MOLECULAR DETECTION OF BACTERIAL VAGINOSIS AND ITS ASSOCIATION WITH MISCARRIAGE IN AL-HILLAH CITY

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ABSTRACT
This study attempt to detect some BV associated bacteria and their associations with miscarriage in vaginosis women. One hundred fifty (150) high vaginal swabs were collected from married vaginosis women, from the hospital of Babylon city and private clinics. Seventy five samples were taken from vaginosis women without miscarriage and Seventy five samples from vaginosis women with miscarriage. Bacterial vaginosis (BV) women was diagnosis as having BV according to Amsel's criteria. The age of patient (15–45) years. The sample was collected by disposable swabs. This swab stored in freezing until were used for molecular analysis, DNA was extracted from these swabs and the 16s rRNA genes of some bacterial vaginosis bacteria detection by polymerase chain reaction technique. Molecular result showed that *G. vaginalis* 139 (92.66%) and *Atopobium vaginae* 116 (77.33%) were more common than other causes of bacterial vaginosis. This result showed that the highest rate of Vaginosis occurred in low education (Illiterate) level 85 (57%) and most cases of miscarriage occurred at the first trimester 45 (60%). Also, the role of the infectious agent with miscarriage investigated, The results indicated a statistically significant difference between multiple miscarriage and single miscarriage of women that infection with *Megasphaera* sp, *Atopobium vaginae*, *G. vaginalis* and *Leptotrichia* sp (p=0.050%, p=0.022%, 0.038%, 0.036% respectively).

Keywords: Bacterial vaginosis, cultivation independent method, miscarriage, BV associated bacteria


INTRODUCTION
Bacterial vaginosis (BV) is a complex clinical syndrome characterized by alterations in the normal vaginal lactobacilli and overgrowth of anaerobic bacteria, including *Gardnerella vaginalis*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mobiluncus species*, *Prevotella species*, and other anaerobes [1,2]. BV is the most common vaginal syndrome affecting women of reproductive age (pregnant and non-pregnant) and it is the
most prevalent cause of vaginal discharge and malodour \[3\]. In pregnancy, BV has been associated with adverse outcomes such as miscarriage, premature rupture of membranes, preterm birth, and low birth weight \[4\]. The massive overgrowth of vaginal anaerobes is associated with increased production of proteolytic carboxylase enzymes, which act to break down vaginal peptides to a variety of amines, which, at high pH, become volatile and malodorous, especially trimethylamine \[5\]. The amines are associated with increased vaginal transudation and squamous epithelial cell exfoliation, creating the typical discharge \[6\]. In conditions of elevated pH, \textit{G. vaginalis} more efficiently adheres to the exfoliating epithelial cells, creating clue cells (i.e., vaginal epithelial cells studded with coccobacillary organisms) \[6, 7\]. Previous study by\[8\] support the primary pathogen model as it is postulated that BV-related bacteria first adhere to the vaginal epithelium, proliferate and then create a dense biofilm. The biofilm is by no means affected by the increase in pH, which may be the result of metabolic events of the amplified bacterial population. A notable feature of BV is the appearance of a polymicrobial biofilm on vaginal epithelial cells. Presence of this biofilm creates a favorable environment for anaerobic bacteria, owing to the presence of an oxygen gradient within the biofilm and makes the infection hard to cure and this result from fact that biofilm producing bacteria need tenfold concentration of antibiotics to get rid when compared with itself but without biofilm \[9\]. Theoretically, the inception of pregnancy complications as a result of BV is due to its potential to favor ascending infections (from the vagina to the chorioamnion) to cause inflammation of the choriodecidual space and activating pathways of labor and subsequently PTD and PTB \[10-12\]. The bacteria associated with septic abortion are usually polymicrobial, derived from the normal flora of the vagina and endocervix, with the important addition of sexually transmitted pathogens \[13\]. There are number of different routes by which an infection may reach the intra-amniotic cavity transplacental spread of infection can occur in women with bacteremia, an infection of the abdominal cavity could spread via the fallopian tubes, but the most common routes is an ascending infection from bacteria colonize the vagina and or cervix \[14\]. Indirect evidence indicated that the most common pathway on intrauterine infection is the ascending route than other route \[15\]. The development of culture-independent molecular techniques like broad-range 16S ribosomal DNA (rDNA) PCR, quantitative real-time PCR (qPCR) assays, denaturing gradient gel electrophoresis (DGGE) and 454 pyrosequencing have improved the detection of uncultivable anaerobes, such as BVAB1, BVAB2, BVAB3, \textit{Eggerthella} spp., \textit{Megasphaera} type 1, \textit{Leptotrichia} spp and \textit{Sneathia} spp from BV-positive women \[16-18\]. The use of 16SrRNA gene sequence-based analyses have frequently been used for the study of the vaginal microbiota, thus revealing the presence of many anaerobic species not previously detected by culture \[16,19\].

**MATERIALS AND METHODS**

**Samples collection**

This study involved (150) samples which were collected from women with bacterial Vaginosis (75 samples from vaginosis women with miscarriage and 75 samples from vaginosis women without miscarriage). All subjects were within 15-45 years of age. The sample include high vaginal swabs from married female were
admitted to the out-patient clinics of Gynecology Babylon Maternity and Pediatrics Hospital during the period from (November 2018 to June 2019). Swabs was immersed in plain tube-containing 5ml of brain heart infusion broth supplemented with 15% glycerol and frozen immediately at -20 °C to be used for molecular diagnosis.

**Molecular diagnosis**

High vaginal swabs total DNA were extracted by using G-Spin™ Total DNA extraction kit (iNtRON, Korea) and done according to company instruction. The concentration and purity of the isolated DNA samples were measured by the Nano Drop spectrophotometer before the performance of PCR. The extracted DNA then stored at -20°C until PCR assay. PCR assay was performed for detection Bacterial Vaginosis associated bacteria by using the primer specific for 16S rRNA according to [20]. The volume of PCR mixture for 16S rRNA amplification was 20μL including premaster mix (Bioneer, Korea), 1μL of each primer (10pmol), 5μL of genomic DNA (5-50ng/mL), and the volume was completed with nuclease-free water. PCR thermocycler condition were done according to [20]. By using conventional PCR thermocycler system is same for each gene that used for detection (Atopobium sp., Eggerthella-like uncultured bacterium, L. iners, Leptotrichia/Sneathia spp., Megasphaera phylo type 1, and Prevotella G1) while different for other bacterial species as was listed in table (1). Products of PCR were examined through electrophoresis, 1.5% agarose gel for 45 minutes and ethidium bromide staining and visualized under UV transilluminator.

**Table 1: Amplification conditions of 16 sRNA genes (20).**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec.</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>62°, 57°<em>, 55°</em>**</td>
<td>30 sec</td>
<td>38 cycle</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>Forever</td>
<td>-</td>
</tr>
</tbody>
</table>

*Annealing Temperature for detection *G. vaginalis*.

**Annealing Temperature for detection *M. curtisii***

***Annealing Temperature for detection other BV associated bacteria species.**

**Statistical analysis**

The result was analyzed by using the statistical software package SPSS 23. Determining the statistical differences among different groups was made using the Pearson Chi-square test and odds ratio with (95%) confidence. Probability of (P≤ 0.05) was considered to be statistically significant.
RESULTS AND DISCUSSION

The result of this study evaluated the effects of BV on different periods of gestation which showed that most cases of miscarriage occurred at the first trimester 45(60%) out of 75 cases of miscarriage was more than second trimester 30 (40%) as shown in figure (1) and there are statically significant between trimester of pregnancy with a P value (p<0.083).

![Figure 1: Incidence of miscarriage case among the pregnant period](image)

Our result in agreement with Some authors who indicated that BV may cause the first trimester miscarriages even though the others stated that BV infection in the early periods of pregnancy may cause the second trimester miscarriage and preterm labor\cite{21,22}. This result unlike previous result conducted by\cite{23} who found there was a statistically significant relationship between BV and second trimester miscarriage (P < 0.05).

Rai and Regan\cite{24} reported that untreated infections going on for a long time without any symptoms cause pregnancy losses. To our opinion, consistent with these results, untreated and asymptomatic BV infection in first trimester or before pregnancy may cause second trimester miscarriage. This result unlike previous study done\cite{25} who found that the prevalence of BV in the early second trimester of pregnancy. The result of our study showed there was a relation between educational level and BV infection. The results revealed that the highest rate of Vaginosis was 85 (57%) out of 150 were in women with a elementary or below educational level, while 38 (25%) out of 150 patients were with middle school also 18 (12%) out of 150 women in high school and the lowest rate of BV was 9 (6%) out of 150 infected women with collage education level as shows in figure (2) and there are statically significant between education level (p≤0.001).
The current result similar with study done by [26] who revealed that 45 (70.5%) out of 60 women were with a low educational level infected with BV. Education promotes increasing awareness, responsibility, knowledge of self-care, healthy lifestyles and behaviours. Low education level usually is associated with low incomes, social class and is related to risks such as unhealthy habits (smoking, alcohol, and drug abuse), violence, weak families and others [27].

**Molecular diagnosis**

DNA was extracted from all 150 collected specimens; conventional PCR was carried out for the detection of specific 16SrRNA genes of eight positive indicators for bacterial vaginosis. After that gel electrophoresis result have showed a total of 295 isolates in vaginosis women with miscarriage and 249 in Vaginosis women without miscarriage belonged to eight different microorganisms have been identified as show in table (2) and produced the specific DNA fragment of molecular weight (558,236,230,208,582,383,210,569 bp) for *Atopobium sp*, *Eggerthella sp*, *Leptotrichia sp*, *Megasphaera sp*, *Mobiluncus curtisi*, *Prevotella sp*, *Gardnerella vaginalis* and *Lactobacillus iners* respectively when compared with allelic ladder; as shown in Figures (3 to 10).

**Table 2: Frequency of detected bacteria in miscarriage and non-miscarriage women.**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>No. In women miscarriagewith</th>
<th>No. In women with out miscarriage</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atopobiumvaginai</em></td>
<td>69(92.00%)</td>
<td>47(62.66%)</td>
<td>116(77.33%)</td>
</tr>
<tr>
<td><em>Eggerthellaspor</em></td>
<td>31(41.33%)</td>
<td>18(24.00%)</td>
<td>49(32.66%)</td>
</tr>
<tr>
<td><em>Leptotrichiaspor</em></td>
<td>63(84.00%)</td>
<td>42(56.00%)</td>
<td>105(70.00%)</td>
</tr>
<tr>
<td><em>Megasphaerasp</em></td>
<td>26 (34.66%)</td>
<td>13(17.33%)</td>
<td>39(26.00%)</td>
</tr>
<tr>
<td><em>M. curtisi</em></td>
<td>16(13.33%)</td>
<td>35(46.67%)</td>
<td>51(34.00%)</td>
</tr>
<tr>
<td><em>PrevotellaG1</em></td>
<td>13(17.33%)</td>
<td>10(13.33%)</td>
<td>23(15.33%)</td>
</tr>
<tr>
<td><em>G. vaginalis</em></td>
<td>67(89.33%)</td>
<td>72(96.00%)</td>
<td>139(92.66%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L.iners</th>
<th>10 (13.33%)</th>
<th>12 (16.00%)</th>
<th>22 (14.66%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>295</td>
<td>249</td>
<td>544</td>
</tr>
</tbody>
</table>

Figure 3: Agarose gel electrophoresis image that showed PCR product analysis for 16S ribosomal RNA gene in Atopobium sp. M (Marker ladder 2000-100bp). Lane (1-10) some Positive Atopobium sp. samples at 558bp product size.

Figure 4: Agarose gel electrophoresis image that showed PCR product analysis for 16S ribosomal RNA gene in Eggerthella sp. M (Marker ladder 2000-100bp). Lane (1-10) some Positive Eggerthella sp. samples at 236bp product size.

Figure 5: Agarose gel electrophoresis image that showed PCR product analysis for 16S rRNA gene in Leptotrichia sp. M (Marker ladder 2000-100bp). Lane (1-10) some Positive Leptotrichia sp. samples at 230bp product size.
Figure 6: Agarose gel electrophoresis image that showed PCR product analysis for 16S ribosomal RNA gene in Megasphaera sp. M (Marker ladder 2000-100bp). Lane (1-10) some Positive Megasphaera sp. samples at 208bp product size.

Figure 7: Agarose gel electrophoresis image that showed PCR product analysis for 16S ribosomal RNA gene in Mobiluncus curtisii. M (Marker ladder 2000-100bp). Lane (1-10) some Positive Mobiluncus curtisii samples at 582bp product size.

Figure 8: Agarose gel electrophoresis image that showed PCR product analysis for 16S ribosomal RNA gene in Prevotella sp. M (Marker ladder 2000-100bp). Lane (1-10) some Positive Prevotella sp. samples at 383bp product size.
The present study agrees with [28] who used 16S rRNA PCR to examine associations between bacterial vaginosis and bacterial morphotypes in infected women. Recent high-throughput sequencing of 16S rRNA genes of the vaginal bacterial communities of pregnant women showed that vaginal microbiome becomes more stable and less diverse as pregnancy progresses, which confers a protective role against ascending infection of the genital tract [29]. However, there are intravaginal microorganisms other than Lactobacillus species in some pregnancies that cause chorioamnionitis. This is because the normal flora (commensal bacteria) that colonize the vagina during pregnancy do not cause inflammatory conditions or vaginitis, such as occurs with infection by pathogenic bacteria. An imbalance in the normal vaginal bacteria is therefore known as bacterial vaginosis (BV). Chorioamnionitis is the main cause of preterm delivery, and various previous reports have stated that chorioamnionitis occurs against a background of BV. When BV causes vaginitis or cervicitis and then progresses to inflammation of all fetal membranes, it can in turn cause premature rupture of membranes and labor [30,31]. Atopobium sp and Gardnerella vaginalis were found in a high percentage of subjects who had bacterial vaginosis, according to bacterium-specific PCR this study was in agreement with study that conducted by [32] who found Atopobium sp and Gardenella vaginalis comprised high percentage (69.79% and 66.67%). Study done by [33] showed that Gardnerella vaginalis was isolated from preterm labor in percentage (20%). Mobiluncus species is observed in vaginal smears but the results with PCR seldom mention an association. Maybe this is because it is not Mobiluncus species seen in...
microscopy but rather other bacterial vaginosis-associated bacteria with the same morphology [16]. However, since these species are an important member in Nugent score they were chosen as potential markers. BVAB2 and *Megasphaera* species are fastidious and strictly anaerobic bacteria which individually are sensitive and specific indicators of bacterial vaginosis but have also together shown promising result in PCR diagnosing bacterial vaginosis [20]. *Megasphaera* spp detected in vaginosis women. Studies done by [34] proposed *Megasphaera* is considered the main cause for (BV) and the main cause for miscarriage [34-37]. Studies done by [17, 38] found a good relationship between the miscarriage and the bacterial vaginosis and discovered that women with first trimester in Belgium have a confidence with 95%. Differences in results between some studies and with those of the current study may due to the Differences in the methodologies employed; for examples, samples were obtained from different part of vagina, from women with different ethic and geographic origin and different PCR amplification conditions were used.

**Association between infectious microorganism and miscarriage**

Twenty eight women out of seventy five miscarriage women with BV had single miscarriage (37.33%) while forty seven (62.66%) women had multiple miscarriages (two or more). Comparison of infection rate between vaginosis women with single miscarriage and vaginosis women with multiple miscarriage following results: *Atobopium* sp the rate was 33.33% % versus 58.66%. The p-value was significant (P=0.022). *Megasphaera* phylotype 1 the rate was 10.66% % versus 24.00%. The p-value was significant (P=0.050). *G. vaginalis* the rate was 33.33% % versus 56.00%. The p-value was significant (P=0.038). *Leptotrichia* sp also show significant (p=0.036) where the rate was 30.66% versus 53.33% as show in table (3).

**Table 3: Association between miscarriage and type of infectious microorganism that causing BV in miscarriagewomen**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>No. In women with single miscarriage</th>
<th>No. In women with multiple miscarriage</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atobopium</em> sp</td>
<td>25(33.33%)</td>
<td>44 (58.66)</td>
<td>0.022</td>
<td>Significant</td>
</tr>
<tr>
<td><em>Eggerthella</em>-spp</td>
<td>11(14.66%)</td>
<td>20(26.66%)</td>
<td>0.106</td>
<td>No</td>
</tr>
<tr>
<td><em>Leptotrichia</em> spp</td>
<td>23(30.66%)</td>
<td>40(53.33%)</td>
<td>0.036</td>
<td>Significant</td>
</tr>
<tr>
<td><em>Megasphaera</em> spp</td>
<td>8(10.66%)</td>
<td>18(24.00%)</td>
<td>0.050</td>
<td>Significant</td>
</tr>
<tr>
<td><em>M. curtisii</em></td>
<td>10(13.33%)</td>
<td>6(8.00%)</td>
<td>0.317</td>
<td>No</td>
</tr>
<tr>
<td><em>Prevotella</em> G1</td>
<td>5(6.66)</td>
<td>8(10.66%)</td>
<td>0.405</td>
<td>No</td>
</tr>
<tr>
<td><em>G. vaginalis</em></td>
<td>25(33.33%)</td>
<td>42(56.00%)</td>
<td>0.038</td>
<td>Significant</td>
</tr>
<tr>
<td><em>Liners</em></td>
<td>6(8.00%)</td>
<td>4(5.33%)</td>
<td>0.527</td>
<td>NO</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>113</strong></td>
<td><strong>182</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Our result show significant association of *Atobopium* spp, *Megasphaera* sp, *Leptotrichia* sp and *G. vaginalis* with multiple miscarriages this result were in agreement with previous study conducted by [33] who found that *Atobopium* sp and *Megasphaera* sp show significant association with multiple miscarriage but didn’t show significant association with *Leptotrichia* sp and *G. vaginalis*. A Study conducted by [19] showed significant
increases in *Atopobium vaginae, Megasphaera spp., Gardnerella vaginalis, Leptotrichia amnionii, and Sneathias anguinegens* in spontaneous miscarriage Korean women compared to women who had never undergone abortion, this result in agreement with our result that found a significant association of *Atopobium vaginae, Megasphaera spp., G. vaginalis, Leptotrichia sp* in miscarriage.

**ETHICAL CLEARANCE**

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

**FUNDING**: Self-funding

**REFERENCES**


