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Isolation of *Atopobium vaginae* in Vaginal and Urine Samples of Iranian Women, the first report

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Abstract

Bacterial vaginosis is not a mono-factorial infection. A synergism of microaerophilic bacteria, *Mycoplasma* spp., and anaerobic bacteria such as *Atopobium vaginae*, *Porphyromonas* spp., *Bacteroides* spp., *Prevotella* spp., and others are involved in these infections. The aim of present study was to determine the prevalence of *Atopobium vaginae* in non-pregnant women suffering from bacterial vaginosis.A total of 102 non-pregnant women who referred to Shahid Akbarabadi hospital in Tehran were tested for bacterial vaginosis. Bacterial culture was performed on Columbia agar containing 2mg/ml Amphotericin B, 30mg/ml Nalidixic acid and 4mg/ml Colistin. Additionally, they were simultaneously cultured on blood agar plates containing fresh human blood and Amphotericin B under anaerobic conditions. Finally, PCR using *Atopobiumvaginae* specific primers were also carried out on extracted DNA from grown colonies as well as from vaginal specimens. From a total of 102 symptomatic women who referred to the hospital, 37% (38/102) were classified as bacterial vaginosis, 43% (44/102) were classified as intermediate status and 20% (20/102) women were asymptomatic. Sixty urine samples were collected. *Atopobium vaginae* were detected in 66% (25/38) of bacterial vaginosis cases and in 10% (4/44) samples with intermediate vaginal flora. It was not detected in asymptomatic women. The bacterium was seen in 10% (3/30) women with urinary tract infection and none in pregnant women.This is the first report of *Atopobiumvaginae* isolation in Iran. The results of this investigation point to a clear association of*Atopobiumvaginae*with bacterial vaginosis. It should therefore be considered as a probable etiological agent.

Keywords: Bacterial vaginosis, Atopobium vaginae, Culture, PCR

INTRODUCTION

Bacterial vaginosis (BV) is considered to be the most common cause of vaginal disorder and imbalance affecting women during their reproductive age[1,2,3,4]. This disorder is strongly associated with alteration in vaginal microflora when hydrogen peroxide-producing Lactobacilli are decreased and replaced by bacteria such as Atopobium, Prevotella spp., Mobillincus spp., Gardnerellavaginalis and Bacteroides spp.[4,5,6].The most common bacteria isolated from BV is Gardnerellavaginalis[6]that grows under appropriate microaerophilic condition and the anaerobic bacterium Atopobiumvaginae. Despite the first work of Leopoldo in 1953 and Gardner and Dukes in 1955 [7], not a singleinfectious agent has been associated as the sole causative agent BV. Ethiopathogenesis of BV is still not fully elucidated [4,7,8].

Bacterial vaginosis is associated with the presence of a dense and lower case forPolymicrobial biofilm, mainly populated by Gardnerella vaginalis with strong adhesion to vaginal epithelium. Bacterial vaginosis is characterized by elevation of vaginal pH and clinical signs such as foamy vaginal discharge, presence of clue cells in wet mount, and fishy odor (positive KOH amine test) [9,10,11]. Based on Amsel criteria, the presence of three of these signs is considered to be a strong indication of BV. However, some patients without BV may reveal similar clinical findings, such as those with Trichomonasvaginalis[10,11,12]. Bacterial vaginosis has also been associated with a long list of complications; such as pelvic inflammatory disease, spontaneous abortion, preterm birth and low-birth-weight infants[1,2,3,4,13]. This study was undertaken to detect the extent of Atopobiumvaginaeassociated BV in nonpregnant women from different age groups who referred toa large general hospital in the Iranian capital, Tehran. The existence of this bacterium in the urine samples of pregnant and nonpregnant women was also investigated.

METHODS

Patients and clinical samples

From November 2014 till October 2015, vaginal samples from 102 non-pregnant women with clear clinical symptoms women of reproductive age were collected. No women received antimicrobial therapy for at least a week before prior to sample collection. Urine samples were also collected from 30 non-pregnant women with urinary tract infection as well as from 30 healthy pregnant women.

Gram stain, Whiff test and pH Measurement

Vaginal dischargewas collected in the following order by a gynecologist: vaginal swabs for direct PCR, which were placed in PBS. Another vaginal swab for Gram stain and observation of clue cells, and another sample for determination of vaginal pH and application of 10% KOH for detection of amine odor (Whiff test).

Diagnosis of BV

The Amsel criteria were used for diagnosis of BV. Samples were classified as BV if three or four of the Amsel criteria were present. The samples were categorized as healthy if less than three of these criteria were seen.

Culture

Atopobiumvaginae is an anaerobic facultative bacterium, which was grown on Columbia blood agar supplemented with 2mg/ml Amphotericin B, 30mg/ml Nalidixic acid and 4mg/ml Colistin in an anaerobic chamber (Gas Pack) at 37˚C for 48-72 hours. The grown colonies were

grey-white in color and non-hemolytic. Additionally, they were simultaneously cultured on blood agar plates containing fresh human blood and 2mg/ml Amphotericin B under anaerobic conditions. Upon staining, Atopobiumvaginae appear as Gram positive, small cocci, and arranged in single cells, in pairs or short chains.

DNA isolation

Total DNA from samples, either grown bacterial colonies or vaginal swabs placed in PBS, were isolated using the YTA Genomic Extraction Mini Kit #YT9040 (Yakta Tajhiz Azma, Tehran, Iran) according to the manufactures guidelines.

Polymerase chain reaction (PCR) assay

A species-specific PCR assay for the detection of Atopobiumvaginae targeting the 16S rRNA gene was arranged [8]. The oligonucleotides used as primers for amplification were AV1-F (5′-TAGGTCAGGAGTTAAATCTG-3′) and AV3-R (5′-TCATGGCCCAGAAGACCGCC-3′) [8]. They were verified for specificity using the BLAST program. The PCR reaction was carried out in a total of volume of 25.0 µl, and the final concentration of the mix for each sample contained: 10 µl Master Mix (containing Taq DNA polymerase, dNTPs, and PCR buffer), 1 µl primer for both primers, 10 µl deionized water, and 4µl of the template DNA.

The amplification protocol was as follow; Initial denaturation at 95˚C for 4 minutes followed by 25 cycles of 95˚C, 55˚C and 72˚C for 1 Minute each, with a final extension at 72˚C for 7 minutes. Following addition of DNA Safe StainTM, the PCR product was electro phrased in 1% agarose gel for 1 hour at 90 V and detected by UV trans illumination (wavelength 254 nm).

Statistical analysis

Data was statistically analyzed for association significance ofAtopobiumvaginaewith bacterial vaginosis and Amsel criteria for diagnosing bacterial vaginosis using spss program. Analysis was performed on MS Office Excel and P value <0.05 was taken as significant.

RESULTS

A total of 102 vaginal swabs from women with symptoms of abnormal vaginal odor or discharge, and also 30 urine samples from women with urinary tract infection and 30 urine samples from healthy pregnant women were registered in this cross-sectional study. All subjects were within 18-35 years of age. In the 102 vaginal samples, 37% (38/102) were found as indicative of BV based on the Amsel criteria [14,15,16,17]. Forty three percent (44/102) were detected as "intermediate vaginal flora". Twenty of the asymptomatic patients had only one or two out of the four clinical criteria and no Atopobiumvaginaewas found, neither by culture or PCR method. Ten percent (4/44) of theAtopobiumvaginae were isolated from patients with "intermediate normal flora". The PCR product was subsequently sequenced and following analysis of the sequence by chromas Program, it was confirmed to be related to Atopobiumvaginae as shown in Fig.1.

Figure 1. Sequence confirm for *Atopobium vaginae*

Of the clinical criteria for bacterial vaginosis, homogenous discharge was present in 31 (82%) while a pHof ≥ 4.5 was found in 34 (89%) cases of bacterial vaginosis. Amine test was positive in all 38 (100%) cases. Clue cells were present in 27 (71%) cases of bacterial vaginosis on wet mount (Table 1).

Table 1. Bacterial vgainosis based on Nugent score and prevalence of *Atopobium vaginae*.

Finding	Nogent Score			P value
	0-3 (n=20)	4-6 (n=44)	7-10 (n=38)	
Homogenous vaginal discharge	0 (0)	29 (65)	31 (81)	0/0009
Vaginal pH>4.5	3 (15)	29 (65)	32 (84)	0/03
Amine positive	0 (0)	24 (54)	38 (100)	0/0001
Clue cells	1 (5)	7 (15)	27 (71)	0/003
Prevalence of *Atopobium vaginae*	0(0)	4 (10)	25 (65)	0/0007

Out of the 38 total BV cases, 66% (25/38) Atopobiumvaginaewere isolated.However, none was isolated from theasymptomatic cases. From the 30 urine samples from women with urinary tract infection, 10% (3/30) Atopobiumvaginaewere isolated. None were isolated from the urine samples of healthy pregnant women. The picture of electrophoresed agarose gel showing the PCR protocol performed directly on the vaginal discharges was shown in Figure 2.

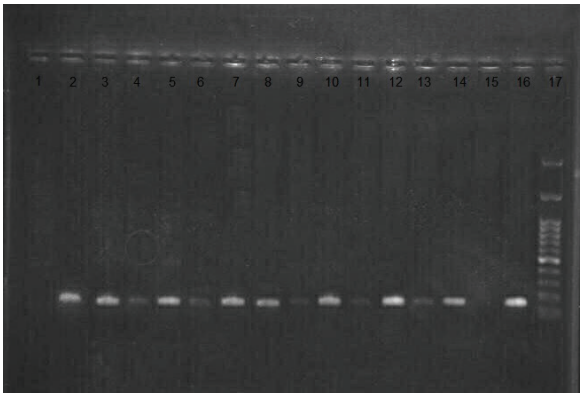

Figure 2. Species-specific PCR assay for the detection of *Atopobium vaginae* in vaginal and urine samples (agarose gel-electroforesis of PCR products of the 16S rRNA gene). The product size is 155 bp. From left to right: 1kb Ladder, control positive, control negative and positive samples.

DISCUSSION

Bacterial vaginosis is a risk factor for obstetric infections and is a cause of mal odorous vaginal secretions. It is linked to various adverse outcomes in women and causes significant gynecological and obstetric morbidity such as preterm birth, sexually transmitted disease, infections following gynecological surgery and pelvic inflammatory disease (PID) [1,3,4].Vaginal lactobacilli can prevent colonization by and growth of other potentially pathogenic bacteria; thereby, reducing the risk of developingBV.

Progression of a reasonable tool for identification of BV is crucial. There is not any standard, credible and objectiveassay for rapid diagnosis of BV. The Amsel criteria and the Nogent score are the two presently attainable diagnostic procedures and are often used in most studies. Most studies which were based on Nugent score, expressed some limitations. It must be performed on a fresh swab, and any postponement in transporting the swab makes the test difficult to perform. It is rarely used by physicians due to the time it takes to read the slides and the need for a skilled technician for interpretation. Additionally, pathogens associated with BV are not identified by such a techniqueas is the case for *Atopobium vaginae* [18]. Recent studies have shown that molecular amplification techniques have both higher sensitivity and specificity for the diagnosis of BV[18,19,20,21] compared to the Nugent score.A few studies examined the association between BV and vaginal bacterial species by molecular methods[22,23,24,25]. The results of this investigation are in agreement with previous molecular approaches that reported a high load of *Atopobium vaginae* in most patients with BV.

It is estimated that 20-30 % of women in reproductive age who refer to sexually transmitted disease (STD) clinics suffer from BV; however, its prevalence can be as high as 50-60 % in high-risk populations[4,9,10].Prevalence of bacterial vaginosis in this study was around 37 %. The prevalence rates of BV were reported to be 31% by Abbai et al in 2015 [11], 39% by Bradshaw et al in 2005 [12], 40% by Mitchellet al in 2012 [13], and 38% by Vicky et al in 2014 [14]. Cross-sectional studies have reported that Atopobiumvaginae was present in 0-20% of women with normal vaginal flora and in 50-78% of women with BV [15,16]. In this investigation, the isolation rate of Atopobiumvaginaein women with BV was 66% (25/38). Fredricks and Marrazo reported the sensitivity and specificity of Atopobiumvaginae for BV to be 96% and 80%, respectively [17]. This bacterium was detected by PCR in 96% of patients with BV [26]. Our data also clearly confirm the involvement of Atopobiumvaginae in BV.This bacterium is aGram-positive anaerobic microbe which cannot be easily isolated by classical microbiological methods. It is hardly detected in healthy women vaginal fluid but is commonly found in patients with BV.This bacterium was isolated with different frequencies such as 50% according to Burton et al[27], 70% according to Ferris et al [28], and more than 95% according to Verhelst et al. and Verstraelen et al [16].

It seems as though, measurement ofAtopobiumvaginae bacterial loadis a good predictor for BV. However, Bradshaw et al[12]claimed thatthe shear detection of Atopobiumvaginaecan be more forecasting of BV than the bacterial load. This difference between our study and that of Bradshaw et al. may be linked to differences in epidemiological characteristics and/or PCR assays. Geographical and/or ethnic origin, pregnancy status, risks of STDs, and prevalence of BV in the population studied by Bradshaw et al. [12]were shown to influence the rate of Atopobiumvaginae in the vaginal flora. Additionally, PCR technical parameters such as DNA target, directly influence the sensitivity of PCR assays. The 16S rRNA target used in this study for Atopobiumvaginae (length, 155 base pairs) was shorter than that used by Bradshaw et al (length, 430 base pairs).It can be hypothesized that our molecular tool is more sensitive.

In another study, Burton et al. [27] used a different set of Atopobiumvaginaespecific PCR primers and detected it in 50% of Canadian BV patients. Probably the most meaningful observation in the present study and that of Burton et al. [27]was that noAtopobiumvaginae was detected in the asymptomatic subjects. The prevalence of Atopobiumvaginaereported by various workers varies from 50-78%. The difference may be due to the fact that different authors have studied different types of population and have regarded different criteria for selecting the cases of BV [25].

This is the first report for detection ofAtopobiumvaginae associated BV in Iranian women. Although PCR seems to be the most sensitive method for detection of Atopobiumvaginae. Gram staining can complement the PCR results. The high prevalence of these bacteria reported in this study in Iranian young women can be very concerning, since BV raise women susceptibility to HPV, HIV and other important sexually transmitted diseases. For this, BV has to be properly and urgently diagnosed in order to be sufficiently treated.

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