ORIGINAL ARTICLES

COMPARISON OF VAGINAL MICROBIAL PROFILES OF WOMEN WITH BACTERIAL VAGINOSIS AND NORMAL VAGINAL FLORA USING POLYMERASE CHAIN REACTION

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SUMMARY

Purpose: *To establish a PCR-based vaginal microbial profile diagnostic system for detection of the most common bacterial vaginosis (BV), associated pathogens including Gardnerella vaginalis, Mobilincus sp., Mycoplasma hominis , Bacteroides sp., Fusobacterium nucleatum and Prevotella sp.* ***Methods:*** *From 70 women, 46 with BV and 24 controls, vaginal microbial profiles were compared by PCR.* ***Results:*** *Among 6 pathogens, Gardnerella vaginalis, Mobilincus sp., Mycoplasma hominis, Bacteroides sp. and Prevotella sp.were found to be significantly high in the vaginal flora of women with BV ($p < 0.05$). Furthermore, co-presence of Gardnerella vaginalis , Mobilincus sp., Mycoplasma hominis , Bacteroides sp., Prevotella sp. and any of the three among these pathogens were significantly higher in BV patients than women with normal vaginal flora ($p < 0.05$).* ***Conclusion:*** *PCR-based sytem for detecting vaginal microbial profile may help to diagnose symptomless BV cases and associated pathogens, which are mostly anaerobes and difficult to culture.*

Key Words: *Bacterial Vaginosis, Polymerase Chain Reaction.*

INTRODUCTION

Bacterial vaginosis (BV) is one of the most common infectious disorders affecting women (1, 2). In the past, BV was termed nonspecific vaginitis, Gardnerella vaginitis or anaerobic vaginosis to differentiate it from other vaginal infections caused by Trichomonas vaginalis or Candida albicans (3). The condition of BV has been described primarily by clinical signs, as described by Amsel et al (4). BV is diagnosed when three of the following four criteria are present: increased homogenous vaginal discharge, raised vaginal pH, detection of "clue cells", and the presence of "fishy odor" after addition of potassium hydroxide to the discharge (amine test).

Women with BV have an increased risk of pelvic inflammatory disease (PID), premature labor and post-surgical infections. BV is considered to be a polymicrobial syndrome mostly caused by anaerobes that are difficult to grow. Although the exact cause of BV is unknown, associated pathogens include Gardnerella vaginalis, Mycoplasma sp, Mobilincus and Ureplasma urealyticum (1, 3). When certain bacteria, especially some non-

culturable or fastidious bacteria, are involved, the actual cause of the disease becomes difficult to identify. The treatment for BV is usually effective, but about 50% of the BV cases are symptomless and thus are not treated. This can create a hidden risk for these women of developing PID or experiencing preterm labor (3, 5, 6).

Since the difficulty in culturing anaerobes has hampered the study of BV with respect to identifying the causative agents, we have sought to establish a vaginal microbial diagnostic system using polymerase chain reaction (PCR). This system can identify the six most common BV-associated pathogens directly from the vaginal swabs without the need to culture them. With this system, BV pathogens can be identified by analyzing microbial profiles between healthy women and BV patients. Therefore, a more sensitive and rapid method for detecting BV pathogens may help to treat BV and subsequent infections.

MATERIALS AND METHODS

The study group and specimen collection: This clinical study was approved by the Ethic Committee of the School of Medicine, Karadeniz Technical University. The vaginal samples were collected at the Family Planning Center from a total of 70 women (46 BV and 24 controls) who were in reproductive-age, non-pregnant and non-diabetic, without a recent history of antibiotic or antifungal treatment. Each patient was asked to fill out a consent form to join this study and a questionnaire about the history of her health status, the use of oral or vaginal contraceptives, past medical conditions or therapies. Vaginal pH was measured with an absorbent pH indicator and two sterile cotton swabs were used for taking vaginal samples. One of the samples was taken with a dry swab for saline wet mount, gram staining and sniff test, and the other swab was placed in a test tube containing thioglucolate transport medium, and sent to the laboratory for analysis. BV was diagnosed according to clinical signs and symptoms, such as the presence of vaginal discharge, a high vaginal pH (>5.5), reduction or lack of vaginal lactobacilli, observation of "clue cells" on saline wet mount, and fishy odor on addition of 10% potassium hydroxide to the discharge.

PCR Analysis: One part of each vaginal sample taken in transport medium was stored at -80°C with 10 % glycerol to maintain vaginal bacteria for future analysis.

The PCR primers were designed according to the bacterial genus-specific 16S rRNA gene sequences from the Gene Bank. Each pair of primers was secreened for its specificity among the stored sequences in the Gene Bank. Moreover, each genus-specific primer was tested using chromosomal DNA from ATCC type strains as templates to ensure the specificity of each sets of primers. The type strains and oligonucleotides for Gardnerella vaginalis ATCC 14018, Mobilincus curtisii subsp. curtisii ATCC 35241, Mycoplasma hominis ATCC 14027, Bacteroides forsythus ATCC 43037, Fusobacterium nucleatum ATCC 10953, and Prevotella intermedius ATCC 25611 were: -GV1; 5'-GCT TGG TGT GAA AGC CCA TC-3'and GV2; 5'- ATG AAG CAA CCC GTT GTA CT-3', MC1; 5'- CTG GAA AGC CAG CAG CTT AA-3' and MC2; 5'- GGC CAT TGT AGC ATG CGT GA-3', MH1; 5'- CAG ACT GAC GGT ACC TTG TC-3'and MH2; 5'-CGT TAG CTG CGT CAG TGA TT-3', BA1; 5'-TGC CGT TGA AAC TGG TAG TC-3' and BA2; 5'-TCA TCC CAA CCT TCC TCA C-3', FN1; 5'- CTA AAT ACG TGC CAG CAG CC-3' and FN2; 5'-CGA CCC CCA ACA CCT AGT AA-3', and PR1; 5'- TTG TTG GGG AGT AAA GCG CC-3' and PR2; 5'-CCA GGT GGG ATG CTT AAT GC-3', respectively.

About 200 µl of the sample in the transport medium was transferred to a sterile eppendorf tube and centrifuged for 30 sec at the highest speed to precipitate the bacteria. For the PCR test, cells in 100 µl lysing buffer (1% Triton-X100 in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) were boiled for 10 min or diluted 10 fold with sterile distilled water. Of each sample, 10 µl was added to a PCR reaction mixture with 1 unit of Taq DNA polymerase, 20 µM of each deoxynucleoside triphosphate (Promega, USA), 20 pmol of each genus specific primers (GibcoBRL, USA) and the PCR reaction buffer to a total volume of 50 µl in a 500 µl micro centrifuge tube. PCR negative vaginal samples were repeated using total DNA as template isolated from the 200 µl sample essentially by the method described by Nath and Galdi (7). The

PCR program was performed as follows: 94°C, 45 sec; 55°C, 30 sec; 72°C, 45 sec for 36 cycles, and a final extension of 7 min at 72°C. The resulting PCR products were analyzed by 2 % agarose gel electrophoresis, stained by ethidium bromide, visualized on an UV illuminator and photographed by a Polaroid camera (Fig.1). Blank controls were used for each PCR assay to rule out possible contamination, defective

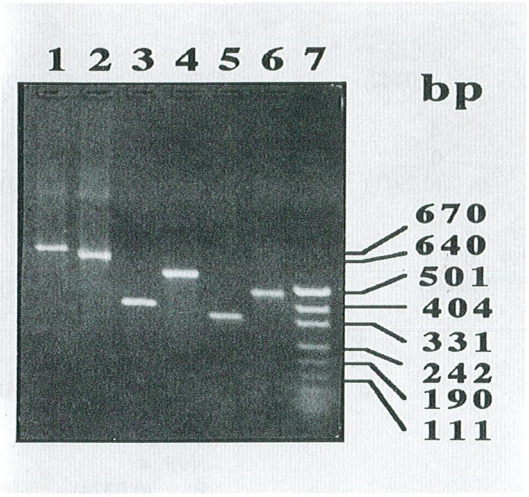

Fig - 1: Detection of BV-associated pathogens by PCR. Lanes 1-6 positive samples of G.vaginalis (670 bp), Mobilincus sp. (640 bp), M. hominis (380 bp), Bacteroides sp. (556 bp), F. nucleatum and Prevotella sp. (437 bp), respectively. Lane 7, molecular size marker.

enzymes or reagents.

For statistical analysis, Epi Info (5.01b version) program (released by CDC and WHO) was used to perform Chi-square and Fisher's exact tests. Significance level was indicated as 0.05 or less.

RESULTS

Vaginal microbial profiles of suspected six BV pathogens between 24 healthy and 46 BV patients were analyzed by PCR. Figure 1 shows an example of positive PCR profile of each bacteria. The presence of each BV-associated pathogen among healthy women and BV patients is summarized in Table 1. When the presence of each pathogen in BV patients and in women with normal vaginal flora G. vaginalis, were compared Mobilincus sp., M. hominis, Bacteroides sp. and Prevotella sp. were present in BV patients more frequently than in women with normal vaginal flora ($p<0.05$). However, the presence of F. nucleatum in both groups showed no significant difference ($p>0.05$).

Among all the pathogens tested, the presence of F. nucleatum together with any other pathogen was not found to be significantly different ($p>0.05$) in both groups. On the other hand, G. vaginalis with Mobilincus sp, M. hominis, and Prevotella sp., Mobilincus sp. with M. hominis, Bacteroides sp. and Prevotella sp. and M. hominis with Prevotella sp. were PCR-detected significantly higher in BV patients than in healthy women ($p<0.05$). Furthermore, detection of three pathogens in the vagina of BV patients and healthy women were compared (Table 2). As a BV indicator bacterium, G. vaginalis, with Mobilincus sp. and M. hominis, with Mobilincus sp. and Prevotella, with M. hominis and Prevotella sp., in addition, Mobilincus sp., M. hominis and Prevotella sp., and any three and more pathogens were co-existent in much higher ratios in BV patients than women with normal vaginal flora ($p<0.05$).

DISCUSSION

BV is defined an altered vaginal microbial profile that is characterized by the dominance of mostly anaerobic bacteria and a significant reduction of lactobacilli morphotypes. It is caused by several microorganisms, including G. vaginalis, certain anaerobes, especially Mobilincus sp., M. hominis, Bacteroides sp., Prevotella sp., Fusobacterium sp., Ureaplasma urealyticum and beta streptococci (8, 9, 10, 11, 12). Among these, G. vaginalis has been considered to be an indicator bacterium of BV. However, G. vaginalis is present in the vagina of healthy women as well as in BV patients. Therefore, detection of G. vaginalis in vaginal fluid can not be used as a diagnostic test for BV. The increased prevalence of G. vaginalis in BV patients suggests that its presence at a high concentration in women with BV may be a prerequisite in the development of BV with other facultative and mostly anaerobic bacteria.

Hillier et al. (13) reported that women with Mobilincus sp. were more likely to harbor G. vaginalis, M. hominis and Neisseria gonorrhea, and less likely to harbor vaginal yeast, and Mobilincus sp., G. vaginalis, and M. hominis were independently associated with a clinical diagnosis of bacterial vaginosis. Among

the BV pathogens, M. hominis has been associated with BV in pregnant and non pregnant women. It was found in 24 to 75 % of women with BV and 13 to 22 % of women without BV with other organisms in the development of BV, the association between each organism and BV was adjusted for co-infection by another organism (Table 1). As shown in Table 1, G.

Table 1: Presence of two BV-associated bacteria together in women with BV and normal vaginal flora.

	G. vaginalis	Mobilincus sp.	M. hominis	Bacteroides sp.	F. nucleatum	Prevotella sp.
G. vaginalis	BV 29 NVF 7 P (0.0146)	27 6 (0.0076)	13 0 (0.0028)	5 0 (0.1571)	14 4 (0.3355)	15 1 (0.0168)
Mobilincus sp.		BV 35 NVF 11 P (0.0235)	15 0 (0.0043)	8 0 (0.0442)	14 4 (0.3355)	17 2 (0.0230)
M. hominis			BV 7 NVF 0 P (0.0018)	4 0 (0.2911)	5 0 (0.1571)	10 0 (0.0123)
Bacteroides sp.				BV 8 NVF 0 P (0.0442)	2 1 (1.000)	3 0 (0.5462)
F. nucleatum					BV 16 NVF 8 P (0.8855)	10 2 (0.1974)
Prevotella sp.						BV 21 NVF 4 P (0.0324)

BV : Bacterial Vaginosis
NVF : Normal Vaginal Flora
P : Significance

(8, 14, 15). Thus, its role in BV is nuclear. Other studies have shown that Bacteroides sp.(8, 10, 16, 17) Mobilincus sp. (8), Fusobacterium sp. (18), and Prevotella sp. as well as Porphyromonas sp., Veillonella sp. and peptostreptococci (8) are associated with BV. The prevalence of Mobilincus sp. in BV has been reported to be 77 % with microscopy and as high as 96 % with culture, and less than 6 % in controls.

In our study, Mobilincus sp. was detected in 76 % of the BV patients and about 45 % of women without BV using PCR. Even though the prevalence of Mobilincus sp. in women without BV was higher by PCR than in other studies, overall its presence in BV patients was significantly higher ($p<0.05$) than controls. This high detection rate of Mobilincus sp. in women without BV by PCR may be due to the high sensitivity of PCR technique compared to the microscopy.

To assess the possibility that each pathogen is associated independently or together vaginalis, Mobilincus sp., M. hominis, Bacteroides sp. and Prevotella sp. were independently associated with BV ($p<0.05$). However, F. nucleatum was not independently associated with BV ($p>0.05$). When each organism was compared with any other pathogen, G. vaginalis with Mobilincus sp., M. hominis and Prevotella sp.;Mobilincus sp. with M. hominis, Bacteroides sp. and Prevotella sp., and finally M. hominis with only Prevotella sp. showed significant association ($p<0.05$) in BV. Further adjustment for these pathogens with BV (Table 2) suggested that co-infection with G. vaginalis, Mobilincus sp. and M. hominis;G. vaginalis, Mobilincus sp. and Prevotella sp; Mobilincus sp., M. hominis and Prevotella sp. in BV was significantly higher ($p<0.05$), as it was with the presence of any three or more pathogens in the same BV patient ($p<0.0009$).

In our BV-associated pathogen detection system based on PCR, in addition to the individual association of G. vaginalis, Mobilincus sp., and M. hominis with BV, each organism was also found to show a positive association with each other and all three together

Table 2: Presence of three BV-associated bacteria together in women with BV and with normal vaginal flora

	BV (n)	NVF (n)	P
G. vaginalis+Mobilincus sp.+M. hominis	12	0	0.0057
G. vaginalis+Mobilincus sp.+Bacteroides sp.	5	0	0.1571
G. vaginalis+Mobilincus sp.+F. nucleatum	12	3	0.3134
G. vaginalis+Mobilincus sp.+Prevotella sp.	15	1	0.0168
G. vaginalis +M. hominis+Bacteroides sp.	3	0	0.5462
G. vaginalis +M. hominis+F. nucleatum	4	0	0.2911
G. vaginalis +M. hominis+Prevotella sp.	8	0	0.0441
G. vaginalis +Bacteroides sp.+F. nucleatum	2	0	0.5428
G. vaginalis +Bacteroides sp.+Prevotella sp.	3	0	0.5462
G. vaginalis +F. nucleatum+Prevotella sp.	7	0	0.0868
Mobilincus sp.+M. hominis+Bacteroides sp.	4	0	0.2911
Mobilincus sp.+M. hominis+F. nucleatum	5	0	0.1571
Mobilincus sp.+M. hominis+Prevotella sp.	10	0	0.0123
Mobilincus sp.+Bacteroides sp.+F. nucleatum	2	0	0.5428
Mobilincus sp.+Bacteroides sp.+Prevotella sp.	3	0	0.5462
M. hominis+Bacteroides sp.+Prevotella sp.	2	0	0.5428
Bacteroides sp.+F. nucleatum +Prevotella sp.	1	0	1.0000
Any three or more bacteria together	26	3	0.0009

in the development of BV. Moreover, the presence of Prevotella sp. with G. vaginalis, Mobilincus sp., and M. hominis was significant ($p<0.05$) individually or as a co-infecting pathogen in BV. While Bacteroides sp. showed positive correlation only with Mobilincus sp. in 17 % of BV cases tested, no individual association or co-presence of F. nucleatum with other pathogens in BV cases was found.

From data obtained in this study, it has been shown that a PCR-based rapid BV diagnosis may help to diagnose BV much faster with a sensitivity compare to other methods without the need to culture BV-associated pathogens, which are mostly anaerobes and difficult to culture in many clinical settings, and help clinicians to design an appropriate treatment regimen. Also, with this PCR system, symptomless or underdiagnosed BV cases may be better diagnosed. Further studies are needed with additional BV-associated pathogens such as beta streptococci, peptostreptococci, propionibacteria, Porphyromonas sp., Campylobacter sp. and U. urealyticum, in a large scale study with more subjects to better understand the multimicrobial nature of bacterial vaginosis.

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