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Facultad de Ciencias Químico Biológicas
Unidad Académica de Medicina
Unidad de Investigación Especializada en Microbiología

Maestría en Ciencias Biomédicas

Análisis de biotipos, factores de virulencia y sensibilidad a antibióticos en cepas de *Gardnerella vaginalis* asociadas y no asociadas a Vaginosis Bacteriana

T E S I S

Que para obtener el título de

Maestría en Ciencias Biomédicas

PRESENTA:

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Directora: Dra. Amalia Vences Velázquez

Codirectora: M. en C. Karen Cortés Sarabia

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UNIVERSIDAD AUTÓNOMA DE GUERRERO
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APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 18 días del mes de junio de dos mil dieciocho se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Análisis de biotipos, factores de virulencia y sensibilidad a antibióticos en cepas de *Gardnerella vaginalis* asociadas y no asociadas a Vaginosis Bacteriana"**, presentada por la alumna Ana Karen Estrada Moreno, para obtener el Grado de Maestría en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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Durante el periodo en que se cursó la Maestría en Ciencias Biomédicas, la C. Ana Karen Estrada Moreno, recibió beca CONACYT con No. De registro 777249

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“Análisis de biotipos, factores de virulencia y sensibilidad a antibióticos en cepas de *Gardnerella vaginalis* asociadas y no asociadas a Vaginosis Bacteriana”

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Dear Dr. Ana Karen Estrada-Moreno,

You have been listed as a Co-Author of the following submission:

Journal: Anaerobe
Title: Biotypes, virulence factors and sensitivity to antibiotics in strains of *Gardnerella vaginalis* associated with normal microbiota and Bacterial Vaginosis
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Thank you,

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Abstract: Introduction: Bacterial vaginosis (BV) is a polymicrobial infection considered as a public health problem that affects women in reproductive age; its main etiologic agent is Gardnerella vaginalis. This bacterium is divided into eight biotypes and produces several virulence factors that favor the development of BV. In addition, an increased resistance to conventional treatment has been reported, which favors the failure to treatment and disease recurrence. Objectives: Analyze the relationship between the virulence factors production, antibiotics resistance and biotypes of G. vaginalis strains associated with normal microbiota (NM) and BV. Materials and methods: We analyzed 150 strains of G. vaginalis; biotyping, biofilm, prolidase, phospholipase C and vaginolysin production were determinated. Additionally, metronidazole and clindamycin resistance was performed by the Kirby-Bauer method and secnidazole by minimum inhibitory concentration (MIC). Results: Biotypes 1 (18%), 2 (13.3%), 5 (17.3%) and 6 (51.3%) were identified. The BV-associated strains produce more biofilm (p=0.026) and have more lithic capacity (p=0.043) that NM-associated strains. No significant difference between virulence factors production and biotypes was observed, with the exception of biotype 2 in phospholipase C production (p=0.0001). BV-associated strains produce more virulence factors in comparison with normal microbiota, especially biotypes 1 and 6, and finally a high resistance rate to metronidazole (100%), secnidazole (95.3%) and clindamycin (90.6%) was observed. Conclusion: A great diversity in the virulence factors production between NM and BV-associated strains was observed, which could contribute during the development of BV; additionally, the high resistance rate may impact in the treatment failure and disease recurrence.

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The authors **Ana Karen Estrada-Moreno, Karen Cortés-Sarabia, Luz del Carmen Alarcón-Romero, Natividad Castro-Alarcón, Eugenia Flores-Alfaro, Isela Parra-Rojas, Amalia Vences-Velázquez** submit the manuscript entitled: **"Biotypes, virulence factors and sensitivity to antibiotics in strains of *Gardnerella vaginalis* associated with normal microbiota and Bacterial Vaginosis"** and declare that they have read and approved the manuscript.

This manuscript is an original research that evaluates biotypes, virulence factor production and antibiotic susceptibility in strains of *G. vaginalis* associated with normal microbiota and bacterial vaginosis. This work provides evidence about the isolation of biotypes 1, 2, 5 and 6 of *G. vaginalis* and the production of biofilm, prolidase, phospholipase C and vaginolysin in strains of this bacterium associated with normal microbiota and bacterial vaginosis. We also, analyzed the antibiotic resistance rate to metronidazole, clindamycin and secnidazole. The phenotypic characterization of *G. vaginalis* strains could provide information for the design of new diagnostic methods and for the better understanding of the pathogenic potential of this bacterium in the vaginal microenvironment.

We expect that you find our work worthy for publication in the Journal of Anaerobes.

Amalia Vences Velázquez, phD
Universidad Autónoma de Guerrero

Highlights

- We isolated biotypes 1, 2, 5 and 6 of *G. vaginalis*.
- Biofilm and vaginolysin production were associated with BV.
- Biotypes 1 and 2 produces more Phospholipase C.
- BV-associated strains produce more virulence factors that NM-associated strains.
- A high resistance rate to metronidazole, clindamycin and secnidazole was observed.

Biotypes, virulence factors and sensitivity to antibiotics in strains of *Gardnerella vaginalis* associated with normal microbiota and Bacterial Vaginosis

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Abstract

Introduction: Bacterial vaginosis (BV) is a polymicrobial infection considered as a public health problem that affects women in reproductive age; its main etiologic agent is *Gardnerella vaginalis*. This bacterium is divided into eight biotypes and produces several virulence factors that favor the development of BV. In addition, an increased resistance to conventional treatment has been reported, which favors the failure to treatment and disease recurrence. Objectives**:** Analyze the relationship between the virulence factors production, antibiotics resistance and biotypes of *G. vaginalis* strains associated with normal microbiota (NM) and BV. Materials and methods: We analyzed 150 strains of *G. vaginalis*; biotyping, biofilm, prolidase, phospholipase C and vaginolysin production were determinated. Additionally, metronidazole and clindamycin resistance was performed by the Kirby-Bauer method and secnidazole by minimum inhibitory concentration (MIC). Results: Biotypes 1 (18%), 2 (13.3%), 5 (17.3%) and 6 (51.3%) were identified. The BV-associated strains produce more biofilm (p=0.026) and have more lithic capacity (p=0.043) that NM-associated strains. No significant difference between virulence factors production and biotypes was observed, with the exception of biotype 2 in phospholipase C production (p=0.0001). BV-associated strains produce more virulence factors in comparison with normal microbiota, especially biotypes 1 and 6, and finally a high resistance rate to metronidazole (100%), secnidazole (95.3%) and clindamycin (90.6%) was observed. Conclusion: A great diversity in the virulence factors production between NM and BV-associated strains was observed, which could contribute during the development of BV; additionally, the high resistance rate may impact in the treatment failure and disease recurrence.

Keywords: *Gardnerella vaginalis*, normal microbiota, bacterial vaginosis, virulence factors, biotypes, sensitivity to antibiotics.

1. Introduction

Bacterial vaginosis (BV) is the most common vaginal infection in reproductive aged women, characterized by the replacement of lactobacilli and overgrowth of anaerobic microorganisms [1]. Its prevalence varies from 5% to 57% based on the geographical distribution and analyzed population [2, 3]. BV has been associated with gynecological and obstetric complications such as pelvic inflammatory disease, endometritis, abortions, and an increased susceptibility to acquire sexually transmitted infections [4-6]

Several bacterial genders have been associated with this condition, among them: *Sneathia/Leptothrix Spp*., *Prevotella bivia*, *Megasphera spp*., *Bacteroides spp*., *Peptostreptococcus spp*., *Ureaplasma spp*., *Atopobium vaginae* and *Mobiluncus spp*.; however, several authors consider *Gardnerella vaginalis* as the main etiologic agent, due to the fact that it is isolated from 48% to 98% of vaginal discharge in women with BV [3, 7]. *G. vaginalis* is a facultative anaerobic cocobacillus, catalase and oxidase negative, from 0.5 to 1.5 μm, without capsule, spores or fimbriae [8]. This bacterium is classified into eight different biotypes based on their metabolic characteristics, and four genotypes (1-4) based on the amplification and restriction of the ribosomal DNA [9, 10]. In patients with BV, a higher frequency of lipase positive biotypes (1-4) and genotypes 1 and 3 has been reported [11, 12]. Nevertheless, no studies have been able to establish a relationship between the presence of a particular biotype or genotype with the development of BV [13-15], so it has been suggested that the virulence factors of *G. vaginalis* could play an essential role during pathogenesis in the vaginal tract.

This bacterium produces several virulence factors involved in the proliferation and invasion of the genital tract; enzymes production such as phospholipase C [13], sialidase [16] and prolidase [17] which mediate lipids, proteins and extracellular matrix hydrolysis, allowing the adhesion to epithelial cells during the beginning of the infection. Additionally, it produces vaginolysin (Vly) a cholesterol dependent cytolysin (CDCs), that promotes bacterial invasion and infection through the lysis of erythrocytes, neutrophils and epithelial cells [18]. Alternatively, *G. vaginalis* produces biofilm that confers resistance to the host immune response, such as hydrogen peroxide (H_2O_2) and lactic acid produced by the lactobacilli, as well as greater tolerance to antibiotics [19, 20].

The conventional treatment of BV is based on the use of antibiotics such as clindamycin (CC), metronidazole (MTZ) and secnidazole (SCZ). MTZ and SCZ belong to the family of 5-nitroimidazoles, and affect bacterial DNA without affecting the lactobacilli microbiota, promoting vaginal resettlement after treatment [27, 28]. While CC, targets bacterial ribosomes inhibiting bacterial protein synthesis [21]. An increased resistance to MTZ and CC has been reported in 24.7% to 76% of the cases for both antibiotics [21, 22]. Recently, the use of SCZ has been proposed as a new alternative treatment for BV [23], and just few studies have been conducted to evaluate its effectiveness in strains of *G. vaginalis* [23-25]. The resistance to treatment with antibiotics has an impact on reincidence, which has been reported in around 30% of the cases of BV during the three months post-treatment [22]. Even so, in Mexico there are no studies that report this situation, and the treatment based on MTZ and CC is still the first choice.

Despite the fact that *G. vaginalis* has been widely associated with the development of BV, particular analysis of biotypes and virulence factors production in strains have failed to establish an association between its presence and BV development. Nonetheless, the

increased resistance to antibiotics used during the conventional treatment, leads to a treatment failure and increased recurrence. Therefore, the aim of this work was to evaluate the relation between biotypes, virulence factors production and antibiotic resistance (metronidazole, clindamycin and secnidazole) in strains of *G. vaginalis* associated with NM and BV.

2. Materials and methods

2.1 Population and sample collection

One hundred and fifty strains of *G. vaginalis* from our biobank were used. Bacterial isolation was performed from vaginal swabs samples of patients who attended to the Servicio de Diagnóstico Integral en la Detección Oportuna de Cáncer Cervicouterino y VPH in the Universidad Autónoma de Guerrero (UAGro). Samples were collected from the vaginal sac fundus by using two sterile swabs, which were placed in physiological saline solution and Stuart transport medium. All patients signed an informed consent based on the Helsinki Declaration of 2013; the project was previously approved by the bioethics committee of the UAGro. The BV diagnosis was carried out based on Amsel criteria, that evaluate clinical parameters such as vaginal pH >4.5, vaginal discharge, amine positive test and the presence of clue cells in pap smear; establishing the diagnosis with the presence of three of the four criteria [36].

2.2 Isolation and preservation of *G. vaginalis*

G. vaginalis isolation was performed on Columbia agar supplemented with 10% of human blood and SR119RE selective supplement (OXOID Cat#1441974). Plates were incubated at 37°C for 48 hours in CO_2 tension. Specific identification includes Gram stain, catalase and

oxidase test. After identification, bacteria were grown in thioglycolate broth and preserved for subsequent test.

2.3 Biotyping

Biotyping was performed by using the classification scheme proposed by Piot *et al.*, [9]. **β-galactosidase:** Culture liquid medium (100 µL) was inoculated in 500 µL of 2-nitrophenyl-β-D-galactopiranoside (Sigma Aldrich Cat # N1127), during 24 hours at 37°C. The presence of a yellow color in the tube was considered positive and negative when the reagent did not turn into any color. **Hippurate hydrolysis:** Culture liquid medium (100 µl) was inoculated in 400 µl of 1% sodium hippurate reagent (Sigma Aldrich Cat # H9380), and incubated at 37 °C for 2 hours. After that, 200 µl of ninhydrin (Sigma Aldrich Cat # 151173) were added and homogenized. The test was considered as positive when the reagent turned purple (indicating the presence of glycine in the mix as a result of hippurate hydrolysis), and as negative when the reagent did not turn into any color. **Lipase**: It was evaluated in egg yolk agar with bromophenol blue as indicator according to the previously reported [26]. The strains were inoculated by streak plate method and incubated at 37°C for 24 hours. The presence of an iridescent halo around the colony was considered as positive and each test was performed by duplicate.

2.4 Phospholipase C

Phospholipase C activity was assessed according to the method previously described [13]. The strains were inoculated by streak plate method in skim milk agar and incubated at 37°C for 24 hours. The presence of a transparent halo around the colonies was considered as positive.

2.5 Biofilm production

Culture liquid medium (20 µL) was inoculated in 180 µL of sterile thioglycolate broth in 96 wells highly hydrophilic flat bottom plates (COSTAR Cat # 3590) and incubated for 24 hours at 37 °C in CO_2 tension. First, growth was evaluated by optical density (OD) to 600 nm, then the medium was discharged and the plate washed with phosphate buffer (PBS) pH 7.0. The plate was air-dried at room temperature during 40 minutes, after we added 200 µL of safranin during 1 minute, the plate was washed with PBS and safranin solubilized with acetic acid at 33%. OD was measured at 650 nm and results classified as; non-producer (<0.1), moderate (0.1- 0.2) and abundant (>0.2), based on the previously reported [13].

2.6 Prolidase

Prolidase was performed by the method previously reported [17]. Briefly, a mix of 50 µL of culture liquid medium with 50 µL of substrate L-proline-p-nitroanilide (Sigma Aldrich Cat # P5328) in 0.1 M sodium acetate pH 5.0 were incubated for 24 hours at 37°C. After, OD was measured at 415 nm. As negative control, the reagent was incubated with sterile liquid medium and results classified as non-producer (<0.1), moderate (0.1-0.2) and abundant (>0.2).

2.7 Vaginolysin

The lytic capacity of used strains was determined by hemagglutination (HA) and percentage of lysis was calculated. In 96 wells "U" plates, 25 µL of culture supernatant of each strain were placed. After that, we added 50 µL of 1 % erythrocytes suspension and the plate was incubated for one hour at room temperature. The supernatant was collected into a new plate and the optical density at 415 nm was obtained from each sample. For analysis, we calculated the percentage of lysis based on the obtained OD, considering as 100% the OD given by erythrocytes suspension at 1% incubated with triton. The reaction

159 was considered as positive when cellular lysis was observed and as negative by the
160 presence of a cellular button. The results were classified in: without lysis (<10 %), low
161 (10.1-49.9 %) and high (>50 %) based on the obtained results.

162 **2.8 Antibiotic resistance**

163 Sensitivity to the antibiotics; clindamycin and metronidazole was carried out by using the
164 disk diffusion method [27]. One inoculum of 3 mL was prepared from a pure culture of *G.*
165 *vaginalis* in physiological saline solution, turbidity was adjusted to 0.5 in the McFarland
166 scale (1.5×10^8 CFU/mL), and inoculated by spread plate method by using a sterile swab in
167 Mueller-Hinton agar plates supplemented with 5% of human blood, let dry for five minutes
168 and then, the sensidisks were placed exerting slight pressure with a considerable distance
169 between them. After 15 minutes, the plates were incubated at 37°C for 24 hours in CO_2
170 tension. After the incubation, halo inhibition diameter was measured and the strains were
171 classified into sensitive or resistant [28]. *G. vaginalis* ATCC 14018 was included as control
172 due to its resistance pattern to these antibiotics is already known.
173 Secnidazole sensitivity was determined by the minimum inhibitory concentration (MIC)
174 method. First, a stock solution was prepared by using the antibiotic Secnidazole (Sigma
175 Aldrich Cat#35382), after the Columbia agar culture medium was prepared at different
176 concentrations of the antibiotic: 0, 2, 4, 8, 16 and 32 (µg/mL) from the stock solution [29].
177 Before culture, an inoculum of *G. vaginalis* was prepared in 3 mL of physiological saline
178 solution and adjusted to 0.5 on the McFarland scale. The bacterium was inoculated in the
179 Columbia medium and incubated at 37°C for 24 hours in CO_2 tension. After incubation, the
180 MIC was recorded and the strains were classified into sensitive (<8 - 16 µg/mL) or resistant
181 (>32 µg/mL) [30]. *G. vaginalis* ATCC 14018 strain was included as control.

2.9 Statistical analysis

Statistical analysis was performed in Stata V.13. A data base was built with the obtained results from each test and analyzed variables. Relative and absolute frequencies were obtained and *p* value calculated with X^2 test. While OR were calculated by logistic regression model and for global analysis of virulence factors, a new variable was built considering the obtained results from each factor and the possible combination between them. In each test, a *p* value <0.05 was considered positive.

3. Results

3.1 Biotyping

A total of 150 strains of *G. vaginalis* were analyzed, of which 50 were associated with BV and 100 to normal microbiota (NM). We identified 4 of the 8 reported biotypes, being the most frequent biotype 6 (51.3%), followed by 1 (18%), 5 (17.3%) and 2 (13.3%), similar frequencies were reported in NM and BV **(Table 1**).

3.2 Production of virulence factors of *G. vaginalis*

In the analysis of virulence factors production, we observed that the abundant production of biofilm was more frequent in BV-associated strains compared with the NM-associated strains (p=0,026 OR=5.17 CI:1.21-22.06). In relation with prolidase production, only 2% of the strains from NM present a high production of this enzyme. While, in BV-associated strains, only 6% present a high production and 26% a moderate production. In the total of analyzed strains, phospholipase C production was detected in only 28.7%, and a high percentage of lysis was more frequently observed in the BV-associated strains compared with the NM-associated strains (*p*=0,043 OR=8.81 CI=1.07-72.28) (**Table 2).**

Subsequently, virulence factors production was associated with each isolated biotype. In relation with biofilm production, we observed that 19.2% of strains belonging to biotype 5 are abundant producers in comparison with 0% of the biotype 1 strains and the low production of the other biotypes. For prolidase production, around 80.7% and 83.1% of strains belonging to biotype 5 and 6 do not produce this enzyme, and only 5% of biotype 2 and 3.7% of biotype 1 strains are abundant producers. Phospholipase C production was predominantly produced by biotypes 1 (51.8%) and 2 (75%), while it was scarcely produced by biotypes 5 (80.7%) and 6 (88.3%). Finally, the highest lithic activity was observed in biotypes 2 (70%) and 5 (73%) (**Table 3)**.

Subsequently, a comparison between the number of virulence factors produced by each biotype in both study groups (NM and BV) was performed. In 3% of the NM-associated strains the production of any virulence factor was observed. The most frequent virulence factor in the total of cases was vaginolysin production alone, followed by the association between Vly+Plc production (20%) and Vly+Bio production (14%), in this group only in few cases we detect the production of three different virulence factors (9%). Biotype 1 is characterized by the production of vaginolysin alone (40%) and combined with prolidase (15%) and phospholipase C (30%), biotype 2 produces Vly combined with Plc (63%), while biotypes 5 and 6 produces Vly in 41.2% and 48% of the cases, respectively. In the BV-associated strains, the production of three or four virulence factors was observed in 30% of the total cases. Being the most frequent Pro+Vly+Bio and Vly+Bio+Plc. In this group, biotype 1 presents the following characteristics: Vly (14.3%), Pro+Vly (14.3%) and Vly+Bio+Plc (22.2%), biotype 2 produces Vly+Plc (22.2%), Pro+Vly+Bio (22.2%) and Vly+Bio+Plc (22.2%), biotype 5 produces Vly (22.2%), Vly+Bio (44.4%) and Vly+Plc (22.2%). Finally, the 48% of strains belonging to biotype 6 produces Vly, and the

combination of Pro+Vly (16%) and Vly+Bio (12%). In just three cases, the production of four different virulence factors was observed, and they belonged to biotypes 1 and 6 (**Table 4**).

3.3 Antibiotic resistance

Finally, we evaluated the antibiotic sensibility of the strains to clindamycin, metronidazole and secnidazole. In the total of the analyzed strains; 100% were resistant to metronidazole, 95% to secnidazole and 90% to clindamycin, without observing differences between both study groups **(Table 5).**

4. Discussion

Bacterial vaginosis (BV) is the most common vaginal infection in reproductive aged women [31], the main etiologic agent is *G. vaginalis,* a Gram variable cocobacillus [5] with eight different biotypes and 4 genotypes [9, 10]. In this study, we report the presence of biotypes 1, 2, 5 and 6 in normal microbiota and BV. Previous studies have analyzed the role of *G. vaginalis* biotypes in the development of BV and reported that biotypes 4, 5 and 7 are the most frequent in the normal microbiota, while biotypes 1, 2, 3, 5, 7 and 8 are associated with BV [13, 32, 33]. The lack of association could be due to the employed biotyping method, that includes the measure of metabolic characteristics and do not consider the production of virulence factors [34]. Alternatively, some biotypes of this bacterium are strict anaerobes, and the isolation by conventional culture methods is not possible, to which the use of molecular methods have been proposed [14].

It has been suggested that the production of virulence factors by *G. vaginalis* vary according to the biotype and its association with BV [34]. We analyzed the production of several virulence factors, between them; vaginolysin, prolidase, biofilm and phospholipase.

The vaginolysin is the main toxin produced by *G. vaginalis*, this protein present high cytotoxic activity, and it is involved in nutrients obtention and bacterial proliferation [35]. In our study we found that the strains associated with BV present a higher lytic capacity (94%), that does not differ between biotypes. Previous studies have shown that infection with this bacterium and the low count of lactobacilli, have an effect on the production of this cytolysin [36] which could explain the higher lytic capacity in the BV-associated bacteria. Additionally, Vly production has been associated with the severity and adverse effects associated with BV [37, 38]. In addition to Vly, we analyzed the production of phospholipase C (Plc) and prolidase. Plc is a lecithinase associated with the lysis of epithelial cells and the compounds of extracellular matrix degradation [39]. No association between Plc production and BV was observed, however, the enzyme production was more frequently produced by biotypes 1 and 2 (52% and 75%, respectively), the production of this enzyme by *G. vaginalis* has been associated with *Pseudomonas spp*. and *Candida spp* co-existence in the vaginal tract, due to the fact that *G. vaginalis* does not possess the coding plasmid for Plc production and it has been stipulated that it could be received from those microorganisms [40]. Nevertheless, prolidase is a proteolytic enzyme that participates in the biofilm cycle formation and in the remodeling of the extracellular matrix [17]. High levels of prolidase, have been found in samples of women with BV, however, in this study, we did not find any relationship. Both enzymes participate in the degradation of a specific compound associated with pregnancy onsets which causes placental tissue damage and abortion [41, 42].

Another factor produced by *G. vaginalis* is biofilm, which are bacterial communities adhered to the epithelial cells surface and it is associated with bacterial proliferation [20]. We observed that the BV-associated strains produce greater amount of biofilm (12%) being

the biotype 5 (19%) the most associated with abundant production. The biotype 5 has been previously associated with NM [32], a high production of biofilm has been associated with the ability to colonize the vagina but not with the ability to develop BV. Biofilm production is important during the initial and maintenance of the infection, due to the physical protection that provides, it also prevents the effect of hydrogen peroxide and lactic acid produced by the lactobacilli of the normal vaginal microbiota [43]. At the same time, auspicious bacterial resistance to antibiotic and BV recurrence until three months after treatment. Furthermore, promotes the genetic material interchange [44, 45].

Studies have shown differences in genes expression between strains of *G. vaginalis*, which has an impact on the production of virulence factors, a wider range of enzymes that degrade cervical mucus and promote infection have been reported in BV-associated strains [46, 47]. In our study, we observed that BV-associated strains produce a greater amount of virulence factors in comparison with NM strains. In addition, some factors such as Plc and prolidase are only produced in conjunction with other factors, while biotypes 1 and 6 in BV-associated strains produce all the analyzed factors in comparison with other biotypes and study group. Previously, biotype 1 has been associated with BV development, which could be related with the high pathogenic potential, suggesting that the vaginal environment, as well as bacterial species of the normal microbiota have an effect on the production of virulence factors of *G. vaginalis* [48, 49].

Finally, we analyzed the antimicrobial resistance of the strains against antibiotics used in the conventional treatment of BV (metronidazole, clindamycin, and more recently secnidazole) [50]. A high rate of resistance to metronidazole (100%) and clindamycin (90%) was observed. Previous studies have reported that *G. vaginalis* strains present high

298 resistant rates to these antibiotics [51, 52, 25]; however, in Mexico according to the Official
299 Mexican Standard NOM-039-SSA2-2002, the treatment of vaginal infections caused by
300 bacteria still remains based on metronidazole and clindamycin as treatment. Due to the high
301 resistance rates to these antibiotics, the conventional treatment should be replaced by
302 antibiotics with greater effectiveness. In relation with secnidazole, we obtained 95% of
303 resistance, an interest fact is that this antibiotic has recently been introduced for the
304 treatment of BV [23, 24, 53]. The metronidazole and secnidazole belong to 5-
305 nitroimidazoles family, while the clindamycin belongs to the lincosamides family; the
306 mechanisms of resistance to antibiotics that belong to this family have not been described,
307 but they suggest that resistance involves the inactivation of antibiotic, intracellular flow
308 pumps or modification of the target site [54, 55]. Therefore, the specific resistance
309 mechanism should be investigated. In addition, the use of members of this family for the
310 treatment of BV should be avoided.

311 Despite the high prevalence of BV among women and the associated obstetric
312 complications, the analysis of the biotypes of *G. vaginalis*, the evaluation of virulence
313 factors production, and antibiotics resistance, are poorly studied. This study presents some
314 limitations in relation with the design and methods used, however, we provide evidence
315 about the production of virulence factors in the strains of *G. vaginalis* to know the real
316 pathogenic potential of this bacterium, also we report a high antibiotic resistance rates. At
317 clinical level, the evaluation of virulence factors could be used as prognosis markers o
318 diagnosis, also the evaluation of antibiotic resistance provides a more efficiency treatment.
319 Further studies are need it in order to completely understand how the production of
320 virulence factors in the vaginal tract is stimulated, and its roles in the symbiotic or

antagonistic relationships that *G. vaginalis* establishes with other bacteria from the vaginal tract or with others biotypes or genotypes of *G. vaginalis*. Another interesting perspective could be discerning the specific mechanisms to antibiotic resistance.

5. Conclusions

This research provides evidence about the production of virulence factors by strains of *G. vaginalis* associated with NM and BV. The BV-associated biotypes, produce a greater amount of virulence factors, evidencing the possible role of these biotypes during the development of the infection. A limitation of the work is that we only analyzed four of the eight biotypes of the bacteria and four virulence factors, nevertheless, as it has been described by other authors the bacterium still has more factors that could contribute to the development of the BV.

In addition, *G. vaginalis* presents a high resistance rate to the antibiotic used for the conventional treatment of BV and to antibiotics of new generation, which suggests that the bacterium is acquiring and/or developing new mechanisms of resistance that constitute a public health problem due to the high frequency of this infection.

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9.- Results

Table 1. Prevalence of biotypes in strains of *Gardnerella vaginalis* associated with NM and BV.

Biotype	Total	NM	BV	*p* value
Biotype 1	27 (18.0)	20 (20.0)	7 (14.0)	**0.586**
Biotype 2	20 (13.3)	11 (11.0)	9 (18.0)	
Biotype 5	26 (17.3)	17 (17.0)	9 (18.0)	
Biotype 6	77 (51.3)	52 (52.0)	25 (50.0)	
Total	150 (100.0)	100 (100.0)	50 (100.0)	

Data reported as: n (%). *p* value: X^2. NM: normal microbiota and BV: bacterial vaginosis.

Table 2. Virulence factors production in *Gardnerella vaginalis* strains associated with NM and BV.

Virulence factors	Total (n=150)	NM (n=100)	BV (n=50)	OR (95%CI)	*p* value
Biofilm					
No producer	104 (69.3)	75 (75.0)	29 (58.0)	1	--
Moderate	37 (24.7)	22 (22.0)	15 (30.0)	1.76 (.80-3.86)	0.156
Abundant	9 (6.0)	3 (3.0)	6 (12.0)	**5.17 (1.21-22.06)**	**0.026**
Prolidase					
No producer	117 (78.0)	83 (83.0)	34 (68.0)	1	
Moderate	28 (18.7)	15 (15.0)	13 (26.0)	2.11 (.91-4.91)	0.082
Abundant	5 (3.3)	2 (2.0)	3 (6.0)	3.66 (.58-22.89)	0.165
Phospholipase C					
Negative	107 (71.3)	73 (73.0)	34 (68.0)	1	
Positive	43 (28.7)	27 (27.0)	16 (32.0)	1.27 (.60-2.66)	0.524
Vaginolysin*					
Without lysis	9 (6.0)	9 (9.0)	1 (1.96)	1	--
Low	44 (29.3)	42 (42.0)	2 (3.92)	0.42 (.03-5.25)	0.508
High	97 (64.7)	49 (49.0)	48 (94.12)	**8.81 (1.07-72.28)**	**0.043**

The data are reported in n (number of strains) and percentage (%). OR: Odd Ratios, CI (confidence interval) and *p* value: logistic regression model. NM: normal microbiota. BV: bacterial vaginosis. *A fictitious case was added to avoid collinearity.

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Table 3. Virulence factors and biotypes of *Gardnerella vaginalis*

Virulence factors	Total (n=150)	Biotype 1 (n=27)	Biotype 2 (n=20)	Biotype 5 (n=26)	Biotype 6 (n=77)	*p* value
Biofilm						
No producer	104 (69.3)	22 (81.5)	13 (65.0)	17 (65.4)	52 (67.53)	
Moderate	37 (24.8)	5 (18.5)	6 (30.0)	4 (15.4)	22 (28.6)	0.051
Abundant	9 (6.0)	0 (0.0)	1 (5.0)	5 (19.2)	3 (3.9)	
Prolidase						
No producer	117 (78.0)	18 (66.7)	14 (70.0)	21 (80.7)	64 (83.1)	
Moderate	28 (18.7)	8 (29.6)	5 (25.0)	4 (15.4)	11 (14.3)	0.633
Abundant	5 (3.3)	1 (3.7)	1 (5.0)	1 (3.9)	2 (2.6)	
Phospholipase C						
Negative	107 (71.33)	13 (48.15)	5 (25.00)	21 (80.77)	68 (88.31)	**0.0001**
Positive	43 (28.67)	14 (51.85)	15 (75.00)	5 (19.23)	9 (11.69)	
Vaginolysin						
Without lysis	9 (6.0)	1 (3.7)	1 (5.0)	1 (3.9)	6 (7.8)	
Low	44 (29.3)	8 (29.6)	5 (25.0)	6 (23.0)	25 (32.5)	0.891
High	97 (64.7)	18 (66.7)	14 (70.0)	19 (73.0)	46 (59.8)	

The dates are reported as: n (%). *p* value*:* test X^2.

Table 4. Association of multiple virulence factors production and biotypes in NM and BV associated strains.

Virulence factors/ Biotypes	Normal microbiota					
	Total	**1**	**2**	**5**	**6**	***p* value**
Any	3 (3.0)	-	-	1 (5.9)	2 (3.6)	
Prolidase	-	-	-	-	-	
Vaginolysin	40 (40.0)	8 (40.0)	-	7 (41.2)	25 (48.0)	
Biofilm	4 (4.0)	-	-	-	4 (7.7)	
Phospholipase C	-	-	-	-	-	
Pro[1] + Vly[2]	8 (8.0)	3 (15.0)	-	3 (17.7)	2 (3.9)	
Pro + Bio[3]	-	-	-	-	-	
Pro + Plc[4]	2 (2.0)	1 (5.0)	1 (9.0)	-	-	
Vly + Bio	14 (14.0)	1 (5.0)	1 (9.0)	2 (11.8)	10 (19.2)	
Vly + Plc	20 (20.0)	6 (30.0)	7 (63.6)	2 (11.8)	5 (9.7)	**0.001**
Bio + Plc	-	-	-	-	-	
Pro + Vly + Bio	6 (6.0)		-	2 (11.8)	4 (7.7)	
Pro + Bio + Plc	-	-	-	-	-	
Vly + Bio + Plc	1 (1.0)	-	1 (9.0)	-	-	
Plc + Pro + Vly	2 (2.0)	1 (5.0)	1 (9.0)	-	-	
Pro + Vly + Bio + Plc	-	-	-	-	-	
Total	100 (100.0)	20 (100.0)	11 (100.0)	17 (100.0)	52(100.0)	
Virulence factors/ Biotypes	**Bacterial vaginosis**					
	Total	**1**	**2**	**5**	**6**	***p* value**
Any	-	-	-	-	-	
Prolidase	-	-	-	-	-	
Vaginolysin	15 (30.0)	1 (14.3)	-	2 (22.2)	12 (48.0)	
Biofilm	-	-	-	-	-	
Phospholipase C	-	-	-	-	-	
Pro[1] + Vly[2]	6 (12.0)	1 (14.3)	1 (11.1)	-	4 (16.0)	
Pro + Bio[3]	-	-	-	-	-	
Pro + Plc[4]	-	-	-	-	-	
Vly + Bio	8 (16.0)	-	1 (11.1)	4 (44.4)	3 (12.0)	
Vly + Plc	6 (12.0)	-	2 (22.2)	2 (22.2)	2 (8.0)	**0.024**
Bio + Plc	-	-	-	-	-	
Pro + Vly + Bio	5 (10.0)	-	2 (22.2)	1 (11.1)	2 (8.0)	
Pro + Bio + Plc	-	-	-	-	-	
Vly + Bio + Plc	5 (10.0)	2 (22.2)	2 (22.2)	-	1 (4.0)	
Plc + Pro + Vly	2 (4.0)	1 (14.3)	1 (11.1)	-	-	
Pro + Vly + Bio + Plc	3 (6.0)	2 (28.6)	-	-	1 (4.0)	
Total	50 (100.0)	7 (100.0)	9 (100.0)	9 (100.0)	25 100.0)	

Data reported as: n (%). *P* value*:* test X^2. [1] Prolidase. [2] Vaginolysin. [3] Biofilm. [4] Phospholipase C. NM: normal microbiota. BV: bacterial vaginosis. A positive result was considerate according to the followed criteria: ≥ 0.1 O.D for biofilm and prolidase and ≥50% percentage of lysis for Vly.

Table 5. Sensitivity to antibiotics in strains of *Gardnerella vaginalis* associated to NM and BV.

Antibiotic	Total (n=150)	NM (n=100)	BV (n=50)	OR (95%CI)	Value *P*
Metronidazole					
Sensitive	0 (0.0)	0 (0.0)	0 (0.0)		
Resistant	150 (100.0)	100 (100.0)	50 (100.0)	--	--
Secnidazole					
Sensitive	7 (4.7)	4 (4.0)	3 (6.0)	1	--
Resistant	143 (95.3)	96 (96.0)	47 (94.0)	.65 (.14-3.03)	0.587
Clindamycin					
Sensitive	14 (9.3)	11 (11.0)	3 (6.0)	1	--
Resistant	136 (90.7)	89 (89.0)	47 (94.0)	1.93 (.51-7.28)	0.328

Data reported as n and percentage (%). OR: Odd Ratios, CI (confidence interval) and *p* value: logistic regression model adjusted by biotypes. NM: normal microbiota. BV: bacterial vaginosis.

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Declarations of interest: none