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Multiplex PCR detection of problematic pathogens of clinically heterogeneous bacterial vaginosis in Bulgarian women

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Background/aim: This study aimed to investigate the correlation between the prevalence of problematic pathogens and the clinical status of women with bacterial vaginosis (BV).

Materials and methods: *Gardnerella vaginalis*, *Atopobium vaginae*, and *Mobiluncus* spp. were detected using a multiplex PCR assay, and their role in the infection of Bulgarian women with clinically heterogeneous BV was evaluated.

Results: The predominant BV-associated pathogen identified was *G. vaginalis* with an incidence of 98.39%, followed by *A. vaginae* (68.05%) and *Mobiluncus* spp. at 17.01%. The coexistence of *A. vaginae* and *G. vaginalis* was more common in women with discharge (in 72.04%) and in patients with chronic recurrent BV than among asymptomatic or newly diagnosed BV cases ($P < 0.05$). *Mobiluncus* spp. was detected mostly in coinfections, in association with *Trichomonas vaginalis*. The coinfections were predominantly related to recurrent BV and with complications ($P < 0.05$).

Conclusion: This is the first study about the correlation between problematic pathogens and clinically heterogeneous BV in Bulgarian women. High frequency of infection with key BV-related pathogens was observed in childbearing women. The incidence was shown to often correlate with coexistent *T. vaginalis*, with severity of infection, and with complicated and recurrent BV after unsuccessful treatments. Screening should be considered in reproductive health programs.

Key words: Bacterial vaginosis, PCR, *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus* spp.

1. Introduction

Bacterial vaginosis (BV) is a leading genital tract infection in reproductive-age women. This very common vaginal disorder occurs when beneficial *Lactobacillus* spp. become replaced by various obligate or facultative anaerobic bacteria, which are normally present in very low numbers or are absent in healthy women (1,2). The abnormal flora has been mostly defined by the presence of high *Mobiluncus* spp. counts (more than 10^4 CFU/mL) visible on Gram staining, with a Nugent score of 9 or 10 (3,4). When the level of lactobacilli in the vaginal niche is 10^7–10^8 CFU/mL, they have antimicrobial properties that inhibit the growth and initial adhesion of *Gardnerella vaginalis* to epithelial cells and its biofilm-forming ability due to production of hydrogen peroxide, bacteriocins, and lactic acid, which maintains low values (about 4.5) of pH (5,6). Bacteria such as *G. vaginalis*, *Atopobium vaginae*, and *Mobiluncus* spp. are recognized as predominant vaginal pathogens and as sensitive indicators in the diagnosis of BV, although many other BV-related pathogens have been found with variable frequency (7–11). *A. vaginae* and *Mobiluncus* spp. are not susceptible to metronidazole, which is a problem for the successful treatment of infection, explaining their key role in the development of chronic and recurrent BV (9,12).

Currently, the causes behind the replacement of normal microbiota by nonbeneficial bacteria are still unknown. When the size of the vaginal lactobacillus population begins to decrease, it results in enhanced virulence of *G. vaginalis*. Only *G. vaginalis* has been shown to exhibit a strong ability to adhere to the vaginal epithelium; *A. vaginae* and *Mobiluncus* spp. can also adhere, but to a lesser extent (4,7,9). Other anaerobes detected in BV-positive women have not shown any adherence, which is a significant marker for their low virulence and their uncertain and erratic participation in this infectious process (10,11). Some epidemiological (13) and experimental data in animal models and in studies with volunteers (14,15) suggest that BV is a sexually transmitted disease, although

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some authors believe that the infection is polymicrobial when there is coexistence of many anaerobes (16). This transmission of infection is more typically caused by a single microbial agent with secondary anaerobic activation, i.e. the pathogenesis is similar to that of *Trichomonas vaginalis* infection (17). *G. vaginalis* forms a significantly thicker biofilm than other vaginal pathogens, which is why it is a predominant species in all BV biofilms (6,17,18). This bacterial species is strongly cytotoxic for vaginal epithelial cells. These data suggest that *G. vaginalis* has a higher virulence potential than other BV-related species. The bioactive agents produced by this bacterium, such as the exotoxin vaginolysin and the hydrolytic enzymes sialidase and prolidase, cause the degradation of mucin and epithelium (9,18,19). The key presence of metronidazole-resistant *A. vaginae* and *Mobiluncus* spp. only at high Nugent scores implicates them as associated with the clinical development of BV (7–9).

The aim of this study was to determine the most problematic and key causative agents of BV using polymerase chain reaction (PCR) and to evaluate the correlation between their prevalence and the clinical status of Bulgarian patients.

2. Materials and methods

2.1. Patients and collection of specimens

Vaginal samples from 538 women from Bulgaria aged 16–45 years were collected between September 2013 and December 2014 and stored at –20 °C for up to 2 days before extraction of total bacterial DNA. Specimens collected from enrolled subjects consisted of 2 vaginal swabs followed by a vaginal lavage. One of the swabs was rolled onto a glass slide, air-dried, and then Gram-stained for microscopic assessment of BV using the Nugent criteria (0–3: normal vaginal flora [NVF]; 4–6: intermediate; 7–10: BV) (3,8). Vaginal lavage was collected by washing the vaginal vault for 30–40 s using a syringe and 5 mL of nonpyrogenic sterile saline. Then 0.5 mL of the sample was placed in a sterile vial and frozen at –70 °C until later use for DNA extraction. The samples were analyzed by molecular genetic methods to determine some key causative agents of vaginal disorder. The inclusion criteria for the examined people enrolled in this study were: sexually active women of reproductive age; no antimicrobial therapy received in the week before the study; negative serological results for *Chlamydia trachomatis*; positive microscopic smears with BV according to the Nugent score (3) for all groups excluding the control group (n = 103) with microscopic diagnosis of NVF (ecosystem with only lactobacilli and visible absence of other bacterial morphotypes). The patients with microscopically detected BV were divided into the following groups based on their clinical status: A and B, asymptomatic (n = 152) and symptomatic with symptoms of discharge (n = 279), respectively; C/D, pregnant (n = 188)/nonpregnant (n = 247); E, women with no complications and no relapse of new-found BV (n = 130); F, people with recurrent symptoms and a tendency to develop chronic BV without coinfection with *Trichomonas vaginalis* (TV) (n = 170); G, recurrent BV with coinfection with TV (n = 78); H, patients with complications of BV such as imminent abortion and premature birth (n = 57). The exclusion criteria were the presence of tumors, amenorrhea, HIV infection, hepatitis B or C, syphilis, gonorrhea, and candidosis.

Informed consent forms were obtained from all participants and were included in their standard medical records. There was no personal patient information in the database. The hospital's ethics committee granted study approval.

2.2. Gram staining and culture method

These methods were performed as previously described (8).

2.3. DNA isolation

Total DNA from vaginal samples was isolated using the DNAsorb-AM nucleic acid extraction kit (AmpliSens) according to the manufacturer's guidelines. DNA isolated in parallel from *G. vaginalis* ATCC 14018 (American Type Culture Collection) was used as a positive control.

2.4. Polymerase chain reaction (PCR) assay

A multiplex PCR assay for detection of the major BV causative agents, such as *G. vaginalis*, *A. vaginae*, and *Mobiluncus* spp., targeting their 16S ribosomal ribonucleic acid (rRNA) genes was performed. All *Mobiluncus* spp.-positive samples were additionally examined by a species-specific PCR test for the identification of *M. curtisii*. The oligonucleotides used as primers for amplification (4,20,21) were synthesized by Alpha DNA (Canada). They were verified for specificity using the Basic Local Alignment Search Tool (BLAST) program available from the NCBI (http//:www.nbi.nlm.nih.gov/BLAST).

PCR was carried out with 10 ng of template DNA, 0.25 μM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 1X reaction buffer, 2 mM $MgCl_2$, and 0.5 U Prime Taq DNA polymerase (Genet Bio) in a total volume of 25 μL. The DNA was amplified using the following protocol: initial denaturation (95 °C for 5 min), followed by 30 cycles of denaturation (95 °C for 45 s), annealing (58 °C and 69 °C for 45 s), and extension (72 °C for 45 s), with a single final extension of 7 min at 72 °C. PCR products were separated in 1% agarose gel for 50 min at 140 V, stained with ethidium bromide (0.5 μg/mL), and detected by UV transillumination (wavelength: 312 nm). The amplification products were identified on the basis of fragment length (4,20,21).

Detection of *Trichomonas vaginalis* (TV) in vaginal samples was done as previously described by Madico et al. (22).

2.5. Statistical analysis

The data were analyzed using the chi-square test and Fisher's exact test for categorical variables. All analytical procedures were performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

Representative multiplex PCR amplicons of BV-associated pathogens are presented in the Figure. The distribution of BV causative agents detected in women with microscopically diagnosed BV and in healthy women with microscopic evaluation of NVF is summarized in Table 1. In women with NVF the incidence of *G. vaginalis* was 5.83%; *A. vaginae* was present in only 0.97%, and no *Mobiluncus* spp. were detected. The most common bacterial pathogen identified, alone or in microbial combinations (Tables 1 and 2), in all patients with microscopic smears with BV was *G. vaginalis*, with an incidence of 98.39%, followed by *A. vaginae* (68.05%), and *Mobiluncus spp.* at 17.01%, particularly *M. curtisii* (9.20%). In symptomatic women, the prevalence of both *A. vaginae* and *G. vaginalis* was very high (72.04%; $P < 0.05$), more than 2 times higher than in asymptomatic ones. Triple infection with *G. vaginalis*, *A. vaginae*, and *Mobiluncus* spp. was observed in 15.19% of the patients with discharge ($P < 0.05$), amounting to a total of 87.23% of cases of multiple (double or triple) infection, as shown in Table 2. About one-fourth of BV-positive samples contained *G. vaginalis* alone; only 1.4% contained *A. vaginae* and 0.23% *M. curtisii* alone. In the other samples, coinfections were demonstrated (Table 2). In the prevailing cases with chronic recurrent BV, presented in Table 3, the PCR results were positive for the 2 leading etiological agents, *G. vaginalis* and *A. vaginae* ($P < 0.05$). The rarer *Mobiluncus* spp. pathogens were also identified in symptomatic patients, but there was high association with more complications such as coinfection with *T. vaginalis* ($P < 0.05$) (Table 3). Coinfections with *A. vaginae* and *Mobiluncus* spp. together only and lack of *G. vaginalis* were not observed, as shown in Tables 2 and 3.

4. Discussion

A significant difference ($P < 0.05$) among women with microscopic diagnosis of BV and NVF was demonstrated in Table 2. The most prevalent etiological agent, *G. vaginalis*, was present in all tested groups of patients with BV and was rarely found in the healthy population (Tables 1–3), which is in agreement with the concept of the leading and initial role of *G. vaginalis* in BV pathogenesis (1,15). New experimental data for significant divergence of 2 different genotypes, biotypes, and the virulence of *G. vaginalis* isolated from healthy and ill persons have been reported in recent years (5,19,20,23). The commensal strains of *G. vaginalis* demonstrate reduced biofilm-forming capacity and cytotoxicity, unlike the pathogenic isolates, which exhibit higher adhesive and aggregative potential (24,25).

We determined that the combination mostly detected (more than 72%) in Bulgarian patients, especially in ones with vaginal discharge, was that of the 2 major pathogens, *G. vaginalis* and *A. vaginae*, and it was

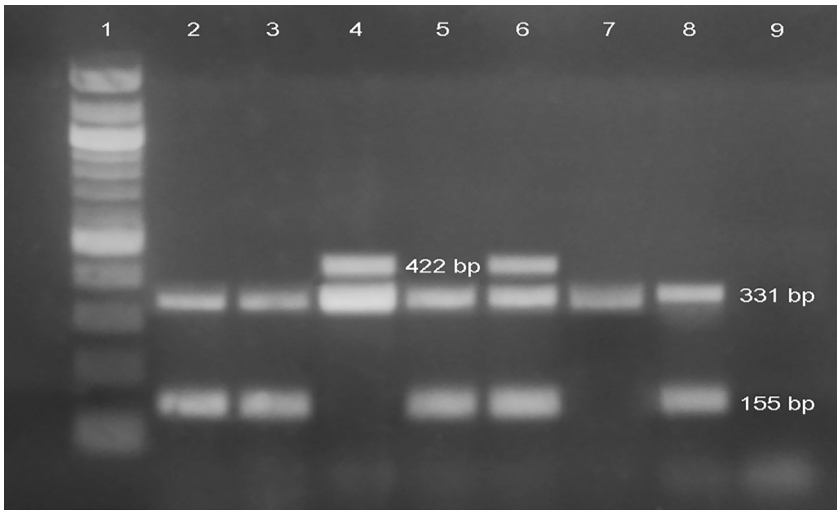

Figure. Multiplex PCR (agarose gel electrophoresis) for detection of *G. vaginalis* (331 bp), *A. vaginae* (155 bp), and *Mobiluncus spp.* (422 bp). Lane 1: DNA marker GeneRule 100 bp Plus DNA Ladder (Fermentas). Lanes 2, 3, 5, and 7 represent the positive clinical samples for *G. vaginalis* and *A. vaginae*; lanes 4 and 6, positive samples for *G. vaginalis*, *A. vaginae*, and *Mobiluncus spp.*; lane 7: *G. vaginalis*-positive sample; lane 9: a negative sample.

Table 1. Prevalence of key vaginal pathogens in women according to microscopic evaluation of BV*.

BV-associated pathogens	Patients		P-value between women with BV and NVF
	With microscopic diagnosis "BV" N = 435 n (%)	With microscopic diagnosis "NVF"** N = 103 n (%)	
G. vaginalis (Gv)	428 (98.39)	6 (5.83)	<0.00001
A. vaginae (Av)	296 (68.05)	1 (0.97)	<0.00001
Mobiluncus spp. (Msp)	74 (17.01)	0	<0.00001

* Bacterial vaginosis.
** Normal vaginal flora.

Table 2. Distribution of BV-associated pathogens among 435 symptomatic and asymptomatic women (2A) and respectively in pregnant and nonpregnant ones (2B).

BV-related pathogens	Group A*	Group B*	P-value*
G. vaginalis (Gv)	99	17	<0.00001
A. vaginae (Av)	2	4	0.612013
M. curtisii (Mc)		1	0.570438
Gv & Av	44	195	<0.00001
Gv & Mc		4	0.256975
Gv & Msp	7	11	0.162942
Gv & Av & Mc		35	0.000905
Gv & Av & Msp		16	0.023971
Total	152	279	

BV-related pathogens	Group C*	Group D*	P-value*
G. vaginalis (Gv)	60	56	0.101815
A. vaginae (Av)	3	3	0.739168
M. curtisii (Mc)	1		0.252416
Gv & Av	101	138	0.809819
Gv & Mc	1	3	0.463855
Gv & Msp	4	14	0.077322
Gv & Av & Mc	13	22	0.484592
Gv & Av & Msp	5	11	0.341983
Total	188	247	

*Group A: Asymptomatic women; Group B: Symptomatic.
*Group C: Pregnant women; Group D: Nonpregnant.
*P-values are a comparison between women with Groups A and B and with C and D.

Table 3. Distribution of BV-associated pathogens in 435 women with different clinical status.

BV-related pathogens	Group E*	Group F*	Group G*	Group H*	P-value* comparison between E and F	P-value* comparison between E and G	P-value* comparison between E and H
G. vaginalis (Gv)	74	37	5		0.000027	<0.00001	<0.00001
A. vaginae (Av)	2	3	1		0.881397	0.8823	<0.00001
M. curtisii (Mc)		1			0.382464		<0.00001
Gv & Av	46	119	37	37	0.00101	0.264777	0.782398
Gv & Mc	1	3			0.462007	0.439234	0.372418
Gv & Msp	3	4	9	2	0.979948	0.009785	0.564982
Gv & Av & Mc		2	18	15	0.217362	<0.00001	<0.00001
Gv & Av & Msp	4	1	8	3	0.101341	0.044078	0.489855
Total	130	170	78	57			

*Group E: Patients with no complications and no relapse of BV.
*Group F: Patients with recurrent BV without coinfection with TV.
*Group G: Recurrent and complicated BV with coinfection with TV
*Group H: Patients with complications of BV such as abortus imminens and premature birth.
*P-values are a comparison between women in Group E and F; E and G; and E and H.

lacking in samples with NVF. *A. vaginae* was identified in 89.61% of symptomatic cases, whereas its incidence in the asymptomatic women was 30.26% and in the healthy women, 0.97%; its incidence was therefore 3 times higher than in asymptomatic women and more than 90 times higher than in women with NVF. Interestingly, there were large differences between the prevalence of *A. vaginae* in the studied groups ($P < 0.05$). Other recent studies in Europe report *G. vaginalis* and *A. vaginae* in 96% and 87% of clinically prominent BV cases, respectively (26). Bradshaw et al. observed that 82% of Australian women with recurrent BV had both *G. vaginalis* and *A. vaginae*, while fewer had *G. vaginalis* alone (27). Ling et al. detected *A. vaginae* in 84% of Chinese women with symptomatic BV (28). In our study, both pathogens, *G. vaginalis* and *A. vaginae*, were found in about 70% of the clinically expressed BV cases and among the patients with chronic recurrent BV. *A. vaginae* has frequently been detected in symptomatic BV-positive cases, most likely because most strains of this microbial agent produce peptidyl peptidase and form ammonia, a substance very favorable for the growth of *G. vaginalis*, which contributes to the smell and irritation associated with vaginal discharge (29). Difficult-to-treat multiple-pathogen infections with *A. vaginae* and *Mobiluncus* spp. in a double or triple combination with *G. vaginalis* were detected in more than 86% of the patients with recurrent and symptomatic BV. These results support the idea of a leading role of both of these BV-associated pathogens, which are considered by many authors as essential markers of this infection (1,18,27). The coinfections were predominantly related to recurrent BV and some complications such as abortus imminens and preterm birth ($P < 0.05$). Other authors have found persistence of both *Mobiluncus* spp. and *M. curtisii* in more than 60% of BV-positive women after treatment with metronidazole (4). Our results showed that *G. vaginalis* alone was detected in 6.5% of the complicated cases and in nearly 57% of the uncomplicated cases. The bacterial loads of *G. vaginalis* and *A. vaginae* infections were higher when the 2 species were present together in vaginal samples than in cases when biofilm was formed by *G. vaginalis* alone. *A. vaginae* has been reported as part of the vaginal ecosystem together with *G. vaginalis*, but not alone (10). The high load of this synergistic bacterial combination causes a more severe infection and poses a significant risk of preterm birth (7,30).

A combination of BV with TV coinfection was found in 17.93% of the examined patients, which was more than the incidence of 13.69% reported in a previous Bulgarian study (8). In most of these samples, all 3 pathogens (*G. vaginalis*, *A. vaginae*, and *Mobiluncus* spp.) were detected, which suggests heavier anaerobic infection. A synergistic effect between protozoa and these anaerobic bacteria was demonstrated. There are data that *Mobiluncus* spp. have never been isolated in pure cultures, but only in mixed cultures with other anaerobes in vaginal samples predominantly from patients with BV or pelvic inflammatory disease, or from amniotic fluid; however, the data's clinical significance is as of yet unclear (4,9,29). An investigation in rhesus macaques revealed that most of the

tested animals carried such microorganisms, especially *M. curtisii*, in their vaginal ecosystem, which were harbored together with *Gardnerella*-like bacteria (9,30). The adherence of *Mobiluncus* spp. by polar attachment via exopolysaccharides of the glycocalyx is increased when the pH increases, e.g., at pH 7.5. After adhesion, the growth of anaerobic organisms appears in the biofilm (9). *Mobiluncus* species have varying sensitivity to metronidazole. *M. curtisii* shows a high level of resistance, which is why it is more difficult to eradicate (9,28,29). The prevalence of antimicrobial-resistant *Mobiluncus* spp. isolated from specimens collected from Turkish women is reported to have become over 80% in recent years (31). The nonsusceptibility of *A. vaginae* to metronidazole, the antibiotic commonly used for treatment of BV, is another problem that reinforces the trends for persistence of vaginal infections (27,29). Some recent data about *G. vaginalis* strains with intrinsic metronidazole resistance show other variants of recurrent BV after frontline therapy (32). Such strains are found in 80%–90% of cases of relapse after treatment with this drug (32). In some cases, 12 months after therapy with metronidazole, *A. vaginae* and *G. vaginalis* may still persist in vaginal samples (27). Treatment with clindamycin reduces the resident microflora, such as lactobacilli, that are resistant to metronidazole (>256 μg/mL) but susceptible to clindamycin (0.023–0.125 g/mL). Only nifuratel and rifaximin have shown strong in vitro activity against the resistant and problematic etiological agents *A. vaginae*, *G. vaginalis*, and *Mobiluncus* spp. without nonbeneficial effects on lactobacilli (12,33,34). New studies have presented data that demonstrate that the known antibiotic therapy alone is not a viable option for the eradication of the BV-related bacteria in biofilm; rather, combinations of antimicrobial drugs, disinfectants, and probiotics are more useful (18,35–38).

To our knowledge, this is the first study that focuses on the correlation between problematic BV-associated pathogens and the clinical status of women with BV in Bulgaria. The predominant etiological agent detected using multiplex PCR in all tested groups was *G. vaginalis*. High frequency of the key combination of *G. vaginalis* and *A. vaginae* was detected in Bulgarian women, more frequently in symptomatic patients than in asymptomatic ones. Taken together, the results from our study indicate an alarmingly high prevalence of causative agents of BV that are problematic for therapy, such as *A. vaginae* and *Mobiluncus* spp. with *G. vaginalis*, and also *T. vaginalis*, in women of childbearing age in Bulgaria. The prevalence of coinfection with 2 or 3 agents in the group of patients with recurrent BV is an acknowledgment of the virulence and the leading role of these agents in the etiology and pathogenesis of BV. Some of the identified pathogens had intrinsic metronidazole resistance. This study supports the idea that screening for such pathogens should be a very useful strategy in the choice of effective therapy as well as in the prevention of relapses and complications of BV, and should be considered in reproductive health programs. Development and evaluation of new methods, new disinfection strategies, and new ways of treatment, especially for recurrent BV infections, are needed.

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