ENDOCERVICAL BIOFILMS IN WOMEN WITH ENDOGENOUS INFECTIONS IN THE LOWER GENITAL TRACT: *IN VITRO* STUDY

BIOPELÍCULAS ENDOCERVICALES EN MUJERES CON INFECCIONES ENDÓGENAS DEL TRACTO GENITAL INFERIOR: ESTUDIO IN VITRO

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ABSTRACT

Introduction: The biofilm is one life form of microorganisms (MOs). On mucous membranes of women without and with endogenous infections, they are part of the normal microbiota and cause pathologies. We have demonstrated previously the participation of biofilms in chronic forms of vulvovaginal candidiasis (VVC), the influence of other microorganisms in its formation and evolution, in bacterial vaginosis (BV) and aerobic vaginitis (AV). **Objective**: To analyze the endocervical biofilms in women with or without vaginal infections (VI) comparing them with vaginal biofilms. **Methods**: We studied 22 women, 9 non-pregnant (NP) and 13 pregnant (P). Each patient was gynecologically evaluated, and a vaginal sample (VS) was taken with an aspersorium and an endocervical sample was taken with cytobrush (CB). We performed a fresh examination, pH determination and amine test. Both samples were inoculated in suitable culture medium. After each one, Gram staining and optical microscopy with crystal violet were performed for the study of BF. These were put into Sabouraud broth. All samples were incubated at 35°C for 20-24 hours. **Results**: We have discovered 9 women without pathology and with normal microbiota (NM) and 13 with vaginal infections (VI): bacterial vaginosis (BV) – 6 (4P); vulvovaginal candidiasis (VVC) – 4 (3P); vaginitis and intermediate microbiota (IMB) – 3 (1E). The notable differences were: inflammatory response in the cytobrush compared to the one found in the vaginal samples of women with vaginal infections (10/13), including women with bacterial vaginosis who did not have inflammatory response in the vaginal sample. In the cytobrush of women with normal microbiota, this response occurred only in 1 case (1/9). It was also observed the formation of microfilms of Gram-positive cocci (mostly *Enterococcus* spp) in the cytobrush of 84.6% (11/13) of the women with vaginal infections and in 66.6% (6/9) of the women with normal microbiota. Among the latter, mixed biofilms were observed in 3 cases with the presence of Gram-positive Bacilli (*Actinobaculum* (anaerobic) or *Actinomyces*). **Conclusion**: Something that called our attention was that the formation of biofilms of *Enterococcus* and other species of *Streptococcus* and *Satphylococcus* in the cytobrush of women with vaginal infections in whose vaginal samples these microorganisms were not observed nor recovered significantly. This is a risk since they can initiate an upper genital tract infection (UGTI). In the 4 P with BV, this risk is added to the risk associated with the BV. The question is whether the complications arising from this in pregnancy are not a result of such behavior. In the women with normal microbiota, the biofilms that have Gram-positive cocci can also represent a notable risk in the moment of performing instrumental procedures.
Keywords: biofilms, vaginal tract, cervical tract, women.

RESUMEN

Introducción: Las biopelículas constituyen una de las formas de vida de los microorganismos (MOs). En las mucosas de mujeres sin y con infecciones endógenas, integran la microbiota normal y desarrollan patologías. Previamente hemos demostrado la participación de las mismas en las formas crónicas de las candidiasis vulvovaginales (CVV), la influencia de otros microorganismos en su conformación y evolución, en la vaginosis bacteriana (VB) y en las vaginitis aeróbicas (VA). **Objetivo:** Analizar las biopelículas endocervicales en mujeres sin y con infecciones vaginales (IV), comparándolas con las BP vaginales. **Métodos:** Estudiamos 22 mujeres, 9 no embarazadas (NE) y 13 embarazadas (E). Cada paciente fue estudiada ginecológicamente y se tomó muestra vaginal (MV) con hisopo y muestra endocervical con citobrush (EC). Se realizó examen en fresco, determinación del pH y prueba de aminas. Ambas muestras fueron inoculadas en medios de cultivo adecuados. A cada muestra se efectuó la coloración de Gram y se realizó la capa celular sobre el dispositivo de vidrio (DV) para el estudio de las BP. Los DV se colocaron en caldo Sabouraud. Todas las muestras se incubaron a 35°C durante 20-24 horas. **Resultados:** Encontramos 9 mujeres sin patología y con microbiota normal (MN) y 13 con infecciones vaginales (IV): vaginosis bacteriana (VB) – 6 (4E); candidiasis vulvovaginal (CVV) – 4 (3E); vaginitis y microbiota intermedia (VMI) – 3 (1E). Las diferencias notables fueron: hallazgo de respuesta inflamatoria en el EC comparada con la encontrada en la MV en las mujeres con IV (10/13), incluyendo a las mujeres con VB que no presentan respuesta inflamatoria en la MV. En el EC de las mujeres con MN dicha respuesta ocurrió sólo en 1 caso (1/9); y formación de BPs de cocos Gram-positivos (la mayoría *Enterococcus* spp) en el EC del 84,6% (11/13) de las mujeres con IV y en el 66,6% (6/9) de las mujeres con MN. En estas últimas se observaron BPs mixtas en 3 casos con la presencia de bacilos Gram-positivos, (*Actinobaculum* (anaerobio) o *Actinomyces*). **Conclusión:** Llama la atención la formación de BP de *Enterococcus* y otras especies de *Streptococcus* y *Satphylococcus* en el EC de mujeres con IV en cuyas MV no se observan ni se recuperan significativamente estos microorganismos. Esto constituye un riesgo ya que las mismas pueden iniciar una infección del tracto genital superior (ITGS). En las 4E con VB, este riesgo se suma al de la VB y cabe preguntarse si las complicaciones derivadas de la misma en la gestación no son el producto de dicho comportamiento. En las mujeres con MN las BP de cocos Gram-positivos podrían representar un riesgo notable para el desarrollo de ITGS en el momento de efectuar maniobras instrumentales.
Palabras clave: biofilmes, tracto vaginal, tracto cervical, mujeres.

INTRODUCTION

In the lower genital tract, the microorganisms (MOs) that compose the normal or usual microbiota can colonize or infect the vaginal mucosa, making biofilms (BF) of single species or mixed ones[1]. Both the colonizing MOs and those producing vaginal and endocervical infections can determine, in pregnant women during pregnancy and at labor, the contraction of congenital or perinatal infections.

A BF is a very dynamic sessile community of MOs, characterized by cells that are irreversibly joined to a substrate or interface between them, saturated in an extracellular matrix of polymerized substances produced by them and that show a changed phenotype with regard to the growth and gene transcription index[2-5]. They can

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have an important role, both in the infections and for protection. In general, the lower genital tract content is studied taking into consideration the planktonic MOs, but not the BF, since we do not know many physiopathological aspects that happen during the colonization and/or infections.

In the vagina, BFs of lactobacilli are responsible for the wider production of lactic acid that decreases the vaginal pH and prevents, thus, the colonization by pathogenic or potentially pathogenic MOs[6].

The usual endocervical microbiota has not been deeply studied and we can assume that it can allow adherence of other MOs that are different from the vagina, due to its more alkaline pH and histological configuration with the cylindrical epithelium tissue. Therefore, in the pathology due to sexually transmitted infections (STIs), like gonorrhea and chlamydia, produced by *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT), respectively, we know that adherence is produced in the cylindrical epithelial cells instead of in those from the vaginal stratified epithelium[7]. The association of NG with the receptor is essential for the invasion of the epithelial cell. However, this can vary since members of the CD66 receptors family were identified for several Opacity (Opa) proteins of NG, which mediate the interaction with phagocytes and the passage through epitheliums[7,8]. CT is linked through bridges of some polysaccharides that are established between the surface of the elementary body and the cellular receptor[9].

Is has not been reliably known if the same happens to endogenous infections, since it was always investigated the vaginal tract and not the endocervical one. Probably, the establishment of BF of other MOs in the endocervix is mediated by similar mechanisms, which according to environmental conditions can be changed by the presence of some adhesins, such as those we have seen in these STI agents.

OBJECTIVE

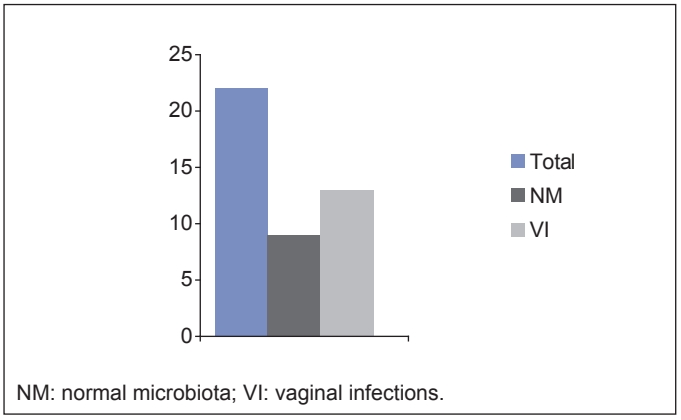
To analyze the behavior of MOs as BF in the endocervix (BFC) in women with and without endogenous vaginal infections (VI) comparing them with the vaginal BF.

METHODS

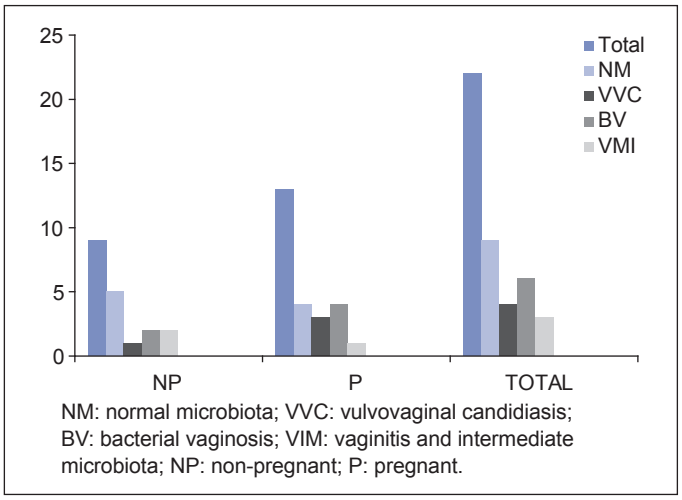
We have studied 22 women, 9 that non-pregnant (NP) and 13 pregnant (P). Each patient was gynecologically studied. It was taken their vaginal sample (VS) with cotton swabs and endocervical sampling with cytobrush (EC). The following exams were performed: wet mount test, pH determination, and amine testing with HOK at 10%. Both samples were inoculated in suitable culture medians: Sabouraud agar, Cystine Lactose Electrolyte Deficient (CLED) agar, trypticase soy agar (TSA). Gram coloration was performed with each sample and the optical microscopy on the glass equipment (GE) for the BF analysis. The GEs were put in Sabouraud broth. All the samples were incubated at 35ºC for 20 to 24 hours.

RESULTS

We found 9 women (5 NP and 4 P) without pathology and NM and 13 with VI: bacterial vaginosis (BV) 6 (4P); vulvovaginal candidiasis (VVC) 4 (3P); vaginitis and intermediate microbiota (VIM) 3 (1P) (**Graphics 1 and 2**).



Graphic 1 – Distribution of patients according to the vaginal content findings.

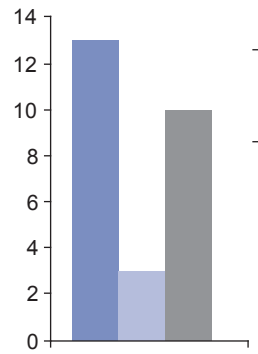


Graphic 2 – Distribution of patients according to their condition and kind of vaginal content.

The most important differences were:

- Inflammatory response in EC comparable to that found in MV in women with VI (10/13), women with BV that did not present inflammatory response in the MV were also included (**Graphic 3 and Figure 1**). In the EC of women with NM, this response happened only in 1 case (1/9).
- Formation of BF of Gram-positive cocci (most of them were *Enterococcus* spp) in the EC of 84.6% (11/13) women with VI and in 66.6% (6/9) women with NM (**Graphic 4**). In the latters, mixed BFs were found in 3 cases with presence of Gram-positive bacilli (*Actinobaculum* (anaerobic) or *Actinomyces*) (**Figures 2 to 4**).

In the cases 12 and 16, one can see the relevant difference on the varied formation of biofilms at vaginal and endocervical levels (**Figures 5 to 12**). In the **Figures 8 and 12**, BF presence of negative *Staphylococcus coagulase* (NSC) (*Staphylococcus epidermidis*) is



	BF Gram positive cocci	
Total of women	22	
Vaginal infections	13	11
Normal microbiota	9	6

Total IV
IR V
IR EC

VI: vaginal infections; IR V: inflammatory response in vagina; IR EC: inflammatory response in the endocervix.

Graphic 3 – Inflammatory response in the vagina and endocervix.

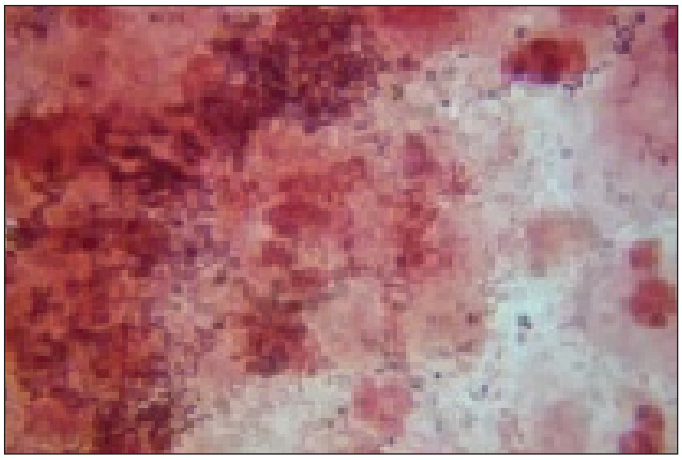
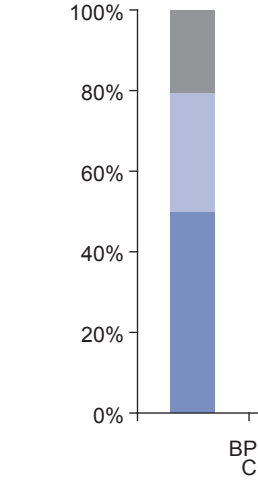


Figure 1 – Bacterial vaginosis – Endocervix – Positive inflammatory answer – Gram staining, 1000x.



Total: total of vaginal infections; BPC C+: biofilms of Gram-positive cocci; NM: normal microbiota; VI: vaginal infections.

Graphic 4 – Biofilms of Gram-positive cocci in the EC of women with vaginal infections and normal microbiota.

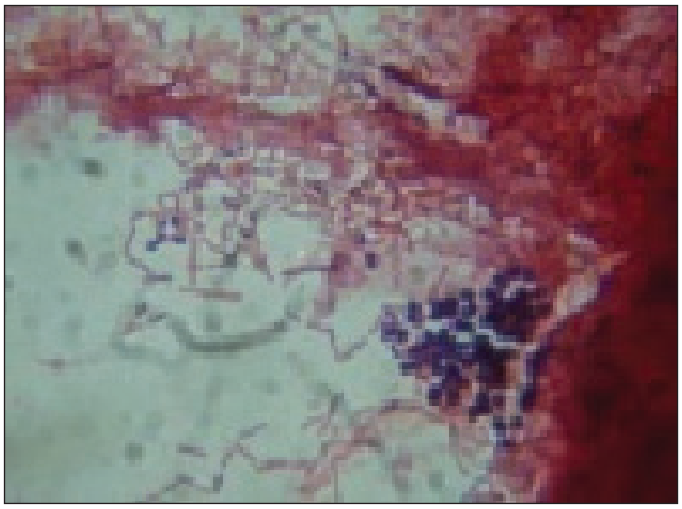


Figure 2 – *Actinobaculum* (anaerobic) or *Actinomyces* – Endocervix – Gram staining, 1000x.

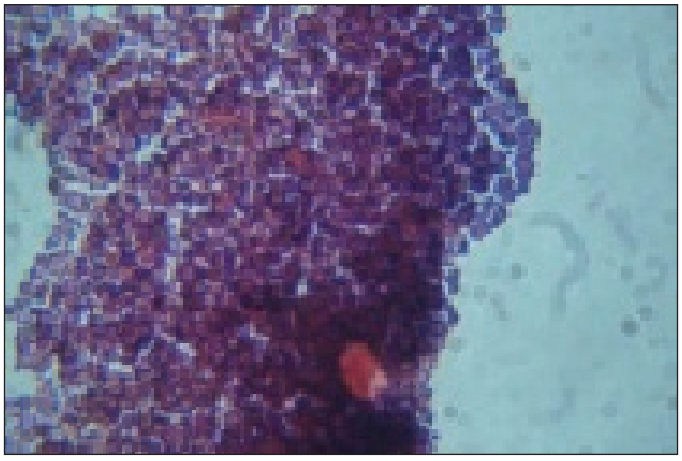


Figure 3 – Endocervical biofilm, Gram-positive cocci. Gram staining, 1000x.

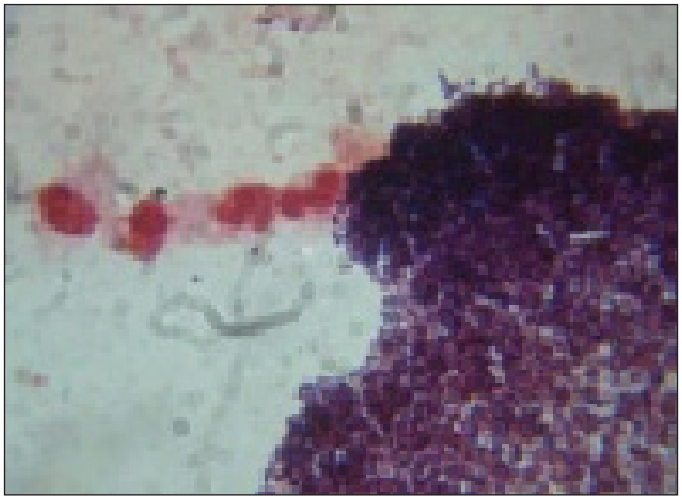


Figure 4 – Endocervical biofilm, Gram-positive cocci. Gram staining, 1000x.

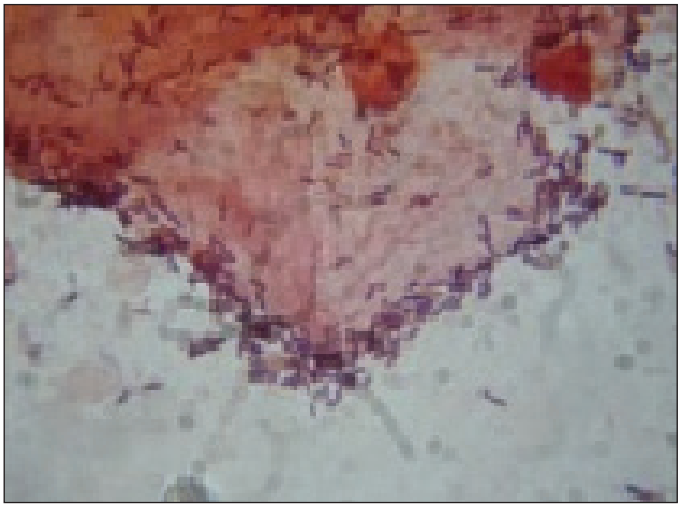


Figure 5 – Case 12: direct vaginal exudate. Gram staining, 1000x.

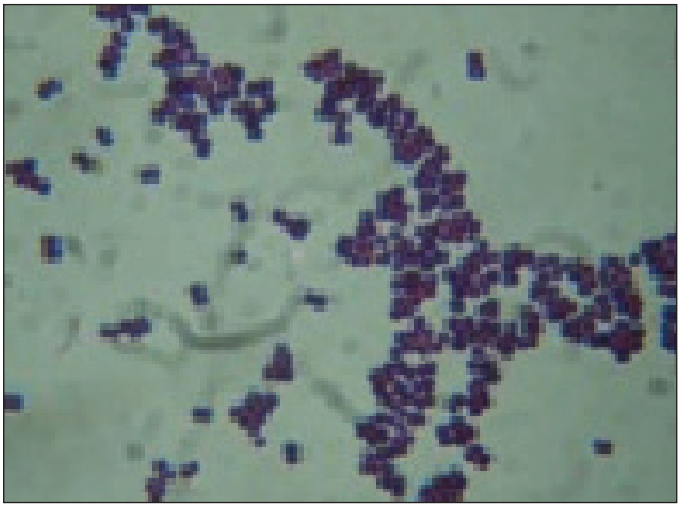


Figure 8 – Case 12: endocervical biofilm. *Staphylococcus epidermidis* (SCN). Gram staining, 1000x.

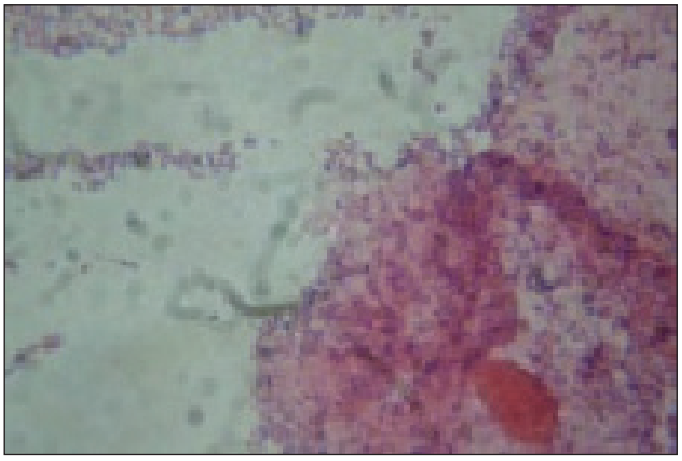


Figure 6 – Case 12: vaginal biofilm. Gram staining, 1000x.

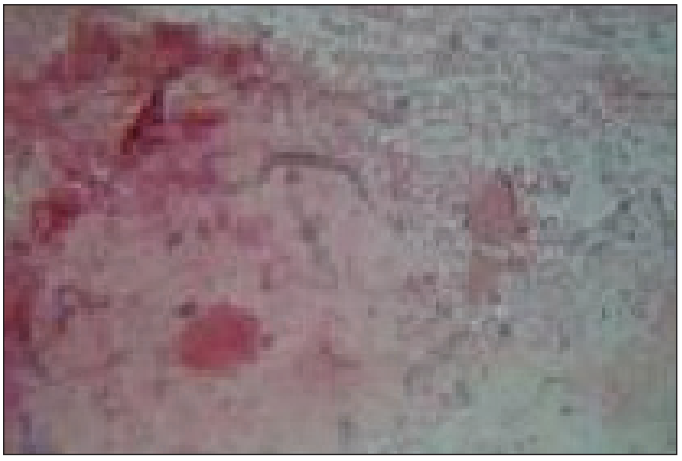


Figure 9 – Case 16: direct vaginal exudate. Gram staining, 1000x.

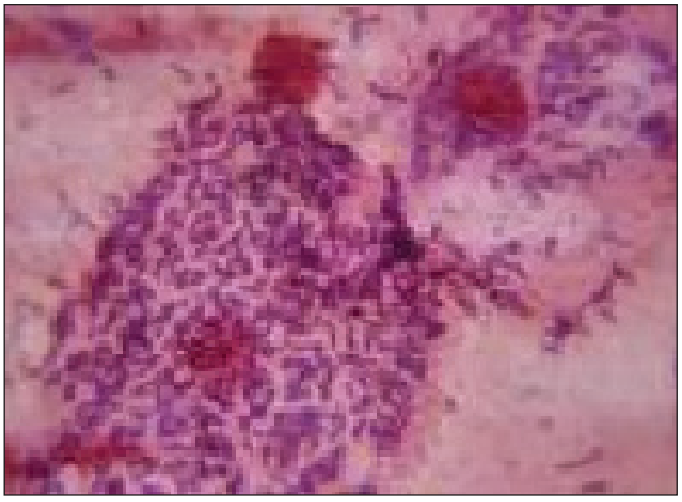


Figure 7 – Case 12: direct endocervical exudate. Gram staining, 1000x.

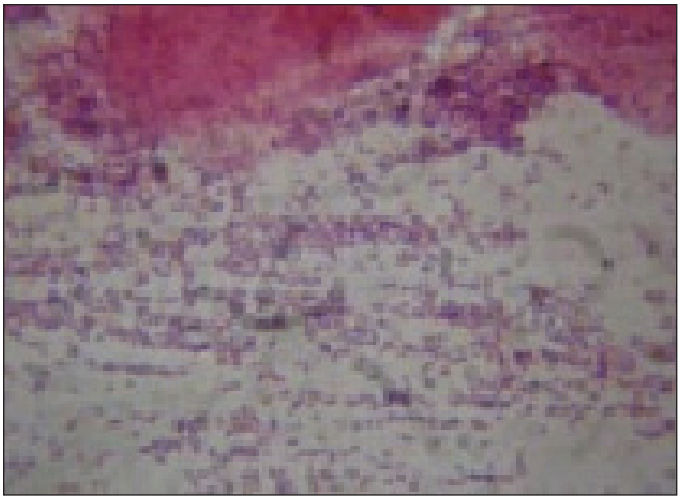


Figure 10 – Case 16: vaginal biofilm. Gram staining, 1000x.

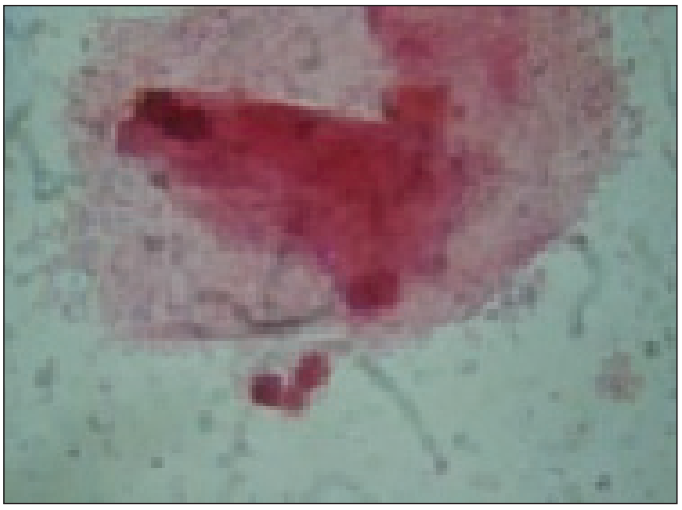


Figure 11 – Case 16: direct vaginal exudate. Gram staining, 1000x.

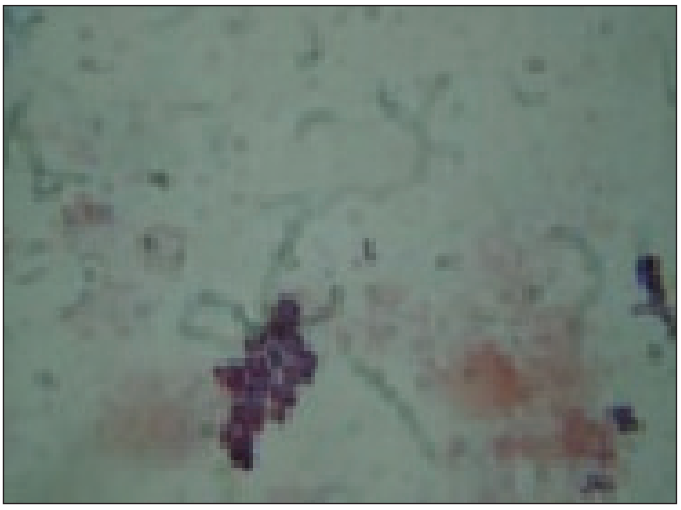


Figure 12 – Case 16: endocervical biofilm. Gram staining, 1000x.

very notable and, nevertheless, these MOs are not seen neither in the vagina nor in their corresponding BF.

DISCUSSION

The formation of BF is an important characteristic of NM constitution from the mucosae in our organism. As it is known, the BFs have two kinds of behaviors in our organism, like NM or a pathogenicity and resistance factor in places that are usually sterile or with prosthesis[10,11]. Their formation participates in the infectious pathology of the lower genital tract together with immunological and allergy factors[12].

We studied the in vitro vaginal BFs both in special equipment and in optical microscopy[13-16]. We have demonstrated that in mixed BFs of yeasts and *Escherichia coli*, the non-albicans Candida species make it easier, due to exuberance of their exopolysaccharide. The bacteria that produce glycocalyx as an adherent material could also have an important role in the pathogenesis of infections by *Candida* spp, not only in the urogenital level but also in other places. These behaviors would explain the almost constant presence of singe species or mixed BFs in the vaginal tract. In the endocervical tract, the possibilities of BFs formation can be different due to some factors, such as the epithelium characteristics, the number of MOs, the pH and the micro-atmosphere. It is known that in the case of NG colonized over the endocervix cells, the anaerobically induced genes and/or those that codify proteins that participate in the anaerobic breathing are needed to form a BF, whereas the genes responsible for the proteins that take part in the aerobic breathing are less abundant in the BF[17]. For all these reasons, the BFs in the endocervix may have a different behavior than the vaginal BFs.

Our findings call the attention to the BF formation of Gram-positive cocci. From the 17 cases of Gram-positive cocci, 11 were of *Enterococcus* spp, 2 of *Streptococcus* spp and 4 of SCN, 3 of which were of women with NM. This is a risk since these BFs may progress to the upper genital tract and create an infection therein, which is usually known as pelvic inflammatory illness or infection (PII) and recently as upper genital tract infection (UGTI). In the 4 P women with BV, this risk is an addition to the one of BV and one may ask if the complications from BV in pregnancy are not the product of the sum of such entities. In women with NM, although Gram-positive cocci BFs happen in lower proportion, they can also represent a risk worthy of consideration for the development of a UGTI when performing instrumental maneuvers.

The inflammatory response in endocervix in BV cases, when absent, could be happening due to the blockage of interleukin (IL)-8 that is in the vagina and not in the endocervix. Although it is increased in the IL-1 β vagina and therefore we expect an inflammatory response, it is not produced through the hydrolytic enzymes of anaerobic bacteria that, together with *Gardnerella vaginalis*, form an abnormal microbiota in the BV[18,19]. We do not know if it happens with such ILs in the endocervix, however there is a different microbiota in the BF with a possible distinct activity compared to them. Data found in literature detail the IL concentrations in cervicovaginal washings and in studies about planktonic MOs[20].

Another theme for discussion with regard to BF from the genital tract is the possible influence in the appearance of late sepsis in newborns. Sepsis in such age range is divided into: early sepsis that is manifested in the first 72 hours for 7 days, and the late one, whose incidence peaks are between the second and third weeks[21]. The Gram-negative bacilli were the most important representations in the 1960s together with emergence of group B *Streptococcus* in early sepsis[22,23]. Recently, Gram-positive MOs represent up to 70% of neonatal sepsis in North America, and SCN is the most prevalent in late sepsis, especially in children born with low gestational age and those with low weight (lower than 1,500 g)[24].

In general, it is confirmed that MOs in neonatal late sepsis come from the environment[25] or the use of central venous catheters, mechanical ventilation, parenteral nutrition or other invasive procedures[26-29]. Few investigators associate the presence of such MOs with a possible colonization from the labor channel.

Detection of SCN BF in the endocervix of a pregnant woman suggests a risk since the MOs that form them may persist, firstly as colonizers and then as infections, in neonates that normally are treated with anti-microbes with difficulties of approaching the BFs.

In the common studies regarding lower genital tract infections and during pregnancy, it is more common to give more attention to vaginal infection and forget about investigating what is happening in the endocervix, with the exception of NG or CT. However, endocervix is part of the labor channel and eventually MOs present may affect the fetus before or in labor.

The described findings allow proposing that further endocervical investigation be performed, independently from the one that is done to investigate NG and CT, especially in women with risks of premature birth or birth of low weight infants.

CONCLUSION

Formation of BF of *Enterococcus* and other species of *Streptococcus* and *Satphylococcus* in the EC of women with VI calls the attention, since in their VM such MOs are not significantly seen or recovered. This is a risk, since they may initiate an infection in the UGTI. In the 4 P with BV, this risk is an addition to that of BV and one should ask if the complications from it in pregnancy are not the product of such behavior. In women with NM, the EC BFs of Gram-positive cocci happen in lower proportion.

Conflict of interests

The authors declared no conflict of interests.

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