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Isolation and Identification of *Bcteroides fragilis* Isolated from Women with Bacterial Vaginosis in Hillah City

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ABSTRACT

Bacterial Vaginosis remains one of the main infections that plaguing women during reproductive age. One of the fastidious anaerobic microorganisms that causes this infection is *Bacteroides fragilis.* Molecular methods may offer advantages over conventional culture due to the rapidity and the properties of these microorganisms. Molecular detection of bacterial vaginosis was done by using specific primer for 16 sRNA and primers for virulence factors; sialidase gene and LuxR gene. It was found that 16 sRNA genes were present in 44 (29.33%) of samples and sialidase gene present in 39(88.6%) and 34 (77.2%) for LuxR gene out of these 44 positive sample.

KEYWORDS: *Bacterial vaginosis* (BV), *Bacteroides fragilis*, polymerase chain reaction, sialidase, quorum sensing

1. INTRODUCTION

Bacteroides fragilis is part of human commensal ,it is an obligate anaerobic, Gram-negative, nonspore forming and rod-shaped bacterium (Kuwahara, 2004). It have role in vaginal infection by effects on vaginal epithelium, lead to facilitate invasion and causing infection (Polanco, 2012).

Culture techniques have long been the first approach in identifying the cause of infection. Several organisms associated with BV are difficult to cultivate, therefore their presence may be missed when using culture techniques and thus, it is not recommended as a diagnostic tool for BV (Sobel, 2000).

The uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates and to study bacterial phylogeny and taxonomy, these genetic marker used for different reasons. These reasons include (i) its presence in almost all bacteria; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene is large enough for informatics purposes (Janda and Abbott, 2012).

Quorum sensing is a bacterial cell–cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs) (Rutherford and Bassler, 2012). Negative bacteria typically use LuxI/LuxR-type QS systems for controlling Quorum Sensing. In these systems, the LuxI homolog is an autoinducer synthase that catalyzes a reaction between S-adenocylmethionine (SAM) and an acyl carrier protein (ACP) to produce a freely diffusible acyl homoserine lactone (AHL) AI (Ng and Bassler, 2009).

Sialidases are hydrolytic bacterial enzymes produced by BV-associated bacteria *Bacteroides fragilis*. Sialidase plays a role in downregulating the innate immune system in BV, since it degrades host defense molecules such as Immunoglobulin-A (IgA) against bacterial enzyme which can be harmful to the maintenance of a healthy vaginal environment. Sialidase-producer strains have higher adherence capacity to epithelial cells and biofilm formation, reinforcing the importance of this enzyme in the pathogenesis of BV and its likelihood of recurrence (Marconi, 2013).

2. MATERIALS AND METHODS

Collection of samples: Samples used in this study were 300 high vaginal swabs collected from 150 women as two swabs for each patients those were in the reproductive age ranging from15 to 45 years old and have bacterial vaginosis.

The patients had symptoms of abnormal vaginal discharge, odor and itching or burning. These women were attended to out-patient clinics of Gynecology and Obstetrics in Babylon Maternity and Pediatrics teaching hospital and Al-Hillah General Teaching Hospital during period from February to October 2016 .The sampling was carried out by specialized gynecologist and under sterile conditions.

Clinical diagnosing: Amsel's criteria were used to diagnosis bacterial vaginosis associated bacteria (BVAB), in women which present in trace amount and have a BV infected according to methods described by (Spiegel, 1983; Eriksson, 2011)

Molecular Analysis of BV Associated Bacteria using PCR Technique:

DNA extraction: Bacterial DNA was extracted from high vaginal swabs collected in phosphate buffer solution by using Geneaid DNA Mini kit (Geneaid /UK).

PCR Analysis: In order to detect the BV associated bacteria by using the primer specific for 16sRNA gene and for virulence factors by using sialidase and LuxR gene according to their references. PCR was used as a diagnostic technique. The reaction mixtures was described in Table.1.

Table.1. The Mixture of Polymerase Chain Reaction

No.	Mixture Contents	Volume (µl)
1	Master Mix	12.5
2	Forward Primer	2.5
3	Reverse Primer	2.5
4	Template DNA	5
5	Nuclase -Free Water	2.5
	Total	25

Molecular Detection of BV Associated Bacteria by using 16sRNA gene and study virulence factors sialidase and LuxR gene by using their genes: The set of PCR reactions was performed for diagnosing BV associated bacteria by using the primer specific 16sRNA gene as well as a virulence factors sialidase and LuxR genes according to their references. The genes were detected by PCR with primers illustrated in Table.2 and the programs were used in PCR analysis illustrated in Table.3, according to their references

Table.2. Oligonucleotide primers sequences and PCR products for detection *B.fragilis*

Gene's Name	Primer Sequence (5´- 3´)	Size (bp)	Reference
16sRNA	F-5' TTCGCTTTTCTGTTTCTGTGT3' R-5'CAGCAACCACCCAAACATTATT3'	555	GeneBank: HE608156.1
sialidase	F-5'TGAAGTTAGTGCCCAGATGCAGG3' F-5'GCTCAGCGCCCAGTATATGACC 3'	528	Gene Bank: D28493.1
LuxR	F-5' GGTCCAGAACCTCAGAAGCA 3' R-5' CATGGTCGCAGCATGCATTT 3'	334	NCBI Ref. Seq. NC_006347.1

Table.3. The programs were used in PCR analysis

Primer	PCR step	Temp.	Time	Repeat	Reference
16sRNA	Initial Denaturation	95C	5min	1	Procedure were designed in this study by using NCBI Gene-Bank and Optimise Writer Protocol online
	Denaturation	95C	30sec	30 cycle	
	Annealing	60C	30 sec		
	Extension	72C	1 min		
	Final extension	72C	5 min	1	
Sialidase	Initial Denaturation	95C	5min	1	
	Denaturation	95C	30sec.	30cycle	
	Annealing	60C	30 sec		
	Extension	72C	30 sec		
	Final extension	72C	1min	1	
LuxR	Initial Denaturation	95C	5min	1	
	Denaturation	95C	30sec.	30 cycle	
	Annealing	60C	30 sec		
	Extension	72C	30 sec		
	Final extension	72C	1 min	1	

3. RESULTS AND DISCUSSION

Clinical Diagnosis of BV: The results of the present study revealed that 300 samples taken from 150 positive bacterial vaginosis patient according to Amsel's criteria. Patients completed necessary questionnaires regarding symptoms, age, pregnancy, demographic data, reproductive and sexual health history including vaginal discharge. All these data will be mentioned to explain the relationship between these factors and BV. Amsel's clinical criteria was considered as one of the most standard method for BV diagnosis (Spiegel, 1983; Eriksson, 2011). Amsel's Criteria revealed that all 150 of women patients gave positive results.

Molecular Analysis of genomic DNA by using PCR technique: The detection of the *B.fragilis* by using specific primers for 16sRNA gene gave an amplicon of (555) bp fragment as shown in Figure.1. It was found that 44 (29.33%) *B.fragilis* isolates were detected molecularly by using specific primers based on 16 sRNA gene that is species – specific gene for differentiation between bacterial vaginosis.

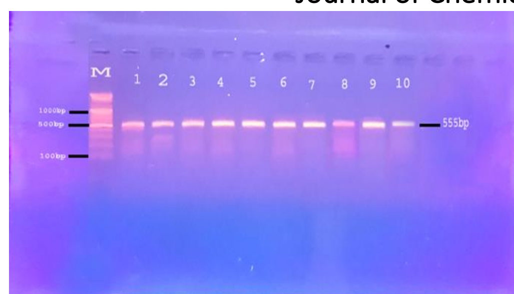

Figure.1. 1.5% Agarose gel electrophoresis at 70 volt for 50 min for 16sRNA gene PCR products visualized under UV light at 280 nm after staining with ethidium bromide.Where M: marker (2000-100bp), lane (1-10) positive isolates with amplicon size (555bp)

The 16S ribosomal RNA is the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The genes coding for it are referred to as 16S rRNA gene and are used in reconstructing phylogenies , gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria (Pereira**,** 2010).

As the widespread use of PCR and DNA sequencing, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in clinical microbiology laboratories. For bacterial identification, 16S rDNA sequencing is important in case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infections. It provided insights into aetiologies of infectious disease, so helps clinicians to prescribe antibiotics and in determining the duration of treatment and infection control procedures (Woo, 2008).

The 44 positive isolates were examined for sialidase enzyme by amplification a primer of (528) bp fragments as shown in Figure.2, the results of detection showing that 39 (88.6 %) positive isolates for sialidase gene.

Figure.2. 1.5% Agarose gel electrophoresis at 70 volt for 50 min for sialidase gene PCR products visualized under UV light at 280 nm after staining with ethidium bromide.Where M: marker (2000-100bp), lane (1-12) positive isolates with amplicon size (528 bp)

This result in agreement with study by (Dongyou, 2011) that defined the neuraminidase enzyme or sialidase as a group of enzymes that cleave sialic acid, a carbohydrate occurring on the surfaces of cells in humans ,animals and microorganisms. It coding by *nanH* gene, as this enzyme is produced by *B.fragilis*, it has been one of the choice methods for detection and identification of this bacteria from clinical specimens.

The detection of *B.fragilis* by using *nanH* gene is identical to what done (Obata-Yasuoka, 2002)s where with depending on this gene used vaginal swabs from bacterial vaginosis infected nonpregnant women ,were detected *B. fragilis* as one causative agents by using PCR method.

While the examined isolates gave 34 (77.2%) positive result for LuxR gene. This gene was detected by using specific primer gave an amplicon of (334) bp fragment as shown in Figure.3.

Figure.3. 1.5% Agarose gel electrophoresis at 70 volt for 50 min for LuxR gene PCR products visualized under UV light at 280 nm after staining with ethidium bromide. Where M: marker (2000-100bp), lane (1, 2, 4, 8, 10, 11 and 12) positive isolates with amplicon size (334bp)

As *B.fragilis* one of bacteria use quorum sensing (QS) to monitor cell density in relation to other cells and their environment. The LuxR system is common among Gram-negative bacteria. The *luxR* gene encodes a transcriptional activator inducible by type I acyl-homoserine lactone autoinducers (e.g., *N*-[3-oxohexanoyl] homoserine lactone and hexanoyl homoserine lactone [C6-HSL]) that was affected on biofilm formation during exponential phase of growth (Pumbwe, 2008).

LuxR transcriptional regulator is a key player in Quorum Sensing (QS), coordinates the expression of a variety of genes, including those encoding virulence factors and antibiotics biosynthesis, motility, nodulation, plasmid transfer, bioluminescence, and biofilm formation (Chen, 2011).

4. CONCLUSION

Bacterial vaginosis is important infection especially that causes with fastidious anaerobic microorganisms.

16 sRNA gene is effective for rapid detection of *B. fragilis.*

Sialidase enzyme, is important virulence factor that assisting the microorganism for causing infection.

Lux R gene is one of important genes that contribute in biofilm formation by bacteria that is responsible for antibiotic resistant and recurrent infections.

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