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Multiplex polymerase chain reaction (M-PCR) for bacterial vaginosis detection

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Abstract:

Bacterial vaginosis (BV) is one of the most common vaginal infections in women of reproductive age. If not treated promptly, the disease can lead to serious complications affecting the fertility and long-term health of women. Research on BV requires effort from a variety of disciplines and its treatment can only be determined by coordinated actions in research and treatment. Currently, BV diagnostic methods are often based on culture techniques and Gram staining. However, molecular biology research is developing and has proved to more advantageous for identifying key pathogens. Combining both methods, we conducted a study to develop a diagnostic procedure using multiplex polymerase chain reaction (M-PCR) for simultaneous and accurate detection of the bacterial species that cause BV, incurring minimal costs and time for testing.

Keywords: **bacterial vaginosis, BV, diagnosis, multiplex PCR, PCR.**

Classification numbers: **3.2, 3.5**

Introduction

Bacterial vaginosis (BV) is one of the main causes of vaginitis among women of reproductive age (15-44 years) [1]. In most cases, BV causes no complications because it is not harmful when properly treated. Sometimes, it can appear and disappear for no apparent reason. However, untreated BV has been associated with serious health problems that affect women's fertility and long-term health. BV may cause increased susceptibility to sexually transmitted infections in non-pregnant woman [2, 3]. It also increases the risk of pelvic inflammatory disease [4], an infection of the female genital tract that causes the womb, fallopian tubes and ovaries to become swollen, increasing the risk of infertility and ectopic pregnancy [5]. In addition, BV affects patients who undergo assisted reproductive technology as it can reduce the likelihood rate in falling pregnant by in vitro fertilisation (IVF) [6]. Therefore, treatment of and screening for BV is essential, especially for pregnant women and patients undergoing assisted reproduction.

Although BV was first described in 1895, the cause of the BV microbial alteration is still not fully understood. All parts of the body have bacteria, and some are beneficial while others are harmful. When there is an imbalance of naturally occurring bacterial flora, harmful bacteria grow in number, and problems can arise. Determining which organisms are truly pathogenic poses many difficulties because of the complexity and variability of the vaginal microflora. However, unlike a typical infection caused by a specific bacterium agent, BV involves multiple pathogens [7]. This is a limited definition of BV, but it is important to note that BV can be defined as an alteration in the normal vaginal microbial ecosystem. The development of new methods, such as polymerase chain reaction (PCR), to analyse complex bacterial systems that are often difficult to culture enables the discovery of new agents, in addition to the common bacterial species known as *Gardnerella vaginalis*, *Mobiluncus* spp. and *Atopobium vaginae.* Several studies have confirmed that when the population level of

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these organisms drops below a critical level, the harmful bacteria may proliferate to become the dominant species in the vaginal microbial ecosystem [8, 9].

Currently, many BV diagnostic methods are based on culture techniques, such as Amsel's criteria [10] and Gram staining [11]. However, conventional microbiological methods only assess the occurrence of pathogens without precisely identifying each species. The modern approach used in bacterial studies came with the invention of PCR, which led to multiplex PCR (M-PCR), real-time PCR, taxon-directed PCR, broad-range bacterial 16S rDNA PCR and fluorescence in situ hybridization (FISH). These novel methods take advantage of the nature of 16S ribosomal RNA (16S rRNA), a gene that is unique insofar as it is present in almost all bacterial species. Bacterial vaginosis is not caused by one single infectious agent, and exclusion of the responsible agents is difficult in treatment studies. To identify these bacteria, therefore, it is essential to determine their antimicrobial susceptibility pattern.

Single PCR (s-PCR) is used to identify 10 common bacterial species that commonly cause BV: *Gardnerella vaginalis, Mobiluncus mulieris, Bacteroides fragilis, Atopobium vaginae, Ureaplasma urealyticum, Megasphaera* type I, BVAB 1, BVAB 2, BVAB 3 and *Sneathia sanguinegens.* Polymerase chain reaction primers are designed to target the variable regions of 16S rRNA and allow amplification of the gene in a wide range of different microorganisms. In this study, we establish a clinical method to detect fastidious microorganisms that cause BV using M-PCR. The diagnosis of BV using M-PCR is clinically effective, and the results can be used for treatment selection for patients.

Materials and experimental methods

Sample collection

The study subjects were 10 common bacterial species that cause BV: *Gardnerella vaginalis*, *Mobiluncus mulieris, Bacteroides fragilis, Atopobium vaginae, Ureaplasma urealyticum, Megasphaera* type I, BVAB (Clostridia-like BV-associated bacteria) 1, BVAB 2, BVAB 3 and *Sneathia sanguinegens* (Table 1).

The biospecimens collected for this study were vaginal fluids collected via intravaginal tampon from patients of the Modular Center at the Military Medical University and the Assisted Reproductive Health Center at 16A Hospital. These patients have tested as positive with by culture for key pathogens, either for one species or for co-infection with several species.

Materials (Table 2)

Equipment and chemicals used in the study include:

- QIAamp DNA mini kit (QIAGEN, Hilden, Germany);

- GoTaq Green Mastermix 2X (ProOmega, USA);

- 0.5X TBE Buffer, 2% and 3% Agarose gel, ethidium bromide;

- Pipette and pipette tips 1000 µl, 200 µl, 100 µl, 20 µl, 10 µl, Eppendorf tube 1.5 ml, glassware;

- Equipment: Heitich Miko 22R Centrifuge, Rotamixer vortex mixer, Thermomixer shaking, Wealtec E-centrifuge (mini centrifuge machine), PCR cabinet, PCR Fast Thermal Cycles 9800;

- Electrophoresis apparatus: horizontal electrophoresis slab gel apparatus;

- UV and visible light spectrophotometer: Gel Doc™ XR+ System.

Genomic DNA extraction

To collect a sample, the doctor scraped the swab firmly against the surface of each sample more than six times. The swab was kept at room temperature after collection. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Standardisation of Single PCR technique (Table 3)

Primers: primers were designed based on the specific 16S rRNA specific region of 10 bacterial species, published in NCBI and examined to ensure the optimal conditions for the PCR procedure.

Table 1. Nucleotide sequences of primers used for PCR.

	Symbol	BV agents	Primer (5' - 3')	Amplicon size (bp)
Group G1	BV4	*Gardnerella vaginalis*	TTACTGGTGTATCACTGTAA CCGTCACAGGCTGAACAGT	330
	BV5	*Megasphaera* type I	GATGCCAACAGTATCCGTCCG CCTCTCCGACACTCAAGTTCGA	211
	BV6	*Bacteroides fragilis*	TTCGCTTTTCTGTTTTCTGTGT CAGCAACCACCCAAACATTATT	842
	BV7	*Atopobium vaginae*	TAGGTCAGGAGTTAAATCTG TCATGGCCCAGAAGACCGCC	155
Group G2	BV2	*Ureaplasma urealyticum*	AGAAGACGTTTAGCTAGAGG ACGACGTCCATAAGCAACT	541
	BV8	BVAB 1	GGAGTGTAGGCGGCACTA CTCTCCGATACTCCAGCTCTA	90
	BV9	BVAB 2	TTAACCTTGGGGTTCATTACAA GAATACTTATTGTGTTAACTGCGC	260
Group G3	BV10	BVAB 3	CATTTAGTTGGGCACTCAGGC ACATTTGGGGATTTGCTTCGCC	160
	BV12	*Mobiluncus mulieris*	ATGGATATGCGTGTGGATGG CCAGGCATGTAAGCCCAAA	80
	BV13	*Sneathia sanguineges*	AATTATTGGGCTTAAAGGGCATC AGTACTCTAGTTATACAGTTTTGTAG	102

Table 2. Components for single PCR.

Component	Volume
GoTaq Green Mastermix 2X	6.25 µl
Deionized water	3.75 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
DNA template	2 µl
Total	12.5 µl

Table 3. PCR thermal cycle.

Temperature	Time	Number of cycles
95^0C	5 min	1
94^0C	45 sec	40
Optimised temperature range	45 sec	
72^0C	45 sec	
72^0C	10 min	1
4^0C	∞	1

To check the specificity of primers and PCR, s-PCR products were electrophoresed in 2% agarose gel for 30 minutes at 110V, then stained with ethidium bromide for 10 minutes and screened under UV light. After checking all primers, we proceeded to develop multiplex PCR to simultaneously identify 10 bacterial species.

Optimisation of the multiplex PCR technique

Multiplex PCR was used to simultaneously detect 10 BV agents. Using the annealing temperatures of standardized s-PCR, we used multiplex primer pairs in the same reaction tube with the same concentration. We then adjusted the concentration gradient by increasing the concentration of weakly active pairs and reducing the concentration of active pairs based on the amplified signal strength of the PCR products in agarose gel.

The M-PCR products were electrophoresed in 3% agarose gel for 45 minutes at 110V, then stained with ethidium bromide for 10 minutes and screened under UV light.

Results and discussion

Standardisation of the single PCR technique

In this study, s-PCR was used to check the specificity of identification of 10 BV agents. After electrophoresis, the optimum annealing temperatures determined were 55^0C (Group G1 and group G2) and 63^0C (Group G3).

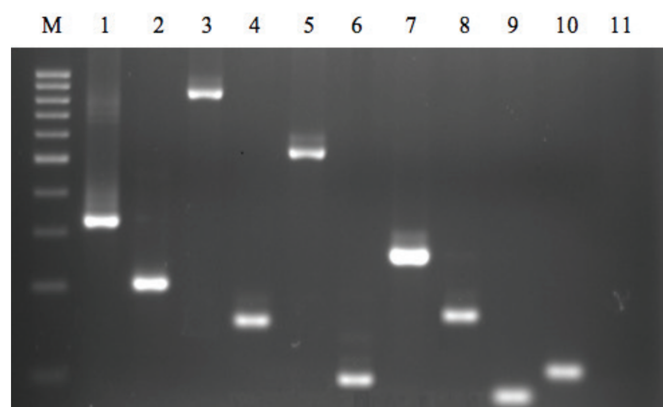

Fig. 1. Electrophoretic analysis of the amplified fragments using s-PCR in 2% agarose gel. Lane M: 100 bp DNA ladder; lane 1: *Gardnerella vaginalis* - BV4 (330 bp); lane 2: *Megasphaera* type I - BV5 (211 bp); lane 3: *Bacteroides fragilis* - BV6 (842 bp); lane 4: *Atopobium vaginae* - BV7 (155 bp); lane 5: *Ureaplasma urealyticum* - BV2 (541 bp); lane 6: BVAB 1 - BV8 (90 bp); lane 7: BVAB 2 - BV9 (260 bp); lane 8: BVAB 3 - BV10 (160 bp); lane 9: *Mobiluncus mulieris* - BV12 (80 bp); lane 10: *Sneathia sanguinegens* - BV13 (102 bp); lane 11: negative control.

The results were obtained as a single band corresponding to each bacterial species without by-products, and the gel electrophoreris image was very clear. Therefore, we decided to set the annealing temperature for G1 and G2 at 55^0C and for G3 at 63^0C.

Currently, there are many traditional methods for determining the presence of BV agents. These include culture, Amsel's criteria, Gram staining and molecular biology techniques. Each method has its advantages and disadvantages. Culture is the classic method but has high requirements regarding the conditions of sample collection and preservation. In addition, some anaerobic species cannot be identified by traditional methods and must be studied using modern molecular biology techniques. With single PCR, we identified 10 common BV agents: BV4, BV5, BV6, BV7, BV2, BV8, BV9, BV10, BV12 and BV13 (Fig. 1). Molecular biology research is developing and has proved advantageous for identifying key pathogens. This successful provides doctors with a basis for treatment with appropriate antibiotics for each pathogen and improves efficiency in testing as well as treatment.

Optimisation of the multiplex PCR technique

After determining the optimum annealing temperature for G1 and G2 at 55^0C and for G3 at 63^0C, we used multiplex primer pairs in the same reaction tube with the same concentration, then adjusted the concentration of each primer pair for M-PCR (Table 4).

Initially, 10 pairs of primers were divided into three groups based on annealing temperature, product size and

product signal strength. Specifically, the assay G1 consisted of BV4, BV5, BV6, BV7; assay G2 consisted of BV2, BV8, BV9 and assay G3 consisted of BV10, BV12, BV13.

Table 4. Multiplex PCR thermal cycle.

Temperature	Time	Number of cycles
95^{0}C	5 min	1
94^{0}C	45 sec	
55^{0}C (assay G1 and G2) 63^{0}C (assay G3)	45 sec	40
72^{0}C	45 sec	
72^{0}C	10 min	1
4^{0}C	∞	1

Based on the result of single PCR procedure, we adjusted the concentration of all primers by comparing to s-PCR products amplicon band combined with the signal strength of each primer pair. We chose the following concentrations as the optimum concentrations (Table 5).

Table 5. Primer concentrations in multiplex PCR.

	BV agents	Primers concentration	Annealing temperature
	BV4	0.3 µl	
	BV5	0.2 µl	
Assay G1	BV6	0.3 µl	55^{0}C
	BV7	0.2 µl	
	BV2	0.3 µl	
Assay G2	BV8	0.2 µl	55^{0}C
	BV9	0.2 µl	
	BV10	0.5 µl	
Assay G3	BV12	0.2 µl	63^{0}C
	BV13	0.5 µl	

In a recent study by Tosheva-Daskalova, et al. [12], three primers were used for the M-PCR reaction to detect three pathogenic bacteria, *Gardnerella vaginalis* - BV4, *Atopobium vaginae* - BV7 and *Mobiluncus* spp. However, in this study, we used four pairs of primers in assay G1 to detect BV4, BV5, BV6 and BV7. The results showed that this assay simultaneously detected more than two pathogens BV4 and BV7 but still ensured the accuracy and quality of the electrophoretic band, resulting in higher test efficiency (Figs. 2-4).

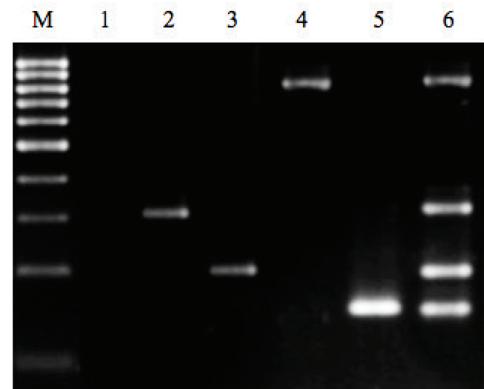

Fig. 2. Electrophoretic analysis of assay G1 in 3% agarose gel. Lane M: 100 bp DNA ladder; lane 1: negative control; lane 2: *Gardnerella vaginalis* - BV4 (330 bp); lane 3: *Megasphaera* type I - BV5 (211 bp); lane 4: *Bacteroides fragilis* - BV6 (842 bp); lane 5: *Atopobium vaginae* - BV7 (155 bp); lane 6: group G1.

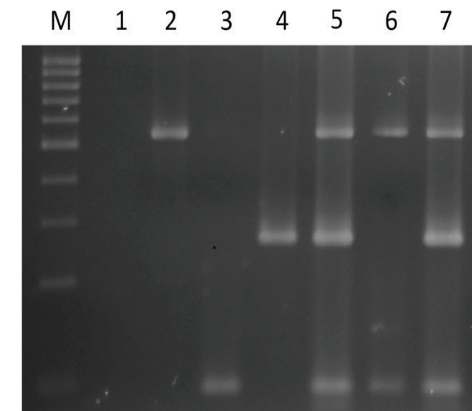

Fig. 3. Electrophoretic analysis of assay G2 in 3% agarose gel. Lane M: 100 bp DNA ladder; lane 1: negative control; lane 2: *Ureaplasma urealyticum* - BV2 (541 bp); lane 3: BVAB 1 - BV8 (90 bp); lane 4: BVAB 2 - BV9 (260 bp); lane 5: group G2.

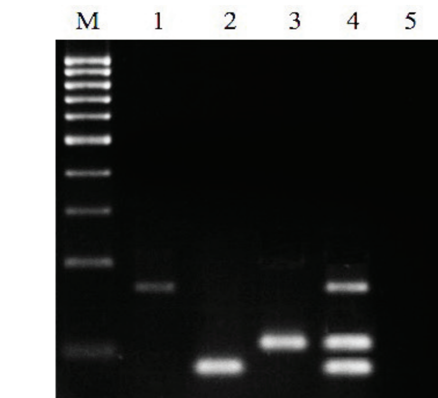

Fig. 4. Electrophoretic analysis of assay G3 in 3% agarose gel. Lane M: 100 bp DNA ladder; lane 1: BVAB 3 - BV10 (160 bp); lane 2: *Mobiluncus mulieris* - BV12 (80 bp); lane 3: *Sneathia sanguinegens* - BV13 (102 bp); lane 4: Group G3, lane 5: negative control.

The M-PCR reaction resulted in a separable band, as in s-PCR, without cross-reactivity between primer pairs. Clinical trials on the biospecimens showed the M-PCR products were successful in identifying bacterial species and can be used to test for BV caused by one species or co-infection with several species. Thus, this research has successfully developed the multiplex PCR process to simultaneously detect 10 common bacterial species that cause BV.

It is not possible to completely replace the classic diagnosis methods. However, this process has many advantages, especially its ability to accurately and simultaneously identify many pathogens as a basis for effective treatment. Besides, about the storage, samples used in PCR only require cold storage, so the conditions are not as strict as those for culture or Gram staining. In the near future, multiplex PCR can be expected to change the way medical laboratories analyse BV samples. This would lead to earlier diagnosis and prevention of possible complications in certain women without the impediments of high cost, long assay times and difficulties in workflow.

Conclusions and future plan

Conclusions

The study has established the success of the multiplex PCR procedure in the simultaneous detection of 10 common bacterial species that cause BV.

It has applied multiplex PCR as a clinical method for diagnosing BV in certain patients and has shown positive results.

Future plan

We plan to continue to optimise the process to create a database for future research on diagnosing BV.

We will use a larger sample size to increase the statistical significance and credibility of the study results.

The authors declare that there is no conflict of interest regarding the publication of this article.

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