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Research Article

# INVESTIGATION OF SOME MICROBIAL AGENTS IN VAGINAL AND ENDOSERVIX SWAP SAMPLES OF 18 – 50 AGED WOMEN WITH VAGINAL DISCHARGE

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***Abstract:*** *Detecting sexually transmitted infectious agents in women by traditional methods is often troublesome. This study aimed to investigate bacterial vaginosis and vaginitis agents by culture, microscopic examination, and molecular methods in women with vaginal discharge complaints. A total of 100 female patients aged 18-50 years, who applied to Dicle University Obstetrics and Gynecology outpatient clinic with the complaint of vaginal discharge, were included in the study. Gram and Giemsa staining, culture, and multiplex polymerase chain reaction (PCR) processes were performed from vaginal and endocervix samples taken with sterile swabs. Gram-stained specimens were evaluated with the Nugent score; the presence of leukocytes with polymorph nuclei, Clue-Cells, and different shaped bacteria. Culture-grown agents were identified at the species level by mass spectrometry. There was at least one microbial agent in 63% of the samples included in the study. According to Nugent scores, 27 specimens were determined positive, and 39 specimens had intermediate values for bacterial vaginosis(BV). Candida species (29%), Streptococcus agalactiae (9%), and Staphylococcus aureus (3%) grew in culture while Mycoplasma hominis (23%), Ureaplasma urealyticum (13%), Trichomonas vaginalis (9% ) and Chlamydia trachomatis (3% ) were detected by multiplex PCR. In our study, it was determined that vaginal discharge could be caused by bacterial, fungal, and parasitic microorganisms as well as bacterial vaginosis agents. It is very difficult, laborious, and necessary to determine dysbiosis and infection in the vagina which has an important microbiome. Gram staining and culture methods are insufficient for the detection of vaginal infection agents. Therefore, it would be beneficial to use molecular methods in addition.*

***Keywords:*** *Bacterial vaginosis, Vaginitis, Candida species., Multiplex polymerase chain reaction, Mycoplasma*

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## 1. Introduction

The vaginal microbiota consists predominantly of lactobacilli in the period between puberty and menopause. Decreased lactobacilli population and changes in microbiota may cause symptomatic problems. The most common dysbiosis of the vaginal microbiota is an anaerobic polymicrobial dysbiosis which is named bacterial vaginosis (BV). Other conditions related to vaginal inflammation are vaginal candidiasis, trichomoniasis, inflammatory and atrophic vaginitis [1]. The predominance of *Streptococcus*, *Staphylococcus*, or *Enterobacterales* bacteria in the vaginal microbiota are also

considered dysbiotic conditions [2]. Our knowledge about various types of dysbiosis and their association with urogenital disease burden increased in the last decades with non-culture-based techniques [3]. Vaginal dysbiosis is related to increased susceptibility and transmission risk to *Human Immun Deficiency Virus (HIV)* and other sexually transmitted infections such as pelvic inflammatory disease (PID). Particularly in women aged 18-50 years, these infections are associated with preterm delivery and an increased risk of maternal and newborn infections [4- 6]. Molecular epidemiological studies have revealed that the lactobacilli-dominated vaginal flora was closely related to an immune-tolerant balanced microbiota. However, studies indicate that not all *Lactobacillus species* were the same in terms of their effect on the microbiota. The presence of *Lactobacillus crispatus (L. crispatus)* was associated with the absence of mucosal inflammation, while *Lactobacillus iners* (*L. iners* ) was reported to accompany anaerobes and pathogens causing dysbiosis[2].

Available epidemiological data for assessing the burden of vaginal dysbiosis and associated clinical conditions are largely based on the Amsel criteria and/or the Nugent score [3]. Molecular studies define bacterial vaginosis as polymicrobial dysbiosis of the vagina[2]. Nugent score and vaginal pH were consistent with molecular studies in demonstrating dysbiosis, whereas Amsel criteria were not. However, it should be noted that Nugent scoring cannot distinguish between *Lactobacillus species*[3]. Additional laboratory methods should be used to detect clinically different dysbiotic conditions. PID was thought to be predominantly caused by *Chlamydia trachomatis (C. trachomatis)* and *Neisseria gonorrhoeae (N. gonorrhoeae)*, and in some cases by BV-associated organisms and *Mycoplasma genitalium (M. genitalium)* [1]. Increasing studies have found that *N. gonorrhoeae, C. trachomatis*, and/or *M. genitalium* were associated with approximately 30% of PID cases. However, BV-associated bacteria or urogenital pathobionts - *Streptococcus agalactiae (S. agalactiae), Staphylococcus aureus (S. aureus),* and *Enterobacterales* - were found in 70% of PID cases [1], [7]. Vaginal dysbiosis and its sequelae are common worldwide. Women with symptoms such as unusual vaginal discharge, unusual odor, and/or vaginal itching receive empirical antibiotic and/or antifungal therapy. However, appropriate diagnostic tests will enable effective treatment to begin sooner [8].

In our study, we aimed to investigate the microorganisms causing dysbiosis, especially BV and vaginitis in women who applied with the complaint of vaginal discharge.

## 2. Materials And Methods

Approval of this study was obtained from the Dicle University Faculty of Medicine Non-Invasive Ethics Committee with the number 141 on 19.02.2018. Informed consent was provided by all the participants of the study.

A total of 100 patients aged 18-50 years, who applied to the Dicle University Hospital, Obstetrics and Gynecology outpatient clinic between 04 July 2018 and 30 August 2018 with vaginal discharge, were included in the study. Patients who had vaginal bleeding and/or used antibiotics at least 72 hours before were excluded. Vaginal and cervical swab samples taken by the obstetrician were transferred to the microbiology laboratory within one hour. Fresh preparations for direct microscopy and Gram and Giemsa staining preparations were prepared from the vaginal swab sample. Vaginal samples were inoculated on Sabouraud dextrose agar (SDA) and 5% Sheep Blood Agar (SBA). One of the cervical swabs was undergone for PCR studies while the other swab was inoculated on SBA and chocolate agar. The SDA and SBA mediums were incubated aerobically while chocolate agars were kept in a 5-10% CO2 environment. After incubation, all grown isolates were identified by MALDI-TOF MS (Matrix-assisted laser desorption ionization time of flight mass spectrometry) with Maldi Biotyper (Bruker Daltonics, USA).

The Allplex™ STI Essential Assay (Seegene, Korea), a commercial multiplex Real-Time PCR test, was used to detect *C. trachomatis, N. gonorrhoeae, Trichomonas vaginalis (T. vaginalis), M.*

*genitalium, M. hominis, U. urealyticum* and *U. parvum* in swab samples. Nucleic acid isolation for Multiplex PCR was performed manually using the RibospinTM vRD (GeneAll Biotechnology, Korea) kit according to the classical spin column method.

Fresh preparations were examined at 400 magnification for the presence of *T. vaginalis* trophozoites, leukocytes, yeasts, and pseudohyphae. *T.vaginalis* trophozoites were also investigated in Giemsa-stained smears.

Gram-stained smears were evaluated according to Nugent scoring (Table 1); the presence of clue cell (clue-cell), budding yeast, and pseudohyphae structures and leukocytes were examined. The Nugent scoring method varies between 0 and 10 according to the relative amount of bacterial morphotypes; smears with a total score of ≥7 are considered compatible with bacterial vaginosis, scores between 0 and 3 are considered normal, and scores between 4 and 6 are considered intermediate values [9-10].

**Table 1.** Evaluation of Gram-stained microscopy of the vaginal swab in the diagnosis of bacterial vaginosis (Nugent scoring) [10]

| Morpho type | Number/field | Score |
|---|---|---|
| **Lactobacillus - like, parallel-sided, Gram-positive rods** | >30 | 0 |
| | 5-30 | 1 |
| | 1-4 | 2 |
| | <1 | 3 |
| | 0 | 4 |
| **Mobilincus-like, curved Gram-negative rods** | 5 | 2 |
| | 1-4 | 1 |
| | 0 | 0 |
| **Gardnerella/Bacteroides - like tiny, Gram-variable coccobacilli/pleomorphic rods** | >30 | 4 |
| | 5-30 | 3 |
| | 1-4 | 2 |
| | <1 | 1 |
| | 0 | 0 |
| **Evaluation of the Nugent Scoring System** | | |
| **Total score:0-3** | Normal | |
| **Total score:4-6** | Intermediate | |
| **Total score:7-10** | Bacterial Vaginosis | |

Study data were analyzed in the SPSS 16.0 program. Chi-square test was used for the relationship between Nugent score, age, clue cell, polymorphonuclear leukocytes (PMNs), and possible causes of dysbiosis. $p<0.05$ value was considered statistically significant. One-way analysis of variance and a Post-hoc Tukey test was applied for age.

## 3. Results

Fifteen of the patients were between the ages of 18 and 25, and 85 of them were between the ages of 26 and 49. At least one microorganism was determined by culture and/or PCR method in 63 swab samples of 100 patients examined, and no agent was detected in 37 of them. Two or more agents were detected in 23 of the patients. The total number of agents isolated from all patients was 94. The microorganisms detected by culture and PCR methods and their distribution according to two age groups are given in Table 2.

**Table 2.** Distribution of Microorganisms Detected by Culture and Multiplex PCR Methods by 18-25 and 26-49 Age Groups

| | **18-25 age group (15 women) n** | **26-49 age group (85 women) n** |
|---|---|---|
| *C. trachomatis*[1] | 1 | 2 |
| *T. vaginalis*[1] | 1 | 8 |
| *M. hominis*[1] | 2 | 1 |
| *U. urealyticum*[1] | 1 | 1 |
| *Candida spp*[2] | 4 | 1 |
| *S. agalactiae*[2] | 0 | 1 |
| *S. aureus*[2] | 1 | |
| *G. vaginalis*[2] | 1 | |

[1] *: Detected by PCR method,* [2]*: Detected by culture method*

According to the Nugent score, 27 of 100 patient samples were evaluated as positive and 39 as intermediate. While 26 of the Nugent score positive patients were between the ages of 26-and 49, one patient was between 18-and 25. Nugent score-age relationship was found to be statistically significant. Nugent score distribution by age is given in Table 3.

**Table 3.** Distribution of Nugent Score by Age

| | **Nugent Scoring** | | | |
|---|---|---|---|---|
| | Negative(n=34) **X̄± SD** | Intermediate(n=39) **X̄± SD** | Positive (n=27) **X̄± SD** | p |
| Age group | 32,4±7,27 | 34,3±8,52 | 37,4±6,9 | 0,046* |

* p<0.05

Clue cells were seen in 84 smear samples (Figure 1) while PMNs were detected in Gram-stained smears of 68 patients (Table 4). *T. vaginalis* was detected in direct examination of 1 smear sample.

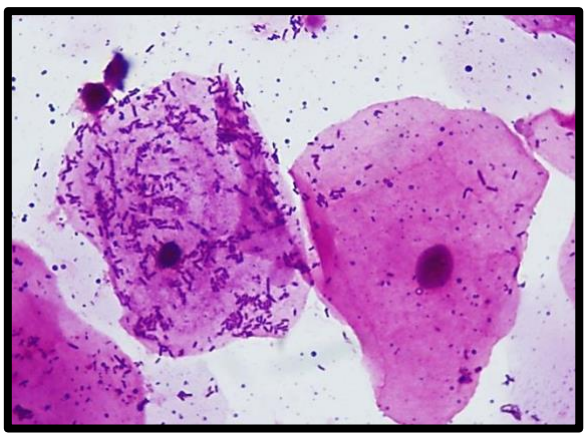

**Figure 1.** Clue cell in a Gram-stained smear

**Table 4.** The presence of clue cells, polymorphonuclear leukocytes (PMNs), and Nugent Score by Age Groups

| | 18-25 age group (n=15) n (%) | 26-49 age group (n=85) n (%) | p | Total |
|---|---|---|---|---|
| **Clue cell** | | | | |
| Negative | 12 (80,0) | 72 (84,7) | | 84(%84) |
| Positive | 3 (20,0) | 13 (15,3) | 0,704 | 16(%16) |
| **PMNs** | | | | |
| Negative | 5 (33,3) | 27 (31,8) | | 32(%32) |
| Positive | 10 (66,7) | 58 (68,2) | 0,899 | 68(%68) |
| **Nugent Scoring** | | | | |
| Negative | 6 (40,0) | 28 (32,9) | | 34(%34) |
| Intermediate | 8 (53,3) | 31 (36,5) | 0,148 | 39(%39) |
| Positive | 1 (6,7) | 26 (30,6) | | 27(%27) |

One-way analysis of variance and a Post-hoc Tukey test was applied for age.
PMNs: polymorphonuclear leukocytes, $p<0.05$ was considered significant

Statistical analysis between vaginal smear Nugent score and presence of *C. trachomatis, N.gonorrhoeae, T. vaginalis, M. genitalium, M. hominis, U. urealyticum, Candida spp.* and *S. agalactiae* are given in Table 5. Culture grown microorganisms are shown in Table 6. *N. gonorrhoeae* and *M. genitalium* were not detected by PCR analysis of any of the patients. Microorganisms detected by PCR analysis are shown in Table 7.

**Table 5.** The relationship between Nugent score and the presence of microorganisms, Clue cells and polymorphonuclear leukocytes (PMNs)

| | NUGENT SCORING | | | |
|---|---|---|---|---|
| | **Negative n (%)** | **Intermediate n (%)** | **Positive n (%)** | **p** |
| *Chlamydia trachomatis* | | | | |
| Negative | 32 (94,1) | 39 (100,0) | 26 (96,3) | 0,329 |
| Positive | 2 (5,9) | 0 (0,0) | 1 (3,7) | |
| *Trichomonas vaginalis* | | | | 0,554 |
| Negative | 32 (94,1) | 34 (87,2) | 25 (92,6) | |
| Positive | 2 (5,9) | 5 (12,8) | 2 (7,4) | |
| *Mycoplasma hominis* | | | | |
| Negative | 30 (88,2) | 31 (79,5) | 16 (59,3) | 0,025* |
| Positive | 4 (11,8) | 8 (20,5) | 11 (40,7) | |
| *Ureoplasma urealyticum* | | | | |
| Negative | 27 (79,4) | 34 (87,2) | 26 (96,3) | 0,150 |
| Positive | 7 (20,6) | 5 (12,8) | 1 (3,7) | |
| *Clue cell presence* | | | | |
| Negative | 32 (94,1) | 37 (94,9) | 15 (55,6) | 0.001** |
| Positive | 2 (5,9) | 2 (5,1) | 12 (44,4) | |
| PMNs presence | | | | |
| Negative | 14 (41,2) | 12 (30,8) | 6 (22,2) | 0,282 |
| Positive | 20 (58,8) | 27 (69,2) | 21 (77,8) | |
| *Candida species* | | | | |
| Negative | 23 (67,6) | 28 (71,8) | 19 (70,4) | 0,927 |
| Positive | 11 (32,4) | 11 (28,2) | 8 (29,6) | |
| *Streptococcus agalactiae* | | | | |
| Negative | 31 (91,2) | 34 (87,2) | 26 (96,3) | 0,445 |
| Positive | 3 (8,8) | 5 (12,8) | 1 (3,7) | |

Chi-square test; * $p<0.05$ ; ** $p<0.01$; PMNs: Polymorphonuclear leukocytes

**Table 6.** Distribution of culture-grown microorganisms in vaginal swab samples

| Microorganisms | Number (%) |
|---|---|
| *Candida albicans* | 21 (21) |
| *Candida dubliniensis* | 2 (2) |
| *Candida glabrata* | 4 (4) |
| *Candida lusitaniae* | 1 (1) |
| *Candida krusei* | 1 (1) |
| *Streptococcus agalactiae* | 9 (9) |
| *Staphylococcus aureus* | 3 (3) |
| *Streptococcus anginosus* | 1 (1) |
| *Gardnerella vaginalis* | 1 (1) |
| *Streptococcus disgalactia* | 1 (1) |
| *Staphylococcus haemolyticus* | 1 (1) |

**Table 7.** Microorganisms detected by PCR Method, presence of PMNs, and clue cell

| Microorganism | (number) | PMNs presence | Clue cell presence |
|---|---|---|---|
| *Mycoplasma hominis* | (23) | 15 | 7 |
| *Ureaplasma urealyticum* | (13) | 8 | 2 |
| *Trichomonas vaginalis* | (9) | 4 | 0 |
| *Chlamydia trachomatis* | (3) | 2 | 0 |

PMNs: Polymorphonuclear leukocytes

## 4. Discussion

The most common complaint of female patients regarding the genital system is vaginal discharge. Infection is one of the leading causes of pathological vaginal discharge. Female genital tract infections can be classified as vulvovaginitis, pelvic inflammatory disease, or exogenous/endogenous infections according to the site of infection. Exogenous genital tract infections are caused by sexually transmitted agents while endogenous infections are caused by endogenous agents after disruption of the vaginal flora. Approximately 90% of women presenting with vaginal discharge have vaginitis; about 40% of vaginitis are reported as BV, 25% as vulvovaginal candidiasis, and 2% as trichomoniasis [11], [12]. Until recently, the diagnosis of bacterial vaginosis was only based on microscopy-based Nugent scoring or Amsel criteria. The development of non-cultural methods has allowed many studies to be conducted and the methods to be compared. Dols JA et al. [13] used the Nugent scoring method with three molecular-based methods in the evaluation of BV. They stated that *L. iners and L. crispatus* or both bacteria clusters were dominant in the vaginal flora of patients without BV, and they especially emphasized that *L.crispatus* was associated with healthy vaginal flora. However, they also stated that *L.crispatus* was not solely responsible for the protection of the flora. In the same study, researchers defined three separate bacterial clusters in BV-positive patients and reported the bacterial profiles in the cluster as anaerobic bacteria dominance with high species diversity[13]. Gram stain and Nugent scoring maintain their importance in the diagnosis of BV, and its sensitivity was reported to be 93-97% [14], [15]. Studies conducted in Turkey reported the prevalence of BV in women with vaginal discharge as 21.2% - 35.8% [16], [17]. The BV rate in our study was 27%, and it was found to be compatible with the results of the studies conducted with the same method. Risk factors thought to be associated with BV include age, age at menarche, duration of menstruation, and sexual activity. BV is most common in the reproductive age which lasts from menarche to menopause [17], [18]. The mean age of patients with a positive Nugent score was found to be 37.4 ± 6.9 and it was statistically significant compared to the

other age groups (P= 0.046). Although Amsel's criteria were reported as a practical method in the diagnosis of BV, it had limitations in diagnosis and was found to be incompatible with molecular methods. The sensitivity and specificity of Amsel's criteria have been reported as 75.0% and 50.8%, respectively [19]. In a study conducted by Şahin N.N [17], it was pointed out that the criteria other than clue cell positivity of Gram staining were not reliable, since it was difficult to evaluate the pH and odor of vaginal discharge in various situations. In the same study, the sensitivity and the specificity of clue cell positivity were determined as 84.7% and 96.5%, respectively for the diagnosis of BV in patients with positive Nugent scoring. In the current study, clue cell absence was found in 94.1% of the Nugent-negative group (P=0), while 44.4% of the Nugent-positive group showed clue cell positivity. In particular, the absence of clue cells was noted as an even more reliable marker for detecting BV negativity.

In our study, *Candida species* were found to be the most isolated microorganisms in vaginal cultures. Of the 29 isolated *Candida spp* isolates, 21 were *C. albicans*. Eight of the patients with vaginal candidiasis were accompanied by BV. Our study was compatible with previous studies. Albayrak et al [20] reported that Candida species grew in 28% of the vaginal cultures of 300 women aged 18-60 years. Kalkancı et al.[21] isolated *Candida spp.* in 16.4% of a total of 567 vaginal swab samples. In another study, *Candida species* were isolated in 266 (33.2%) of 801 patients, and the most frequently isolated species was *C. albicans* (61.7%)[22]. Vulvovaginal candidiasis has been reported to be the most common dysbiotic condition after BV. It was reported that 75% of women have experienced vulvovaginal candidiasis at least once in their lifetime[7]. Since susceptibility profiles differ according to Candida species, empirical treatment is not recommended unless there is a positive vaginal culture and clinical findings[23].

*T. vaginalis* is a sexually transmitted protozoan that causes infection in the vagina. The infection may spread through the lymph nodes to the hypogastric area of the pelvis. The increased numbers of PMNs seen on the fresh smear samples of vaginal discharges indicate inflammation[23]. Although the direct microscopic examination is a fast and cheap method with high specificity, its sensitivity varies between 58-and 82%[20]. The sensitivity of the method varies with the time from sampling to examination, the number of parasites, and the experience of the examiner [24]. In the current study, among 9 (9%) samples detected by PCR, only one was detected by direct examination and two by Giemsa staining. The prevalence of trichomoniasis ranges from 3% to 40%. The socio-economic level of the studied groups and the methods used are the main factors affecting the prevalence. In studies conducted in Düzce, Ankara, and Hatay, the prevalence of 13.4%, 4.9%, and 2.18% were reported, respectively [25–27].

Aerobic or facultative anaerobic vaginitis was first defined by Donders in 2002 as a type of vaginal infection caused by aerobic bacteria. Although its pathogenesis remains unclear, colonization of *Escherichia coli, Streptococcus spp., Enterococcus faecalis*, *Staphylococcus spp. (S.aureus,* and Coagulase-Negative Staphylococci such as *S. epidermidis*) have been reported in these patients. Detecting aerobic vaginitis is important to prevent complications (such as rupture of membranes, preterm labor, and chorioamnionitis in pregnant women, pelvic inflammatory disease in non-pregnant women) and to apply the appropriate treatment[28]. Studies investigating the frequency of *S. agalactia* / *Group B Streptococcus* (GBS) in our country varied according to the patient groups included in the study and whether the patients were pregnant or not. Pregnant women were not included in our study, and GBS was grown in the culture of 10% of the vaginal samples. In a study conducted in İstanbul, vaginal cultures of women using and not using an intrauterine device (IUD) were evaluated. GBS grew in 6% (3/30) of the vaginal cultures in the IUD using group and in 4% (2/28) of the vaginal cultures of those who did not use the IUD [29]

Molecular methods contribute to the easy and rapid identification of microorganisms that do not grow easily in culture media or that require special growth media. In our study, we detected *M.hominis* in 23 of 100 samples, *U. urealyticum* in 13, *T. vaginalis* in 9 and *C. trachomatis* in 3 samples by multiplex PCR method. A study in Australia investigated bacterial and viral cervicitis agents by multiple PCR. In 233 cervical specimens from 175 patients, *U. parvum*, *M. hominis*, *U. urealyticum*, *M. genitalium*, and *C. trachomatis* were detected in 57%, 13.7%, 6.1%, 1.3%, and 0.4%, respectively[30]. Large and controlled studies are needed on the role of agents detected by molecular methods in infections. The prevalence of genital mycoplasma infection has been reported as 30-40% in various studies. Co-infection was frequently reported in genital infections[31]. Our study was compatible with previous publications. Among the samples of symptomatic patients in our study, PMNs were observed in 16 of 23 samples with *M. hominis* while PMNs were not detected in 7 samples. In the current study, *U. urealyticum* (in 5 samples), *T. vaginalis* (in 5 samples), *Candida spp*. (in 4 samples), and *C. trachomatis* (in 1 sample) were detected simultaneously with *M. hominis*.

The strength of our study was the investigation of multiple infectious agents using the molecular method in addition to culture and microscopic methods. The limitation of our study was the small number of samples obtained over a two-month period. Another limitation was the inability to culture some agents that required specific culture media, such as *Mycoplasma* and *Ureaplasma species.*

## 5. Conclusion

This descriptive study revealed that various bacterial, fungal, and parasitic agents may cause vaginal discharge. Multiplex PCR methods will facilitate the detection of agents - especially those that can't be cultured or require specific media- in routine diagnostic laboratories. The regional distribution of the agents and their presence in the urogenital flora may differ. More comprehensive studies are needed on the microbiota distribution of the agents and their ability to cause infections.

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**Ethical statement**

The current study was carried out in accordance with research and publication ethics rules. Approval of this study was obtained from the Dicle University Faculty of Medicine Non-Invasive Ethics Committee with the number 141 on 19.02.2019.

**Conflict of interest**

The authors have no conflict of interest.

**Authors' Contributions**

NA conducted the study, EA provided samples from patients, Rİ and SY carried out conventional processing of samples, CS carried out molecular tests, F.Ç analysed the test reports and NÖ wrote the article.

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