

Original Article**Cervicovaginal microbial flora in methenamine silver staining method***Noushin Afshar Moghaddam\**, *Farahnaz Mardanian\*\**, *Mojgan Mokhtari\****Abstract**

**BACKGROUND:** Vagina like all other mucosal organs owns its especial bacterial/microbial flora. Though may be pathogen in other circumstances, members of vaginal normal flora do not cause disease on healthy vaginal mucosa. In this study, we tried to determine the relationship between microscopic findings on Methenamine silver stained cervicovaginal smears and clinical symptoms.

**METHODS:** A total of 389 cervicovaginal smears were examined cytologically from April to August 2005, among which 103 satisfactory smears of patients who were normally menstruating were subsequently selected. The originally Papanicolaou-stained smears were stained with Methenamine silver method. The cervicovaginal flora in symptomatic and asymptomatic patients was classified into four groups. The relationship between the type of genital flora and the presence of *Candida* or *Actinomyces* spp was also determined. Data were analyzed with SPSS software using Chi-square test.

**RESULTS:** In 103 evaluated patients, 46 (44.7%) were symptomatic and the rest were asymptomatic. The most prevalent genital microbial flora in both symptomatic (21.7%) and asymptomatic (37.9%) patients was type II (*Lactobacilli*). Microbial frequency differences were significant for types II ( $P = 0.034$ ) and III ( $P = 0.039$ ) in both groups. Coexistence of microbial flora of type I ( $P = 0.02$ ) and type IV ( $P = 0.033$ ) with *Candida* was statistically significant. Coexistence of all types of microbial flora with *Actinomyces* was not proved significant.

**CONCLUSIONS:** Symptomatic women, except those with potential pathogens, tend to have *Lactobacillus* flora. Therefore, it is advisable that all *Lactobacilli* types be investigated through microbiological methods in symptomatic patients. In silver stained slides, there was a clear relationship between the type of vaginal microbial flora and the presence of *Candida* spp.

**KEY WORDS:** Microbial flora, cervicovaginal smears, methenamine silver, symptomatic, asymptomatic.

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A large number of organisms gain access to vagina, but their proliferation is controlled by several interrelated mechanisms. As a result, normal vagina maintains a microbial equilibrium dominated by anaerobes, which varies according to age, hormonal status, and sexual activity<sup>1,2</sup>.

Thus, it would be difficult to define what exactly normal vaginal flora is. Some organ-

isms are frequently encountered in vagina without being associated with clinical manifestations of diseases, and are considered to be non-pathogenic inhabitants<sup>3-6</sup>. Under certain adverse conditions, the microbiologic milieu of vagina is altered and pathogenic organisms predominate, causing vaginitis<sup>7-9</sup>. Proper identification of the various organisms responsible for gynecologic infections usually requires

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microbiologic cultures. However, examination of adequately prepared vaginal smears provides reliable data which can be used to determine the etiology of such infections<sup>3</sup>. Substituting vaginal direct smear for microbiologic culture gives the advantage of being in the same pattern of natural habitat<sup>10</sup>. However they stain weakly with the conventional Papanicolaou method and may not be favorably visualized. Therefore, a silver staining method was developed. Bacteria and fungi stain distinctly black and can be studied in greater detail and it is possible to define their relationship to cells<sup>11</sup>.

In this study, we tried to determine the genital microbial flora in cervicovaginal smears of symptomatic and asymptomatic women using silver staining method and evaluated the relationship between the presence of *Candida* or *Actinomyces* spp with the type of genital flora.

## Methods

This descriptive-analytic study was carried out in the Cytopathology and Gynecology Departments of Al-Zahra Hospital, Isfahan, Iran from April to August 2005. A total of 389 cervicovaginal smears were examined cytologically and 103 satisfactory smears<sup>12</sup> of patients with normal menstruation were selected. The patients were subdivided into two groups, namely symptomatic and asymptomatic groups. In this way, the sampling method was convenient and non-randomized.

The patients were considered symptomatic if they had symptoms such as vaginal itching, burning, dryness and abnormal discharge. Smears were stained with Methenamine silver staining method. The originally Papanicolaou-stained smears did not need to be decolorized with this method and could be stained directly. Bacteria and fungi stained black and could be easily studied in greater details besides their relationship to cells<sup>13</sup>.

Since the bacteria were well stained, the cervicovaginal floras in symptomatic and asymptomatic patients were classified into four types: Type 1, lactobacillus predominant: The

bacteria were rod-shaped, slender and long. There was an abundance of bacteria outside the epithelial cells, often with marked cytolysis of the glycogen-rich intermediate squamous cells. No coccoid bacteria were detected.

Type 2, lactobacillus flora: The number of lactobacilli was much lower than in type 1. There was no cytolysis. In some smears, limited numbers of coccoid bacteria could also be found. Type 3, mixed flora: A mixture of lactobacilli and various other short, plump or round bacteria could be observed. Occasionally clue cells, squamous cells covered with cocci, could be found. Type 4, coccoid predominant: There was an abundance of coccoid bacteria in the background of the smear, covering epithelial cells (clue cells)<sup>11</sup>.

Data were analyzed in SPSS using Chi-square test. P values of <0.05 were assumed to indicate statistical significance.

## Results

Of the 103 patients evaluated in this cross sectional study, 46 (44.7%) were symptomatic while the rest (55.3%) were asymptomatic. In the symptomatic group, microbial flora included: type I: 3 (2.9%), type II: 22 (21.4%), type III: 12 (11.7%) and type IV: 9 (8.7%).

Frequencies of all types of genital microbial flora in both symptomatic and asymptomatic groups are compared in table 1. Microbial frequency differences were significant in type II ( $P = 0.034$ ) and type III ( $P = 0.039$ ) in patients of both groups. There was no significant difference in microbial flora between type I ( $P = 0.326$ ) and type IV ( $P = 0.112$ ) (figures 1, 2).

In this study, the relationship between the type of genital flora and the presence of *Candida* or *Actinomyces* spp was also determined (tables 2 and 3). Coexistence of microbial floras of type I ( $P = 0.02$ ) and type IV ( $P = 0.003$ ) with *Candida* was statistically significant but there was no significant coexistence of microbial floras of type II ( $P = 0.326$ ) and III ( $P = 0.112$ ). Coexistence of no types of microbial flora with *Actinomyces* was significant (figures 3, 4).

**Table 1.** Comparison of genital microbial flora in symptomatic and asymptomatic patients.

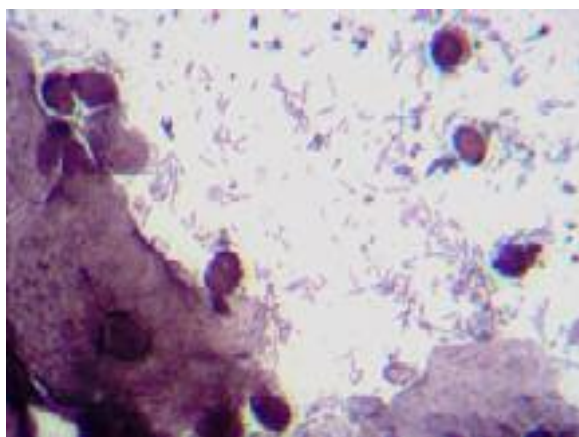
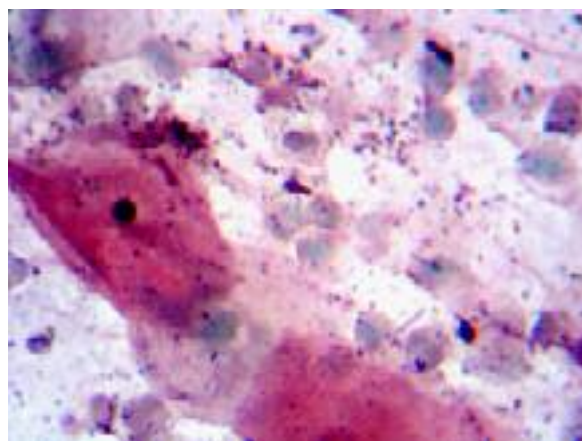
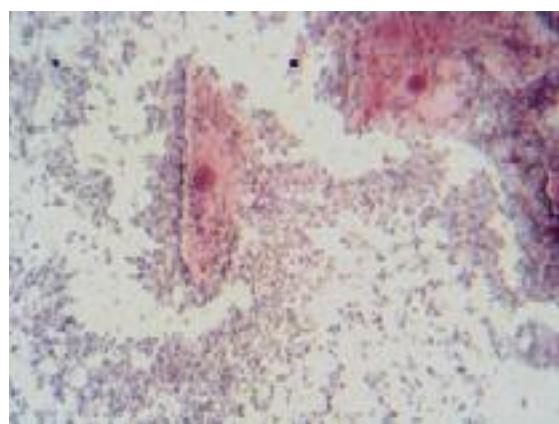
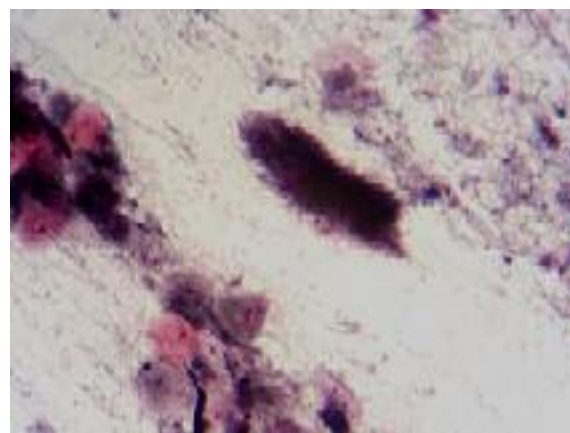
Microbial flora	Symptomatic Frequency		Asymptomatic Frequency	
	No	%	No	%
Lactobacillus predominant (type I)	3	2.9	7	6.8
Lactobacillus (type II)	22	21.4	39	37.9
Mixed (type III)	12	11.7	6	5.8
Coccioid predominant (type IV)	9	8.7	5	4.9

**Table 2.** Genital microbial flora in the presence of *Candida*.

Microbial flora	Frequency	
	No	%
Lactobacillus predominant (type I)	5	8.8
Lactobacillus (type II)	17	29.8
Mixed (type III)	7	12.3
Coccioid predominant (type IV)	6	10.5

**Table 3.** Genital microbial flora in the presence of *Actinomyces*.

Microbial flora	Frequency	
	No	%
Lactobacillus predominant (type I)	0	0
Lactobacillus (type II)	0	0
Mixed (type III)	2	66.66
Coccioid predominant (type IV)	1	33.33

**Figure 1.** High-power (\*400) photomicrograph showing Lactobacillus predominant with marked cytolysis.**Figure 3.** High-power (\*400) photomicrograph showing yeast of *Candida* spp.**Figure 2.** High-power (\*400) photomicrograph showing Coccioid predominant with clue cells.**Figure 4.** High-power (\*400) photomicrograph showing wool-ball with thin radiating threads of *Actinomyces*.

## Discussion

In this study, silver staining method was used to achieve a more obvious microscopic view of genital microbial floras and to evaluate their association with organisms such as *Candida* and *Actinomyces* spp<sup>14</sup>. *Actinomyces* are very well outlined in black, displaying the well-known arrangement into balls of wool with thin radiating threads protruding outward. In the silver-stained smears, it would be no longer difficult to differentiate these bacteria from the so-called Pseudoactinomycotic radiate granules, since the latter do not stain with silver<sup>15</sup>. *Actinomyces* is a member of the natural flora in oropharynx. Vaginal contamination may be due to orogenital contact, using intrauterine devices (IUDs) and other foreign bodies such as tampons<sup>16,17</sup>. This organism is non-pathogenic in most cases and has no significant clinical value. Some gynecologists recommend removal of IUD for its possible pelvic complications<sup>18-20</sup>.

Three patients in our study suffered from *Actinomyces* infection, all of whom were using IUD. Two had mixed flora (66.66%). In one the flora was coccoid predominant (33.33%). In a similar study, Boon et al reported mixed flora as the most prevailing microbial flora in *Actinomyces* contaminations<sup>14</sup>. Therefore, it seems that the growth of this pathogen is not related to vaginal PH. In the silver stain, both pseudohyphae and spores of *Candida* spp stain black; of course spores stain more intensely than the hyphae. Recurrent candidal infection is a clinical problem and antifungal treatments may have only limited success<sup>11,21</sup>. It is therefore of interest to have a closer look at the associated bacterial flora to devise new approaches. *Candida* grows in acid PH, while its growth decreases in alkaline environments<sup>11</sup>. Hence, candidal infection is expected to be found more frequently with type I flora (lactobacilli predominant) which makes a favorable environment owing to cytolysis and acid PH<sup>14,22</sup>. However, *Candida* showed a statistically significant coexistence with type IV microbial

flora (coccoid predominant) in our study. Findings of Mendoza are in agreement with ours<sup>7</sup>.

The most prevalent genital microbial flora was type II (lactobacilli) in symptomatic women. Lactobacilli are often considered to be commensal or beneficial members of human microbial ecology and are therefore not reported in routine cytology reports. However, not all may be physiologic. Thus, we suggest that all lactobacilli types should be investigated through microbiological methods in symptomatic patients<sup>4</sup>. On the other hand, symptoms may be due to hormonal or mechanical factors. As expected, the most prevalent microbial flora was type II (lactobacilli) in the asymptomatic group. Coccoid predominance (4.9%) in this group should not be ignored. Coccoid flora included a bacterial mixture of *Gardnerella vaginalis*, bacteroides, anaerobic cocci, *Mobiluncus* species and even *Mycoplasma hominis*. Although it is impossible to distinguish them on Papanicolaou smears based on morphology<sup>14,23</sup>, diagnosis is particularly important in pregnancy as there is a risk of chorioamnionitis and preterm delivery<sup>24-26</sup>.

## Conclusions

Using the methenamine silver staining method, we could show that lactobacillus flora is associated with symptoms. Symptomatic women other than those infected with potential pathogens tend to show lactobacillus flora. Therefore, we suggest that all lactobacilli types should be investigated through microbiological methods in symptomatic patients. In silver stained slides there is a clear relationship between the type of vaginal flora and the presence of candidal infection.

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## References

1. Donders GG, Bosmans E, Dekeersmaecker A, Vereecken A, Van Bulck B, Spitz B. **Pathogenesis of abnormal vaginal bacterial flora.** *Am J Obstet Gynecol* 2000; 182(4):872-878.
2. Donders GG. **Microscopy of the bacterial flora on fresh vaginal smears.** *Infect Dis Obstet Gynecol* 1999; 7(3):126-127.
3. Ramzy I. Gynecologic infections. In: Ramzy I, editor. *Clinical Cytopathology and Aspiration Biopsy* (Hardcover). McGraw-Hill Professional; 2000. p. 53-72.
4. McLean NW, Rosenstein IJ. **Characterisation and selection of a Lactobacillus species to re-colonise the vagina of women with recurrent bacterial vaginosis.** *J Med Microbiol* 2000; 49(6):543-552.
5. Demirezen S. **Cytolytic vaginosis: examination of 2947 vaginal smears.** *Cent Eur J Public Health* 2003; 11(1):23-24.
6. Donder GG, Vereecken A, Bosmans E, Dekeersmaecker A, Salembier G, Spitz B. **Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis.** *BJOG* 2002; 109(1):34-43.
7. Mendoza-Gonzalez A, Sanchez-Vega JT, Sanchez-Peon I, Ruiz-Sanchez D, Tay-Zavala J. **[Frequency of Gardnerella vaginalis vaginosis and its association with other pathogens causing genital infections in the female].** *Ginecol Obstet Mex* 2001; 69:272-276.
8. Marrazzo JM, Koutsky LA, Eschenbach DA, Agnew K, Stine K, Hillier SL. **Characterization of vaginal flora and bacterial vaginosis in women who have sex with women.** *J Infect Dis* 2002; 185(9):1307-1313.
9. Abu Shaqra QM. **Bacterial vaginosis among a group of married Jordanian women: occurrence and laboratory diagnosis.** *Cytobios* 2001; 105(408):35-43.
10. Prey M. **Routine Pap smears for the diagnosis of bacterial vaginosis.** *Diagn Cytopathol* 1999; 21(1):10-13.
11. Boon ME, Gray W. Normal vulva, vagina and cervix: hormonal and inflammatory conditions. In: Gray W, McKee GT, editors. *Diagnostic Cytopathology*. London: Churchill Livingstone; 2003. p. 677-688.
12. Spires SE, Banks ER, Weeks JA, Banks HW, Davey DD. **Assessment of cervicovaginal smear adequacy. The Bethesda System guidelines and reproducibility.** *Am J Clin Pathol* 1994; 102(3):354-359.
13. Pritt B, Mount SL, Cooper K, Blaszyk H. **Pseudoactinomycotic radiate granules of the gynaecological tract: review of a diagnostic pitfall.** *J Clin Pathol* 2006; 59(1):17-20.
14. Boon ME, Marres EM, Hoogeveen MM, Goedbloed AF, Milios J. **Visualization of vaginal flora in cervical smears using a modified microwave silver-staining method.** *Histochem J* 1998; 30(2):75-80.
15. Bhagavan BS, Ruffier J, Shinn B. **Pseudoactinomycotic radiate granules in the lower female genital tract: relationship to the Splendore-Hoeppli phenomenon.** *Hum Pathol* 1982; 13(10):898-904.
16. Aydin A, Erkilic S, Bayazit YA, Kocer NE, Ozer E, Kanlikama M. **Relation between actinomycosis and histopathological and clinical features of the palatine tonsils: a comparative study between adult and pediatric patients.** *Rev Laryngol Otol Rhinol (Bord)* 2005; 126(2):95-98.
17. Kayikcioglu F, Akif AM, Haberal A, Faruk DO. **Actinomyces infection in female genital tract.** *Eur J Obstet Gynecol Reprod Biol* 2005; 118(1):77-80.
18. Dokic M, Begovic V, Loncarevic S, Vulovic R, Dimitrijevic J, Popovic S et al. **[Actinomycosis--a multidisciplinary approach to a clinical problem].** *Vojnosanit Pregl* 2004; 61(3):315-319.
19. Lawson E. **Systemic actinomycosis mimicking pelvic malignancy with pulmonary metastases.** *Can Respir J* 2005; 12(3):153-154.
20. Wai CY, Nihira MA, Drewes PG, Chang JS, Siddiqui MT, Hemsell DL. **Actinomyces associated with persistent vaginal granulation tissue.** *Infect Dis Obstet Gynecol* 2005; 13(1):53-55.
21. Eschenbach DA. **Chronic vulvovaginal candidiasis.** *N Engl J Med* 2004; 351(9):851-852.
22. Demirezen S. **The Lactobacilli--Candida relationship in cervico-vaginal smears.** *Cent Eur J Public Health* 2002; 10(3):97-99.
23. Mikamo H, Sato Y, Hayasaki Y, Hua YX, Tamaya T. **Vaginal microflora in healthy women with Gardnerella vaginalis.** *J Infect Chemother* 2000; 6(3):173-177.
24. Yudin MH. **Bacterial vaginosis in pregnancy: diagnosis, screening, and management.** *Clin Perinatol* 2005; 32(3):617-627.
25. Lamont RF, Sawant SR. **Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth.** *Minerva Ginecol* 2005; 57(4):423-433.
26. Boggess KA. **Pathophysiology of preterm birth: emerging concepts of maternal infection.** *Clin Perinatol* 2005; 32(3):561-569.