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A study on the frequency of vaginal species of *Mycoplasma genitalium*, *Gardnerella vaginalis* and *Neisseria gonorrhoeae* among pregnant women by PCR technique

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ABSTRACT

Bacterial vaginosis or non-specific vaginitis describes the disease caused by a change in the normal Flora of the vagina, which leads to the elimination of Lactobacilli, generating hydrogen peroxide and excess growth of bacteria, particularly anaerobic bacteria. This disease is the most prevalent infection of the female genital tract, and the rate of frequency of anaerobic bacteria, specifically vaginal species of *Gardnerella* and *Mycoplasma*, is 100 to 1,000 times higher than that of healthy individuals. To determine the rate of frequency of *Gardnerella vaginalis*, *Mycoplasma genitalium* and *Neisseria gonorrhoea*e, which are present in bacterial vaginosis. samples of vaginal secretions of pregnant women referred to the Women's Clinic in the Tonekabon Township were obtained. In order to detect the presence of *Gardnerella vaginalis*, *Mycoplasma genitalium* and *Neisseria gonorrhoea*e, the samples were studied using the Polymerase Chain Reaction (PCR) method. After obtaining the data, the results were analysed using the Chi-square (x2) test. Of the 44 samples tested, 3 cases were found to contain *Gardnerella vaginalis* (6.81 percent), 2 cases to contain *Neisseria gonorrhoea*e (4.54 percent), and 10 cases to contain *Mycoplasma genitalium* (22.72 percent). Statistical analysis showed that *Mycoplasma genitalium* was significantly related to the consequence of abortion. However, there was no relationship between infections caused by *Gardernerella vaginalis*, *Mycoplasma genitalium* and *Neisseria gonorrhoea*e with premature delivery and hospitalization of the newborn in the Neonatal Intensive Care Unit (NICU). Considering the findings, it seems that a low percentage of the studied populations were afflicted by the bacterial vaginosis.

1. Introduction

Bacterial vaginosis consists of a change in the normal flora of the vagina, which is accompanied by a reduction of Lactobacilli, particularly strains generating H2O2 and their replacement with a large number of bacteria including *Gardnerella*, *Mycoplasma*, *Neisseria*, anaerobic gram-negative bacilli and peptostreptococcus spp. and Mobiluncus spp. (Brook et al., 2003).

Replacement of *Gardnerella vaginalis*, *Mobiluncus* and genital *Mycoplasma* with lactobacilli are the preeminent attributes of bacterial

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vaginosis (Ryan et al., 1999). Prevalence of bacterial vaginosis in the women included in this study is in the range from 10 to 25 percent. Pregnant women afflicted by the bacterial vaginosis are exposed to an increased risk of premature rupture of embryonic membranes, premature delivery, chorioamnionitis, endometritis after giving birth by caesarean section and spontaneous abortion, with the danger of premature delivery increasing as much as 5 times (Donders et al., 2000; Hay et al., 1994; Kurki et al., 1992). Based on this, screening tests are suggested for pregnant women at stages of 24 to 29 weeks of pregnancy (Gibbs et al., 2008). Causes of this vaginosis are unknown. But, it is assumed that vaginosis occurs due to repeated alkalization of the vagina (vaginal shower). In this state, reconstruction of Lactobacilli is difficult (Cunningham et al., 2010). *Gardnerella vaginalis* is available naturally in the genital system of women. But, it may grow in certain conditions and lead to the occurrence of disease. From a serological viewpoint, this bacterium differs from natural organisms of the women's genital urinary urethras and also, from organisms of the generating agent of vaginosis. Secretions of the vagina infected with these bacteria often smell of fish and a few other anaerobic bacteria, in addition to *Gardnerella vaginalis*, are also found in the vagina. In this state, the vagina's pH is less than 4.5 (Brook et al., 2003).

*Neisseria gonorrhoea*e (Gonococcus), the causative factor of gonorrhoeae, also creates urethritis in women. Gonococcus attacks mucous membranes of the urogenital duct, eyes, anus, and throat and develops acute vaginal secretions which may be led to attack the tissue. Initial infection in women occurs in the mucous membrane of the uterus where Gonococcus reaches the urethra and leads to the vaginal secretions in the mucosa. After initial infection, pelvitis, fibrosis and occlusion of the uterine duct occur. About 20% of the women afflicted by intra-uterine infections resulting from this bacterium often become infertile. The chronic inflammation of the cervix or inflammation of gonococcal rectum is often asymptomatic (Brook et al., 2003).

Mycoplasma genitalium is related to vaginosis, endometritis, Pelvic Inflammation Disease (PID), pyelonephritis, prostatitis, delivery fever and fever after abortion, repeated spontaneous abortion, newborn birth with low weight and newborn meningitis (Jensen et al., 2004; Manhart et al., 2003). Diagnosis of these microorganisms was accomplished through culture. However, their isolation using this technique was met with some difficulties, including the length of time of isolation which doesn't allow for a fast and simple diagnosis of genital infections (Lind et al., 1984). Of effective methods of diagnosis, Polymerase Chain Reaction (PCR) was used. This method is fast and specific and able to differentiate among various species of the studied bacteria. However, the purpose of this study has been to survey the rate of infection of pregnant women referred to hospitals of Tonekabon Township compared to *Gardnerella vaginalis*, *Mycoplasma genitalium* and *Neisseria gonorrhoeae*.

2. Matherials and Methods

2.1. Study method

This study comprised 44 pregnant women referred to one of the hospitals of Tonekabon, Mazandaran where vaginal sampling was performed using a sterile cotton swab by a gynecologist. Samples were placed in a tube containing the phosphate buffer saline and transferred, within ice, to the microbiology laboratory of the Islamic Azad University of Tonekabon Branch, and kept in a freezer until the process of DNA extraction could be performed.

2.2 DNA extraction

1 µl of the tested samples was entered into 1.5 ml microtubes and centrifuged in 15,000 rpm for 30 minutes. Lysis buffer and proteinase K were then added to these microtubes and incubated in a 55^{o}C warm water bath. Then the phenol-chloroform method was used to extract the bacteria's DNA (Vatani et al., 2005).

2.3. Polymerase Chain Reaction (PCR)

After extracting the DNA, PCR was carried out in order to identify the specific genetic fragments of the desired bacteria. PCR master mix for each sample included 10 µl of master mix, 10 µl of mineral oil and 5 µl of extracted DNA. Timing and thermal programming of PCR was carried out on the basis of the order observed in Table 1.

Table 1. Steps, time span and temperatures used in the polymerase chain reaction (PCR)

Step	Time/cycles	Temperature
Initial Denaturation	90 sec	94°C
Denaturation	50 sec	94°C
Annealing	50 sec	64°C
Extension	50 sec	72°C
Final Extension	2min	72°C

2.4. Agarose gel Electrophoresis:

On completion of the final extension, 1.5% of agarose gel was used to study the gene products. 1 µg/ml of Ethidium bromide was used to sight the bands. The gel was then studied under a transiluminator apparatus.

2.5. Statistical analysis

The obtained data was analyzed by SPSS software (18) and Chi-square (x^2) test with the rate of P less than 0.05 considered to be significant.

3. Result

Out of 44 samples tested, 3 cases of *Gardnerella vaginalis* (6.81%), 2 cases of *Neisseria gonorrhoeae* (4.54%), and 10 cases of *Mycoplasma genitalium* (22.72%) were reported to be positive. Figures 1 to 3 suggest the identification of gene fragments resulting from these bacteria.

The number of cases of affliction of the infections studied in different age groups was determined, with the results shown in the Table 2.

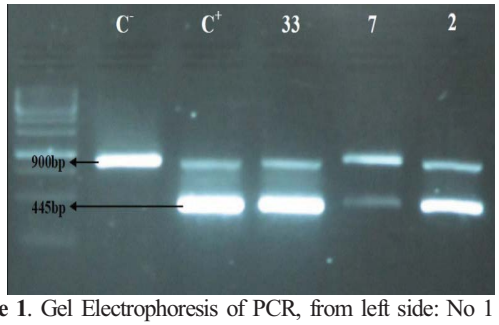

Figure 1. Gel Electrophoresis of PCR, from left side: No 1 of 1Kbp size marker, No 2 of negative control, No 3 of positive control of 445 bp fragment, No 4, 5 and 6 are positive samples of patients infected by *Gardnerella vaginalis*.

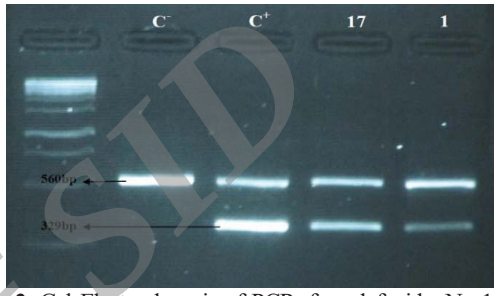

Figure 2. Gel Electrophoresis of PCR, from left side: No 1 of 1Kbp size marker, No 2 of negative control, No 3 of positive control of 329 bp fragment, No 4 and 5 are positive samples of patients afflicted with *Neisseria gonorrhoeae*.

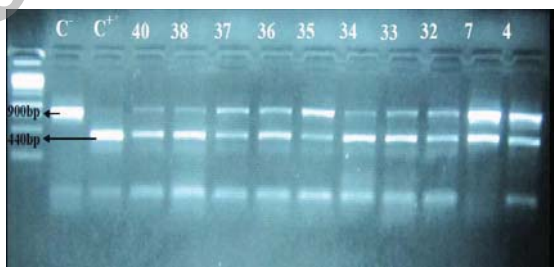

Figure 3. Gel Electrophoresis of PCR, from left side: No 1 of 1Kbp size marker, No 2 of negative control, No 3 of positive control of 440 bp fragment, numbers 4 to 13 are the positive samples of patients infected by *Mycoplasma genitalium*.

Table 2. Age distribution of the patients through affliction with infections of the genital system

Age	Number	*M. genitalium*		*G.vaginalis*		*N. gonorrhoeae*	
		+	-	+	-	+	-
15-20	2	1	1	0	2	0	2
20-24	11	2	9	2	9	1	10
25-30	18	4	14	0	18	1	17
30-35	10	3	7	0	10	0	10
35-40	2	0	2	1	1	0	2
40-45	1	0	1	0	1	0	1
Total	44	10	34	3	41	2	42
Percentage of infection		22.72%		6.81%		4.54%	

A number of cases of affliction with these bacteria and the relationship of the infections with premature delivery were studied. Among the 44 sample cases, 3 cases of abortion and one case of premature delivery were reported. In the one case of premature delivery, no bacteria were identified. However, the presence of *Mycoplasma* was reported in the three cases of abortion. The only significant relationship was between the existence of infection with *Mycoplasma genitalium* and the abortions ($P<0.05$). Infections caused by *Gardnerella* and *Neisseria* did not show any significant relationship despite the premature delivery and abortion.

4. Discussion

In the various studies, the frequency of *Gardnerella vaginalis* in the women with an unnatural secretion from the vagina reached up to 71% (Cunningham et al., 2001). Yet, this organism is a part of the normal flora of the vagina among 20 to 40% of the healthy women (Murray et al., 1990). Studies have shown that infection with this bacterium is asymptomatic in 55% of the women (Baeten et al., 2001). The patients suffering from bacterial vaginosis may have diversified symptoms or more than half of them may be asymptomatic (Franklin et al., 2000; Dadhwal et al., 2000; Purwar et al., 2001). The studies carried out on the pregnant women afflicted by bacterial vaginosis show that the risk of complications, including chorioamnionitis, premature rupture of membranes, premature delivery, birth of a low-weight newborn and endometritis followed by caesarean increases several times over (Dadhwal et al., 2000; Purwar et al., 2001).

Some studies carried out in Iran represent a high prevalence of bacterial vaginosis in some regions (Keshavarz et al., 2001). Studies of our group in the Tonekabon Township showed that, out of 44 cases, 3 cases of *Gardnerella vaginalis* (6.81%), 2 cases of *Neisseria gonorrhoeae* (4.54%) and 10 cases of *Mycoplasma genitalium* (22.72%) were evaluated to be positive.

In all cases studied, there was only one case of premature delivery, which lacked any sort of bacterial infection considered by our group, and no significant relationship among infections of *Gardnerella*, *Mycoplasma* and *Neisseria* with premature delivery was observed. Also, no significant relationship was observed between bacterial vaginosis and premature delivery in a study conducted on the vaginal secretions of the pregnant women referred to the hygienic and therapeutic center of Qazvin from 2008 to 2010 (Abutorabi et al., 2012).

Research conducted by Carey et al, showed that bacterial vaginosis has no influence on premature delivery (Carey et al., 2000). Nevertheless, in a study carried out on 130 cases referred to the therapeutic center at Kerman Medical Sciences University in 2002, a significant relationship could be shown between bacterial vaginosis and premature delivery (Gibbs et al., 2008). Studies by Kurkinen et al., on pregnant women lacking the premature delivery showed that bacterial vaginosis can lead to premature rupture of the water sack and premature delivery (Kurkinen- Räty et al., 2000). A study carried out on 4,430 pregnant women by Kiss et al showed that treatment of bacterial vaginosis significantly decreases the rate of premature deliveries (Kiss et al., 2004). In another study, Leitich et al studied more than 100 cases of occurrence of premature delivery and observed that a noticeable rate of bacterial vaginosis among these premature deliveries exists and therefore, they assumed a direct relationship between these two factors (Leitich et al., 2003). The results obtained by the current study showed no significant relationship between *Gardnerella vaginalis* and *Neisseria gonorrhoeae* and premature delivery. However, considering the low number of samples studied, these results are not highly supported through a statistical point of view.

Among the referrer cases considered in our study, 3 cases of abortion occurred. These cases of abortion were significantly related to the infection of *Mycoplasma genitalium* but were not significantly related to other studied organisms. *Mycoplasma genitalium* is the causative agent of genital system infections and plays a role in the occurrence of various diseases of the genital organ, fertility disorders, disease and mortality of neonates. Therefore, diagnosis of these organisms is a very significant problem which must be taken into consideration, and an appropriate diagnostic method is to be applied in order to identify these organisms. With regard to results obtained from the study of PCR, 10 cases were afflicted by *Mycoplasma*

genitalium [22.72 percent]. Certainly, all cases of the positive *Mycoplasma* were identified in our test because superiority of method of usage of PCR over culture has been shown in various studies. Luki et al carried out a study using the PCR technique and culture in order to isolate *Ureaplasma urealyticum*, *Mycoplasma hominis* and *Mycoplasma genitalium* in the clinical samples obtained from 47 exposed-to-risk pregnant women and 8 new-born neonates which, regarding *Mycoplasma genitalium*, four samples were positive using the PCR method and had a negative result in the culture (Luki et al., 1998). In other tests carried out by Yoon et al, in order to identify *Ureaplasma urealyticum*, it was specified that about 28% of patients using the PCR method were positive in the samples of amniotic liquid while the positive digit, through method of culturing the samples, was only 16% (Yoon et al., 2000).

In several cases in which diagnosis of the desired organism in one infection is required, usage of the culture method wastes time, even though culture of samples, including *Gardnerella vaginalis* is of no importance and doesn't assist in diagnosis of the vaginosis because it exists in half of the healthy individuals as well. Of course, regarding other factors, especially regarding *Mycoplasma* infection, time of culture can last several weeks, while application of PCR method reduces this time to only a few hours.

Conclusion

The results of this study showed that *Gardnerella vaginalis*, *Neisseria gonorrhoeae* and *Mycoplasma genitalium* are present in the vaginal samples of pregnant women of the studied group, meanwhile, only 1 case of premature delivery and 3 cases of abortion were observed. Premature delivery didn't show a relationship with any of the bacterial infections, but the relationship of *Mycoplasma genitalium* with abortion was significant. Therefore, infection of *Mycoplasma genitalium* in the abortion must be taken seriously and certainly treated after diagnosis. At this time, diagnosis through PCR method can be of much significance from the viewpoint of timing, cost-effectiveness and earlier commencement of treatment.

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