Qualitative and Quantitative Detection of Some Bacterial Vaginosis Pathogenic Markers Using Real-Time PCR

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Abstract. Bacterial vaginosis is a vaginal condition characterized by an abnormal vaginal discharge due to an overgrowth of normal bacteria in the vagina. Women with bacterial vaginosis also have fewer than the usual population of vaginal bacteria, i.e lactobacilli. In this study vaginal samples from 75 women were taken to detect and quantify two putative positive-indicator of bacterial vaginosis namely, Atopobium vaginae and Megasphaera type1, in addition to a negative-indicator of bacterial vaginosis (Lactobacillus crispatus) using real-time polymerase chain reaction technique. The prevalence of bacterial vaginosis was 34.66% according to scoring system, the most dominant species in patients and in women without the syndrome was L. crispatus (80.76 and 79.59% respectively), but its concentration of 16S rRNA genes was significantly higher in bacterial vaginosis negative women (2.8×10^8 vs. 4.74×10^7 copy/swab). In bacterial vaginosis patients, the highest rDNA concentration was for Megasphaera (3.10×10^8 16S rRNA copy/swab).

Introduction

Bacterial vaginosis (BV) can simply be defined as a disturbance in the vaginal ecosystem in which the predominant lactobacilli are replaced by an overgrowth of vaginal commensal organisms (1). It may be transient or become persistent. BV is recognized as the most cause of abnormal vaginal discharge in women of childbearing age (2).

Over the years a number of different methods have been used to diagnose bacterial vaginosis, of these is polymerase chain reaction (PCR) which has broadly utilized for detection and diagnosis of bacterial vaginosis and the associated organisms. Several studies have used broad-range 16S rRNA gene PCR to characterize the community of vaginal bacteria (3,4,5). These molecular studies have discovered a large number of novel, fastidious, or uncultivated bacterial species. However, methods such as broad-range 16S rRNA gene PCR with denaturing gradient gel electrophoresis or sequence analysis of cloned amplification products are not sensitive for detecting infrequent or minority species in a bacterial community (6).

Other studies have targeted species-specific region of the 16S rRNA gene (7,8), such sensitive methods have provided more accurate and reliable facts toward understanding of the vaginal bacterial flora especially in disturbed cases like bacterial vaginosis. PCR-based assays to detect BV-related organisms could be useful in several settings, for example PCR can potentially detect and quantify genital tract organisms such as mycoplasmas that are not detected by commercial tests, also such
methods could be used on stored specimens (9). In addition, since it is possible to develop quantitative PCR assays for a specific types of organisms in the genital tract this can helpful in the discrimination between normal and abnormal vaginal niche as some microorganisms, e.g. *Gardnerella vaginalis*, found in both health and disease (10). Furthermore, the use of multivariate analysis of the quantity of a set of BV-associated marker organisms present in the vaginal samples represents the best approach to obtaining a truly objective and accurate option for diagnosis women with this condition (7,11).

**Materials and methods**

A total of 75 woman aged between 15-49 years, whom visiting the outpatient department in the Educational Hospital of Maternity and Pediatrics, in addition to some private clinics in Al-Diwaniyah city, were enrolled for comparison of vaginal flora between women with and without bacterial vaginosis using quantitative polymerase chain reaction technique. Specimens were collected from the lateral vaginal wall and posterior fornix, and they were assessed for bacterial types using Nugent's scoring system (12).

qPCR assay was performed for detection and copy number quantification of two bacterial vaginosis positive indicators ( *Atopobium vaginae* and *Megasphaera-type1* ), in addition to *Lactobacillus crispatus* ( as a negative indicator for BV). First Genomic DNA was extracted from vaginal swab samples using a commercial genomic DNA extraction kit ( Geneaid/USA ) and it was done according to manufacturer’s instructions.

Primers targeting 16S ribosomal RNA genes were designed using NCBI GenBank data base and Primer 3 online. Specificities of all designed primers were confirmed by BLAST searches in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). The primers were provided by Bioneer company (Korea). Primers sequences are shown in table (1).

**Table (1) Primers used in real time PCR**

| Bacterium                    | Primer Sequence         | PCR Product Size | GenBank Code  |
|-----------------------------|-------------------------|------------------|---------------|
| *Atopobium vaginae*         | ATGTTGCCAGCGGTTAAAGC    | 99bp             | AJ585206.2    |
|                            | AGGACATAAGGGGCGTATGAC   |                  |               |
| *Megasphaera-type1*         | TGAAATCGAGTGCAAACGG     | 87bp             | AY271952.1    |
|                            | TCGGTATTAGCCAGCGGTTTC   |                  |               |
| *Lactobacillus crispatus*   | CAGGTCTTGACATCTAGTGC    | 107bp            | HQ716720.1    |
|                            | AACCCCAATCTCAGCAC       |                  |               |

qPCR master mix was prepared for detection and copy number calculation of bacterial vaginosis indicator bacteria from vaginal samples by preparation of genomic DNA standard curve for *16S rRNA* gene (13).

The reaction mixture for *16S rRNA* genes of target bacteria in a total volum of 50 μl was as follow: DNA template (2.5 μl), *16S rRNA*- F. primer (1 μl), *16S rRNA* -R. primer (1 μl), 2X Greenstar master mix (Bioneer/Korea) (25 μl), and DEPC water (20.5 μl).

Miniopticon Real-Time PCR system ( BioRad/USA ) was used with the following conditions: initial denaturation (95°C, 1 min, 1 cycle.), denaturation (95°C, 10 sec.), annealing/extension (55°C, 30 sec., 45 cycles), and melting (60-95°C, 5 min, 1 cycle).
Sham (no DNA template) PCR controls were run with each qPCR assay, these controls consisted of all necessary PCR reagents for amplification except template DNA and were used to monitor for bacterial contamination of the PCR reagents. After the end of each PCR run, melting curve analysis was performed from 60-95°C (0.1°C S⁻¹) for detecting non-specific PCR products and primer-dimer coamplification.

Results and discussion

Evaluation of staining smears revealed that 26 women had bacterial vaginosis i.e., a prevalence rate of 34.66%, eleven of these cases were associated with the detection of Candida sp. in stained smears. The remaining 49 subjects were without BV according to scoring system, however, ten of these women had Candida sp.

Results obtained with q-PCR showed that L. crispatus was the dominant species among women with and without BV, where it was isolated from 80.76 % and 79.59 % of these two groups respectively (table 2).

| Organism      | Women without BV* | Women with BV* |
|---------------|--------------------|----------------|
|               | Number            | Percentage%    | Number            | Percentage%    |
| A. vaginae    | 26                | 53.06 (Aa)**   | 19                | 73.07 (Ab)     |
| Megasphaera-1 | 29                | 59.18 (Aa)     | 19                | 73.07 (Aa)     |
| L. crispatus  | 39                | 79.59 (Ba)     | 21                | 80.76 (Ba)     |

* Chi-square was used to calculate the differences in percentages of isolation at p < 0.05.

**The capital letters refer to the vertical reading while the small letters refer to the horizontal reading. The similar letters indicate no significant differences and the different letters indicate significant differences.

A high abundance of lactobacilli and a high number of Lactobacillus 16S rRNA gene copies in patients with BV have been previously reported (9,14,15). For example the reported percentage of L. crispatus isolation reached to 83% (16), and even to 92% in BV-positive women (17).

Detection of L. crispatus in most of women without BV supports many of the previous studies which reported that this species is the most common Lactobacillus species in the vagina of healthy fertile women than in that of posmenopausal women (18,19,20).

Absence of L. crispatus in more than 20 percent of women without BV may due to the dominance of other Lactobacillus species rather than L. crispatus, such as L. gasseri, L. jensenii, or L. iners as these species are highly recovered from vagina of healthy women (16,21).

In addition, colonization of healthy vagina with organisms rather than Lactobacillus; e.g., Atopobium, Gardnerella, Peptostreptococcus, Prevotella, Pseudomonas, and Streptococcus, alone or in combinations; has been previously reported (4,18,22,23), and it has been suggested that, alone, the absence of Lactobacillus does not define an unhealthy state, i.e. vaginosis; on the other hand the presence of solely or a combination of these non Lactobacillus species does not define an unhealthy state (18).

In women without BV, Megasphaera type1 was the most prevalent non Lactobacillus species (59.18%), followed by A. vaginae (53.06%).

Megasphaera type1 has been recovered from 29 women without BV. This bacterium has been previously isolated from women whom had no infection with a high prevalence (15,24,25), where it was detected even in more than 50% of normal vaginal specimens (8).
Sequencing of 16S rRNA of vaginal samples revealed that the uncultivated Megasphaera-like phylotypes represent new species in this genus and that they may be uniquely adapted to genitourinary environment (26).

A. vaginae, which has been isolated from 53.06 percent of BV-negative women, has been previously recorded in such a subjects group at a prevalence sometimes acceded that obtained in this study (8,15,25).

In BV patients, A. vaginae and Megasphaera-1 were isolated at a percent of 73.07% for both. These bacteria have been reported to be etiological agents of BV that detected at high frequencies (11,25,27).

Quantitative Real-Time PCR (qPCR) of 16S rRNA gene copies of the three target bacteria was based on 10 fold dilution DNA standard curve.

Results showed that in BV-negative women, L. crispatus (as a negative predictor of BV) concentration was significantly higher than that of non Lactobacillus species with a mean of 2.80×10^8 copies of 16S rDNA per swab (table 3), this concentration was also higher than that in BV-positive group (4.74×10^7 copy/swab). This result is expected as lactobacilli were found to be the most predominant organisms in healthy women using such technique, i.e. qPCR (7,8,28).

| Organism       | Women without BV* Mean ± SE (copy/swab) | Women with BV* Mean ± SE (copy/swab) |
|----------------|----------------------------------------|--------------------------------------|
| A. vaginae     | 2.68×10^7 ± 4.16×10^6(Aa)**             | 6.80×10^7 ± 3.74×10^7 (Aa)           |
| Megasphaera-1  | 1.06×10^7 ± 3.97×10^6(Aa)               | 3.10×10^7 ± 7.35×10^7 (Bb)           |
| L. crispatus   | 2.80×10^8 ± 3.99×10^7(Ba)               | 4.74×10^7 ± 1.78×10^7 (Ab)           |

* Differences in 16S rRNA concentrations between women with and without BV was determined by t-test, while differences among the same subjects group was estimated using ANOVA with LSD.

**The capital letters refers to the vertical reading while the small letters refers to the horizontal reading. The similar letters indicates no significant differences and the different letters indicates significant differences at p < 0.05.

SE Standard error.

The concentrations of Megasphaera type1 in BV-positive women were significantly higher than those in women without BV, 3.1×10^8 vs. 1.06x10^7 copy/swab, (Table 3-4). Megasphaera-1 concentration was found to be higher in BV patients than Megasphaera-2, and thus to have a stronger association with BV (5).

Although it was confirmed by qPCR that the presence of A. vaginae is diagnostically more valuable marker for bacterial vaginosis than G. vaginalis (4,17), in our study there was no significant difference in the DNA copies number of A. vaginae between BV-negative and BV-positive women, but it was higher in the latter group (6.80×10^7 copy/swab).

It was suggested that subjects with BV had an adherent biofilm that is primarily composed with three bacterial groups, of which G. vaginalis is the most prominent where it constitutes 60-90% of the biofilm mass. Atopobium accounts for 1-40% of biofilm mass, and lactobacilli are present between 1 and 5% in only 20% of the biopsy samples. This adherent bacterial biofilm presents in 90% of BV patients, while only 10% of women with normal vaginal microflora have a similar biofilm (29).
The determination of the exact bacterial number is difficult due to factors affecting the amplification reaction and variation of the copy number and the ribosomal content of cells in different bacteria (30).

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