, corroborating the proposition that the molecular methods are the most sensitive and accurate.

G. vaginalis has some virulence factors already well established. Among them, cytolytic toxin acts as a hemolysin and belongs to the family of cholesterol-dependent cytolysins, VLY. It has been hypothesised that VLY is involved in the pathogenesis of BV, leading to cell death [10]. Patterson *et al.* [19] and Randis *et al*. [20] examined the cytotoxic activity of anaerobic bacteria associated with BV and found that only *G. vaginalis* was able to induce the lysis of vaginal epithelial cells, whereas other bacteria studied did not

cause detectable cytological alterations. It is accepted that VLY is not a single virulence factor of *G. vaginalis*. It has been suggested that the sum of virulence characteristics, such as adherence properties, mucin degradation, biofilm formation, and sialidase activity confers the pathogenic potential to *G. vaginalis* [19]. In our study, of the 204 isolates submitted to PCR for detecting the *vly* gene, 201 had the gene. However, 3 negative samples were isolated from patients with BV. We did not evaluate other virulence characteristics in these isolates, and we could not establish a correlation between *vly* gene detection and pathogenicity. To date, there are no data available to compare with our data. The data observed in this study may suggest the need for further studies in vaginolysin characteristics considering genetics, biochemistry, and physiology of the VLY protein.

The CDC-recommended treatment for bacterial vaginosis is metronidazole or clindamycin, which are available in oral and intravaginal preparations [21]. However, other drugs of the nitroimidazole class have been explored as an alternative to metronidazole for the treatment of BV [22].

In this study, the treatment of patients was not evaluated. Antimicrobial susceptibility was evaluated among the isolated *G. vaginalis* samples against selected antimicrobial drugs of clinical and microbiological interest. Based on the results, we found high rates of susceptibility to clindamycin. Nagaraja [23] and Teixeira *et al*. [14] observed 76% and 100% (MIC 90% < 0.0625 μg mL^{-1}) of the samples to be sensitive to clindamycin. In spite of the sensitivity of the samples, wide variation was observed (from 0.0625 to 1,024.0 μg mL^{-1}), similar to that found by Nagaraja [23] (0.016–>256 μg mL^{-1}). However, the MICs 50% and 90% are low, within the limits of sensitivity to clindamycin. Thus, the result can be considered favorable, since the antimicrobial successfully treats BV.

Regarding the susceptibility profile of metronidazole, a high level of resistance was observed. Austin *et al*. [22] observed 68% resistance, with an MIC 50% of 32.0 μg mL^{-1}, MIC 90% > 256.0 μg mL^{-1}, and a variation of 2.0–>256.0 μg mL^{-1} (highest concentration tested). Teixeira *et al*. [14] found 70% resistance to metronidazole, and MIC 90% > 512 μg mL^{-1} (highest concentration tested).

Previous studies have related resistance to metronidazole and tinidazole. Goldestein *et al*. [24] had demonstrated 20% resistance of *G. vaginalis* to metronidazole, and the same group reported 29% resistance to metronidazole in 2002. Austin *et al*. [22] related resistance in *G. vaginalis* isolates, with 54% resistant to tinidazole and 68% resistant to metronidazole (470 isolates). Nagaraja [23] found 34 (68%) strains resistant to metronidazole, which was a very high rate in the population studied. Our results corroborate the literature and point out antimicrobial resistance against drugs empirically used in chemotherapy.

Ampicillin is not routinely used for the treatment of BV due to its ineffectiveness in eradicating *G. vaginalis*. This is probably due to the inactivation of ampicillin by beta-lactamases produced by other vaginal anaerobes and not specifically by *G. vaginalis* [25,26]. A high rate of ampicillin resistance and intermediate resistance was observed. In relation to ampicillin/sulbactam (a beta-lactamase inhibitor), resistant bacteria samples were not observed, although a small percentage was classified as intermediate resistance. This result is similar to that found by Goldstein *et al*. [26], who observed 100% sensitivity to this antimicrobial. Our findings suggested that the phenomenon of resistance observed among strains of *G. vaginalis* may be related mainly to the production of beta-lactamase.

Conclusions

In this work, the presence or absence of recurrence among patients was not verified, and it is known that the use of antibiotics may eliminate the pathogenic microorganism and also destroy most of the microorganisms responsible for maintaining the balance of the healthy microbiota. Furthermore, the lack of parameters for the correct diagnosis of BV and the empirical use and misuse of antibiotics aggravates the problem of recurrence and resistance. The data presented here are alarming with respect to resistance to antimicrobial agents used in the treatment of BV. Overall, this study focused on antimicrobial resistance of *G. vaginalis*, in both BV and non-BV women, and on detecting the *vly* gene, a virulence determinant hypothesized to be involved in the pathogenesis of BV. Further prospective studies are needed to better address the role of *G. vaginalis* in BV pathogenesis, and regional drug-susceptibility studies should be used to minimize the bacterial resistance phenomena.

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