Journal of Medical Bacteriology

Determination of the Prevalence of *Gardnerella vaginalis*, *Trichomonas vaginalis* and *Athophobium vaginalis* in Shahid Akbarabadi Hospital of Tehran with Multiplex PCR

Neda Housseini [1], Farzaneh Housseini [1], Robab Rafiei Tabatabaei [1], Ali Shivaee [2]*

[1] *Department of Microbiology, North Tehran branch, Islamic Azad University, Tehran, Iran.*

[2] *Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran.*

ARTICLE INFO	ABSTRACT
Article type: Research Article	***Background***: More than 30 bacterial, viral, and parasitic pathogens have the potential for transfusion (sexually transmitted), an infectious group called sexually transmitted infections (STIs). *Atofobium vagina*, *Gardnerella vaginalis* are one of the most common causes of the disease. *T. vaginalis* is also the most common protozoa among vaginosis causing agents. This study aimed to determine the prevalence of these factors in conjunction with the multiplex PCR method.
Article history: Received: 24 Jun 2019 Revised: 02 Aug 2019 Accepted: 27 Aug 2019 Published: 12 Sep 2019	***Methods***: In this study, among 320 women who referred to Shahid Akbarabadi Hospital in Tehran, 70 samples of people with symptoms of vaginosis who had not taken antibiotics at least one week before the visit were selected. The results of the Amsel criteria and Nugent score tests indicated only 27 cases as bacterial vaginosis. After DNA extraction from the specimens, a multiplex PCR was performed on the samples.
Keywords: *A. vaginalis, G. vaginalis, T. vaginalis, multiplex PCR.*	***Results***: The results of the multiplex PCR showed that in 27 cases, 14 cases of bacterial vaginosis only in terms of *A. vaginalis*, 21 cases were only *G. vaginalis* and 10 were positive for both bacteria and in the case of *T. vaginalis* only 1 positive sample was observed among all samples of vaginosis, which was also positive in the *A. vaginalis*. ***Conclusion***: The results of this study showed that multiplex PCR could be of great help in the diagnosis of bacterial vaginitis diseases.

*Corresponding Authors: Farzaneh Housseini, Department of Microbiology, North Tehran branch, Islamic Azad University, Tehran, Iran.

Tel: +98-21-22950723, *E-mail:* farzaneh953@yahoo.com.

Introduction

More than 30 bacterial, viral, and parasitic pathogens have transfusion potential, forming an infectious group called sexually transmitted infections (STIs). *Gardnerella* is one of the bacteria associated with bacterial vaginosis. *Gardnerella* vaginitis is the most common bacterial vaginosis in reproductive age (1).

G. vaginalis is observed in 30-50% of women without bacterial vaginosis symptoms, and in 70% of women without bacterial vaginosis has been seen by PCR. Studies show that *G. vaginalis* alone cannot be an etiologic factor in bacterial vaginosis. The epidemiology of *T. vaginalis* infection is different from other infections that cause genital secretion in women. First, age distribution is a distinct infection of *Chlamydia* or *Neisseria gonorrhoeae*. In chlamydial and gonococcal infections, the prevalence is highest in women aged 15-25 years, while *Trichomonas vaginalis* infection seems to peak at a later stage in life (between 40-50 years) (2, 3). This is the difference in the distribution of information about STI control programs and the targeting of screening efforts. The specific age distribution of infection in men has not been studied sufficiently. Regarding the clinical importance of the bacteria, the PCR method, if correctly performed and has high speed and sensitivity can detect the disease in the early stages and also can identify patients without symptoms (9).

Athophobium vaginalis is also an absolute anaerobic bacteria recently reported in connection with bacterial vaginosis (3). According to the studies of Burton, about 50% (5, 6), according to Ferris studies, about 70% (7) and more than 95% have been observed in Verhelst et al. In people with bacterial vaginosis with symptoms, this bacterium with *G. vaginalis* has been seen in the biofilm of the vaginal mucosa (8).

In bacterial vaginosis, the number of lactobacilli is severely reduced, and the number of gram-negative anaerobic bacteria and other bacteria such as *G. vaginalis*, *A. vaginalis*, *Mobilncus*, *Fusobacterium*, *Bacteroides*, and *Prevotella* are increased.

T. vaginalis is one of the most common non-viral sexually transmitted infections (STIs) worldwide. In 2008, the World Health Organization estimated global 276.4 million new cases of *T. vaginalis* globally among adults aged between 15 and 49 years old (4).

Athophobium vaginalis is also an absolute anaerobic bacteria recently reported in connection with bacterial vaginosis (3). According to the studies of Burton, about 50% (5, 6), according to Ferris studies, about 70% (7) and more than 95% have been observed in Verhelst et al.

In bacterial vaginosis, the number of lactobacilli is severely reduced, and the number of gram-negative anaerobic bacteria and other bacteria such as *G. vaginalis*, *A. vaginalis*, *Mobilncus*, *Fusobacterium*, *Bacteroides*, and *Prevotella* are increased. Regarding the clinical importance of the bacteria, the PCR method, if correctly performed and has high speed and sensitivity can detect the disease in the early stages and also can identify patients without symptoms (9).

Regarding the mentioned problems, Multiplex PCR (Multiplex Polymerase Chain Reaction) method is used today as a complementary and rapid method for the diagnosis of even a bacterium. Multiplex PCR is an essential diagnostic method that can directly detect contamination and form the basis of this research (10). This study aimed to evaluate the prevalence of *T. vaginalis*, *A. vaginalis* and *G. vaginalis* in patients with bacterial vaginosis using multiplex PCR.

Materials and Methods

Clinical samples

The study Samples included women with bacterial vaginosis symptoms who referred to Shahid Akbarabadi Hospital. In this study, among

320 women who applied to Shahid Akbarabadi Hospital in Tehran, 70 samples of people with symptoms of vaginosis who had not taken antibiotics at least one week before the visit were selected. Specimens were collected from 18 to 36 years old. From each patient, three vaginal swabs were taken, the first swab was pulled straight to direct smear, the second swab was cultured on a particular medium, and the third swab was transferred to a sterile medium containing buffered phosphate (PBS). Also, the pH bar was used to measure the vaginal pH and 10% potassium hydroxide for performing the Whiff test. Amsel criteria and Nugent score were used for diagnosis of bacterial vaginosis in the subjects (11).

DNA extraction

DNA's prepared from cultures and extraction was carried out using the kit of extracted DNA from bacteria supplied by Yekta Tajhiz Co. according to the kit instructions (YTA Genomic Extraction Mini Kit for Blood/Cultured Cell#YT9040).

Primer design

In this study we designed primers. Primer sets specific for each of the three species were designed based on the DNA sequences of the 16S rRNA. Oligonucleotide's of primer were synthesized and purified after synthesis on a DNA synthesizer (380; Applied Biosystems, Norwalk, Conn.) The sequences were as follows: for GV F 5' GCAAGCCTTTTGGGTGAGTG 3'; for GV R 5' TTTCGCTTCTCAGCGTCAGT 3'; for ATO-VAG F 5' CTTTGCAACCAACAATGACACC 3'; for ATO-VAG R 5' CAGCGCATACGGT AAGCGTAC 3'; for TV F 5' GAGTTAGGGTA TAATGTTTGATGTG 3' and for TV R 5' AGAATGTGATAGCGAAATGGG 3'. The sizes of the PCR products predicted with these primers were 303 bp with primer set GV F/R, 218 bp with primer set ATO-VAG F/R, and 337 bp with primer set TV F/R. For checking the specificity of the primer design, the targeted DNA sequence was aligned with the 16S rRNA sequences of other microorganisms present in vaginal samples. To ensure the accuracy of the results, positive and negative controls were also used in this study.

Table 1. Frequency of patients with age-related bacterial vaginosis in *A. vaginalis, T. vaginalis* and *G. vaginalis*.

age	Nom	*A. vaginalis*	*G. vaginalis*	*T. vaginalis*
18-23	14	8 (%57.1)	11 (%52.4)	0 (%0)
23-30	8	4 (%28.6)	6 (%28.6)	1 (%100)
30-36	5	2 (%14.3)	4 (%19)	0 (%0)
Total	**27**	**14**	**21**	**1**

Multiplex PCR

Multiplex PCR was performed with 2- µl culture DNA preparations. The three primer sets, GV F/R, ATO-VAG F/R and TV F/R were combined for the simultaneous detection and identification of the species. The optimal concentration of primers was 0.5 pmol. The contents of the PCR tube include: PCR Master Mix (2x):12 µl, Primer Mix: 3 µl (GV: 0.5F 0.5R, ATO-VAG: 0.5 F 0.5 R, TV: 0.5 F 0.5 R), DNA: 2 µl and Water: 8 µl (Total Volume: 25 µl). Cycling times were 5 m at 94 °C (to initial denaturation: activate a small fraction of the heat-activated DNA polymerase), followed by 30 cycles of denaturation at 94 °C for 45 s, annealing beginning at 60 °C for 45 s, extension at 72°C for 45 s, and Final Extension at 72 °C for 1 min.

PCR products (20 ml) were shown by electrophoresis in 1% agarose gels at 100 v for 30 min with Tris-borate-EDTA buffer (pH 8.3) and visualized with DNA Self Stain (5 µl). The gel was then ejected under the Ultraviolet Transilluminator device, which emits 254 nm UV rays and the presence or absence of a PCR product with positive control and marker was examined.

Statistical analysis

The collected data were analyzed by SPSS software to determine the mean difference between groups. Crosstabs and Chi-Square tests were performed to evaluate the qualitative variables and to compare the percentage of variables by SPSS-16 software.

Result

Among 320 women referred to Shahid Akbarabadi Hospital in Tehran, 70 samples of people with symptoms of vaginosis who had not taken antibiotics at least one week before the visit were selected. The results of Amsel criteria and the Nugent score showed that only 27 cases were introduced as bacterial vaginosis.

The results of Multiplex PCR in this study showed that out of 27 cases of bacterial vaginosis, 14 cases (51.8%) were only for *A. vaginalis*, 21 cases (78%) for G.vaginalis and 10 cases (37%) for both bacteria were positive, and only 1 positive sample (3.8%) was found in the case of *T. vaginalis*, among all isolated samples. In this sample, the *A. vaginalis* was also vaginal.

Also, the prevalence of G.vaginalis and *A. vaginalis* in 10 cases (37%) and prevalence of *A. vaginalis* and *T. vaginalis* in 1 case (3.8%) were observed. The prevalence of *G. vaginalis* and *T. vaginalis* was not found in any of the samples.

The results showed that the frequency of vaginosis in the age range of 18-23 years old was the most among all, and in the age range of 30-36 years old was the least among all. Also, in patients between the ages of 18-23, the frequency of *G. vaginalis* was the most among all and *T. vaginalis* wasn't reported.

In patients between the ages of 23- 30, the frequency of *Gardnerella vaginalis* was the most and *T. vaginalis* was the least. In patients aged 30-36 years, the rate of *Gardnerella vaginalis* was the most and *T. vaginalis* wasn't observed. Further details are given in Table 1.

Discussion

This study is the first study in conjunction with *G. vaginalis*, *T. vaginalis* and *A. vaginalis* in patients with bacterial vaginosis symptoms in Iran. The results of Multiplex PCR in this study showed that out of 27 cases of bacterial vaginosis, 14 cases were only for *A. vaginalis*, 21 were just for *G. vaginalis*, and 10 for both bacteria were positive, and for *T. vaginalis* only 1 sample Positive results were observed among all isolated samples, in which the *A. vaginalis* was also positive. Several studies have reported synergism between these two bacteria and *T. vaginalis*. Recent studies have shown that biofilm formation in epithelial cells in women with bacterial vaginosis accounts for more than 90% of the *A. vaginalis* and *G. vaginalis*, and these two bacteria are associated with biofilm formation (12).

The prevalence of G. vaginalis in various articles was reported from 68-100%. Also, the prevalence of *A. vaginalis* of bacterial vaginosis samples is 78-80%, and in a healthy person, it has been said below 20% (13, 14). This study showed that *A. vaginalis* is 51% and *G. vaginalis* is 78%, and the prevalence of these two agents is 37%, the percentages obtained are within the stated range of the above articles. One of the reasons for this difference can be a difference in health

behavior. It is noteworthy that, according to studies by Datcu et al., *A. vaginalis* is more specific in developing bacterial vaginosis than *G. vaginalis*, and according to these studies, Datcu reported the prevalence of *G. vaginalis* in women with bacterial vaginosis 100% (13).

33 cases of bladder biopsy were taken from cystitis in 2001 by Agarwal in the diagnosis of *G. vaginalis* DNA in cystitis; biopsy specimens such as urine samples were used that may have been infected with normal flora of the vagina to be DNA extraction was done, and PCR was performed using specific primers for *G. vaginalis*. In patients with cystitis, no positive cases of *G. vaginalis* were isolated (15). In a study Eykyn and Macfadyen in Australia, in the urine of 15.9%, and Mc Dowall et al. study 18% of healthy pregnant women who developed suprapubic aspiration were able to separate the *G. vaginalis* (16, 17). Cultivation methods due in no small amount of bacterial diversity present in the vaginal environment, there can be different sensitivities in the diagnosis of this agent, which could be one reason for the differences in the results of this study.

In this study, we sued three methods (Gram stain, culture, and PCR) for the diagnosis of *G. vaginalis*. In microscopy and Gram stain, 57% of bacterial vaginosis in women with symptoms and 14% of women without symptoms was observed. It has been concluded that microscopic and PCR methods are an excellent way to detect *G. vaginalis* associated with bacterial vaginosis. In a study by Kathelin et al. in 2014 to the epidemiology of infections transmitted sexually using DNA was extracted from 100 samples of vaginal swabs in 48 cases (48%) vaginalis was positive, the study almost matches and in our study, 78% of *G. vaginalis* was found (18).

A. vaginalis is observed in 50-78% of women with bacterial vaginosis (13, 14, 18), which is consistent with the current study. In a study done by Marrazzo by PCR method (19), out of 73 women samples, 27 had bacterial vaginosis, and

46 were healthy. Women with bacterial vaginosis had clinical symptoms. The sensitivity and specificity of *A. vaginalis* in 96% and 80% bacterial vaginosis reported that our findings are close to this range (20, 21).

It is difficult to detect vaginal atopyobia from vaginal fluid in healthy women, but in patients with bacterial vaginosis, according to Burton's studies, about 50%, according to Frreis studies, is estimated to be about 70% and more than 95%, according to Verhelst's (6). A survey by Cartwright et al. in 2012 from 402 vaginal Swab specimens using qPCR method found 63.9% bacterial vaginosis. 98.1% of *G. vaginalis* was isolated from bacterial vaginosis, and 98.2% of the samples were reported positive for *A. vaginalis* (22).

In a study conducted by Burton et al. in 2005 about the examination of *A. vaginalis* in women referring to the women's clinic, 55 women were taken and analyzed by PCR for the presence of *G. vaginalis*, *Bacteroides* and *Mobilncus* they got. In 40% of the cases, *G. vaginalis* and *A. vaginalis* were seen, and 50% of the *A. vaginalis* was positive (6). The prevalence of *T. vaginalis* has been reported below 20%, and the results of this study have also been published in this range (6).

In the diagnosis of this factor, different diagnostic methods have different sensitivities and specificities. In a study by Ashshi et al (2015) on isolated samples of people with STIs, it can be shown that from 135 samples isolated from symptom women, Multiplex Real-Time PCR can be performed with Taqman with high sensitivity and specificity in the diagnosis of a factor, including *T. vaginalis* (23).

Also, in a study by Piperaki et al. (2015), comparing two methods of culture and PCR on isolated samples of women with symptomatic urinary tract infections, and asymptomatic women showed that vaginalis *T. vaginalis* showed that the method PCR could provide 100% sensitivity to more accurate results than a 69% sensitivity culture method (24).

In a study done by Madeline et al. in 4646 women with bacterial vaginosis, it shows that about 3.1% of people with *T. vaginalis* are infected. The result of this study is close to our study results (25). A survey by Bruni et al, (2017) on *T. vaginalis* showed that the incidence of *T. vaginalis* in 499 cases of women with bacterial vaginitis was 2.4% by culture and 1.2% by color observation. Also, the results of molecular experiments showed an incidence of 4.2% for this parasite among the samples. This indicates high sensitivity and specificity for molecular tests to detect these protozoa. The results obtained in this study are in line with the results of Bruni studies, and the extent of the difference can be due to the number of patients and the choice of patients when entering the study (26).

In this study, all patients with bacterial vaginosis at the age of fertility were between 18-36 years old. The majority of patients with an average age ranged from 18-23 years old, which could be since more samples were collected from this age range.

As mentioned above, there are contradictory reports on the results of various studies concerning bacterial vaginosis of *G. vaginalis* and *A. vaginalis*. However, the cause of these contradictions can be due to multiple factors such as: the selection of patients, small sample size, the use of different diagnostic methods, differences in the geographical distribution of populations, the presence of interventional factors such as social, economic, sexual activity, etc., the rate and the intensity of bacterial colonization in the genitourinary tract and the involvement of genetic factors.

Conclusion

According to the result of this study, a relatively high percentage of women with bacterial vaginosis have been infected with *G. vaginalis* and *A. vaginalis*. Considering that genital infections caused by these bacteria in the

absence of diagnosis and treatment continue to persist and can lead to dangerous consequences, such as pelvic inflammatory infections and infertility, therefore, women with clinical signs with rapid and accurate methods such as Multiplex PCR may be necessary.

Ethics approval and consent to participate

Not needed.

Conflict of interest

All contributing authors declare no conflicts of interest.

References

1. Catlin BW. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clin Micro Rev* 1992; **5**(3):213-37.
2. Gergova RT, Strateva TV, Mitov IG. *Gardnerella vaginalis*-associated bacterial vaginosis in Bulgarian women. *The Braz J Infect Dis* 2013; **17**(3):313-8.
3. Polatti F. Bacterial vaginosis, *Atopobium vaginae* and nifuratel. *Curr Clin Pharmacol* 2012; **7**(1):36-40.
4. Greenwood J, Pickett M. Transfer of *Haemophilus vaginalis* Gardner and Dukes to a New Genus, *Gardnerella*: *G. vaginalis*

(Gardner and Dukes) comb. nov. *Int J Syst Evol Microbiol* 1980; **30**(1):170-8.

5. Burton JP, Devillard E, Cadieux PA, et al. Detection of *Atopobium vaginae* in postmenopausal women by cultivation-independent methods warrants further investigation. *J Clin Microbiol* 2004; **42**(4):1829-31.

6. Burton JP, Chilcott CN, Mohammed AQ, et al. A preliminary survey of *Atopobium vaginae* in women attending the Dunedin gynaecology out-patients clinic: Is the contribution of the hard-to-culture microbiota overlooked in gynaecological disorders? *Aust N Z J Obstet Gynaecol* 2005; **45**(5):450-2.

7. Ferris MJ, Masztal A, Martin DH. Use of species-directed 16S rRNA gene PCR primers for detection of *Atopobium vaginae* in patients with bacterial vaginosis. *J Clin Microbiol* 2004; **42**(12):5892-4.

8. Verhelst R, Verstraelen H, Claeys G, et al. Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae*, *Gardnerella vaginalis* and bacterial vaginosis. *BMC Microbiol* 2004; **4**(1):16.

9. Den Boer J, Yzerman E. Diagnosis of Legionella infection in Legionnaires' disease. *Eur J Clin Microbiol Infect Dis* 2004; **23**(12):871-8.

10. McDonough EA, Barrozo CP, Russell KL. A multiplex PCR for detection of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis* in clinical specimens. *Mol Cell Probes* 2005; **19**:314-22.

11. Goodfellow M, Kämpfer P, Busse HJ, et al. Bergey's manual of systematic bacteriology: The Actinobacteria: Springer; 2012.

12. Datcu R. Characterization of the vaginal microflora in health and disease. *Dan Med J* 2014; **61**(4):B4830.

13. Trost E, Götker S, Schneider J, et al. Complete genome sequence and lifestyle of black-pigmented *Corynebacterium aurimucosum* ATCC 700975 (formerly *C. nigricans* CN-1) isolated from a vaginal swab of a woman with spontaneous abortion. *BMC genomics* 2010; **11**(1):91.

14. Agarwal M, Dixon R. A study to detect *Gardnerella vaginalis* DNA in interstitial cystitis. *BJU international* 2001; **88**(9):868-70.

15. Macfadyen D, Heffernan JF. Urine testing for isoniazid in the supervision of out-patient oral chemotherapy for pulmonary tuberculosis: The failure of a routine service. *Bull World Health Organ* 1967; **36**(5):847.

16. McDowall T, Jackling B, Natoli R. Are we there yet? Changing perceptions of accounting as a career preference. *Int J Learn* 2012; **18**(4):335-52.

17. Jobe KA, Downey RF, Hammar D, et al. Epidemiology of sexually transmitted infections in rural southwestern Haiti: the Grand'Anse Women's Health Study. *Am J Trop Med Hyg* 2014; **91**(5):881-6.

18. Demirezen S. Bacterial vaginitis: general overview. *Mikrobiyoloji bulteni* 2003; **37**(1):99-104.

19. Downey RF, Hammar D, Jobe KA, et al. Epidemiology of sexually transmitted infections in rural Haitian men. *Int J STD & AIDS* 2015; **26**(10):710-5.

20. Marrazzo JM. Interpreting the epidemiology and natural history of bacterial vaginosis: are we still confused? *Anaerobe* 2011; **17**(4):186-90.

21. Cartwright C, Bryndon D, Lembke K. Diagnosis of bacterial vaginosis, semiquantitative, multitarget PCR assay. *J Clin Microbiol* 2012; **50**(7):2321.

22. Ashshi AM, Batwa SA, Kutbi SY, et al. Prevalence of 7 sexually transmitted organisms by multiplex real-time PCR in Fallopian tube specimens collected from

Saudi women with and without ectopic pregnancy. *BMC Infect Dis* 2015; **15**(1):569.

23. Copeland A, Sikorski J, Lapidus A, Nolan M, Del Rio TG, Lucas S, et al. Erratum to: Complete genome sequence of *Atopobium parvulum* type strain (IPP 1246 T). *Stand Genomic Sci* 2010; **2**(3):361.

24. Sutton M, Sternberg M, Koumans EH, et al. The prevalence of *Trichomonas vaginalis* infection among reproductive-age women in the United States, 2001–2004. *Clin Infect Dis* 2007; **45**(10):1319-26.

25. Bruni M, Silveira M, Stauffert D, et al. LB1. 61 Prevalence of *Trichomonas vaginalis* among women attending a university clinic in Pelotas, Brazil using microscopy, culture and the aptima tv assay. BMJ Publishing Group Ltd; 2017.