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Molecular Assessment of the Bacterial Diversity in Women with Breast Infections

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Abstract:
In the present study, a total of 100 clinical samples were collect and processed from patient suffering from breast infection or mastitis carried out in two main place in Babylon province i.e. Al-Hilla General Teaching Hospital (breast cancer consolation) and outpatient clinics, from November (2017) to April (2018), which they were diagnosis as breast infection or mastitis by the physician after the medical tests included the clinical examination of the patient and U/S, mammography).
Also PCR amplification test for universal gene ,*uni678*gene, *uni1870*gene . and *sau* gene were used for direct detection of bacterial isolates from clinical samples and the result showed that out of 100 samples ,only (37,40)sample contain gene to universal primers ,*uni-678*(210) bp and *uni-1870*(438)bp respectively .
Polymerase chain reaction (PCR)used for detection virulence genes that coding to hemolysin of *S.aureus* isolates ,it was found that positive results were showed for *hla,hlb, ,hlg* and *hld* that with percentage ,(45.4%,(32%),(36%)and (54%) respectively .

INTRODUCTION

Mastitis is an infection of tissue of the breast that occurs most frequently during the time of breastfeeding. It can occur when bacteria, often from the baby's mouth, enter a milk duct through a crack in the nipple.1
Breast infection most commonly occurs one to three months after delivery of a baby, but they can occur in women who have not recently delivery as well as in women after menopause. Other causes of infection include chronic mastitis .2
Hormonal changes in the body can cause the milk ducts to become clogged with dead skin cells and debris. The clogged ducts make the breast more open to bacterial infection. The infection tends to come back after treatment with antibiotics .1
Sometimes a breast abscess can complicate mastitis. Noncancerous masses such as abscesses are more often tender and frequently beneath the skin. The edge of the mass is usually regular and well defined.3,4 .
Bacterial breast infections typically involve the fatty tissue in the breast. Swelling, localized pain, redness, and fever are the most common symptoms of a bacterial infection. When a breast infection causes a collection of pus, it is called an abscess. Most bacterial breast infections respond to antibiotics but in more severe cases .
Non-lactational mastitis occurs in women with weakened immune systems, including women who have had lumpectomies with radiation therapy and women with diabetes. Some infection-like symptoms are a sign of inflammatory breast cancer, but this is very rare .5
Mastitis is a multi-etiological disease involving blend of different pathogens. Although *Staphylococcus aureus* was consider as a major mastitis pathogen, many other opportunistic pathogens are known to cause mastitis including, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus agalactiae* and *Klebsiella pneumoniae* .6
The success of *S. aureus* as a pathogen is attributable, in part, to the diverse range of virulence factors produced. The virulence factors facilitate the invasion and colonization of host tissue, evasion of the hosts' immune defence mechanisms, aid in acquisition of nutrients and dissemination of the bacteria within the host tissue.7 .Among the vast array of virulence factors produced are numerous enzymes and cytotoxins, such as coagulase, collagenase, exfoliative toxins, hemolysins, hyaluronidase, leukocidins, lipases, nucleases and staphylokinase.8
Now a days Coagulase negative staphylococci (CNS) are emerging as major mastitis pathogens worldwide.9
coagulase Negative Staphylococci, *Staphylococcus aureus*, *Streptococci viridans*, *Enterococcus faecalis* and Group B streptococci were the most abundant bacteria isolated from the breast milk of mastitis suffering women. 10
Mastitis is often treated with antibiotics. But nowadays development of multiple resistance against antibiotics in different bacteria has led to treatment failure. Reason to the treatment failure is due to indiscriminate use of antimicrobials without checking its' in vitro sensitivity to the causing bacteria . 11
The application of culture-independent molecular techniques, and particularly those based on *16S rRNA* genes, allowed a complementary biodiversity assessment of the human milk microbiome. The use of such techniques confirmed the dominance of staphylococci and streptococci, the relatively frequent presence of lactic acid bacteria and bifidobacteria, and the existence of DNA belonging to other bacterial groups.12
So the aim of this study to the detection of the main causes of bacterial breast infection using molecular methods.

MATERIALS AND METHODS

Ethical approval:
The necessary ethical approval from ethical committee in Al-Hilla General Teaching Hospital (breast canser consulation) and out patient clinics was obtained. moreover, agreement from the family and patients for sampling and carrying out this work was obtained ".
Patients Specimens:
This study includes a total of 100 specimen from patients who admitted to Al Hilla General teaching hospital and outpatient clinics for during the period from November 2017 to April 2018.
Milk and abscess specimen were collected from patients that admitted the Breast cancer consultation with specific symptom as redness and pain in the breast that are suspected to breast infection according to the diagnosis of physician by sterile clean method .
Patient specimen collection:
These Specimens were collected by physician depending on the following steps:
1-localization of the site of the abscess by clinical examination or by ultra sound.
2-Sterilazationof the area by povidine iodin 10% and usage of syring 10cc of 21 or 23gaug needle to get the sample of abscess.

3-If a sign of mastitis whith out evidence of abscess formation on clinical background and U/S confirmation the sample from milk of breast.

4- Taken nipple discharge milk as a sample after sterilization of the nipple , by sequeeze of the nipple to get sample, which was put in the tube or swap.

5-Pour the milk or abscess in plain tube contain to phosphate buffer to prevents dry form.13

Direct Extraction of DNA from clinical samples

This method was applied according to the genomic DNA purification kit that supplemented by the manufacturing company (Geneaid, UK). The suspension containing DNA was stored at-20 C until used as template for PCR.

Amplification of specific primers in this study:

A number of primers that were used in this study whether for bacterial identification, study of virulence factors; all these primers are listed in **Table-1**, with their thermal conditions. PCR mixture consists of a pair of specific primers of each gene. A single reaction mixture contained 2.5µl of forward, 2.5µl of reverse, 5µl of extracted DNA, 12.5µl of master mix and 2.5µl of nuclease free water. PCR amplification was confirmed by agarose gel electrophoresis by visualization against UV light. Electric current was allowed to pass at 70 volt for 50min. UV trans-illuminator was used 280 nm for the observation of DNA bands, and the gel was photographed using digital camera.14

RESULT AND DISCUSSION

Direct Detection of bacteria by molecular detection (PCR)

DNA was extracted from all (100) samples collected from infected women, conventional PCR was carried out using these DNA from the amplification of specific primer (Uni678,Uni 1087,sau).after the gel electrophoresis showed that Out of 100samples ,only (37 , 40)samples contain gene to universal primers ,Un i -678(210) bp and uni1870 (438)bp respectively ,fragment when compared with allelic ladder as show in figures (1, 2). which tested against six bacterial species *E. coli, S. aureus* ,S. agalactiae ,*S. dysgabectiae, S. paroutens* ,*S. uberis*.

Table 1: The Primer Sequences and PCR Condition

Genes	Primer sequence (5′-3′)	Size (bp)	PCR condition	Reference
UNI 678	F- AGTGGAATTCCATGTGTAGC R- GAGTGCTTAATGCGTTAGCT;/P|	210	94ºC 10min 1x 94ºC 2min 57ºC 1min 35x 72ºC 1min 72ºC 10min 1x	Rainard *et al* ., 2003
UNI 1870	F- TGGAAGGTTAAGAAGAGTGG R- GCCTCCGTTACCTTTTAGGA	438	94ºC 10min 1x 94ºC 1min 55ºC 1min 30x 72ºC 1min 72ºC 10min 1x	Rainard *et al* ., 2003
Hla	F-GCCTCCGTTACCTTTTAGGA R-CTTTCCAGCCTACTTTTTTATCAGT	327	94ºC 10min 1x 94ºC 1min 64-68ºC 1min 30x 72ºC 1min 72ºC 10min 1x	Mehr otra, *et al 2000*
Hlb	F-GTGCACTTACTGACAATAGTGC R-GTTGAGTAGCTACCTTCAGT	465	95ºC 10min 1x 95ºC 30sec 60 ºC 1min 40x 72ºC 30sec 72ºC 10min 1x	Mehr otra *et al., 2000*
Hld	F-AGAATTTTTATCTTAATAATTAAGGAAGGAGTG R- TTAGTGAATTTGTTCACTGTCTCGA	93	94ºC 10min 1X 94ºC 1min 57ºC 2min 30x 72ºC 1min 72ºC 10min 1X	Haenni *et al., 2001*
Hlg	F- GTCAYAGAGTCCATAATGCATTTAA R- CACCAAATGTATAGCCTAAAGTG	867	95ºC 2.5min 1x 94ºC 30sec 55ºC 1min 30x 72ºC 30sec 72ºC 7min 1x	Mehr otra *et al ., 2000*
Sau	F- ATAAGAGATGGCGGTACTAAA R-TAAGGCGATTACACGTTACT	530	95ºC 3min 1x 95ºC 2min 54ºC 1min 30x 72ºC 1min 72ºC 10min 1x 94ºC 1min 55ºC 1min 30x 72ºC 1min 72ºC 7min 1x	Smyth *et a*l2005

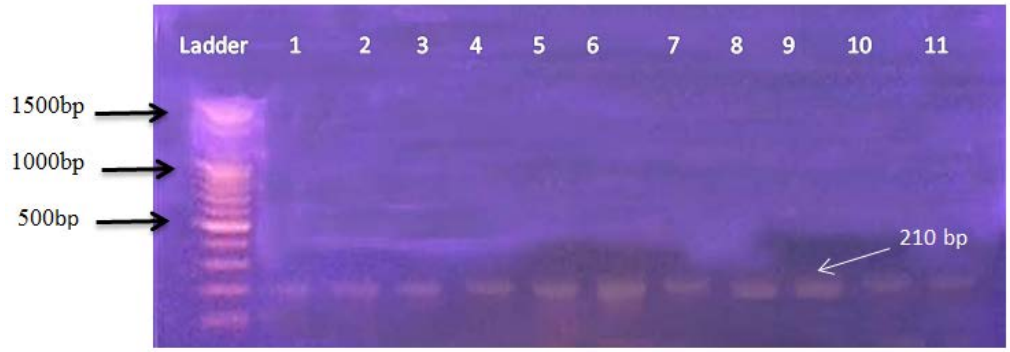

Figure 1: Agarose gel electrophoresis at 80 volt for 30 min. for *uni-678* gene in *S. aurous*. PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp this positive for this gene with amplicon size210 bp.

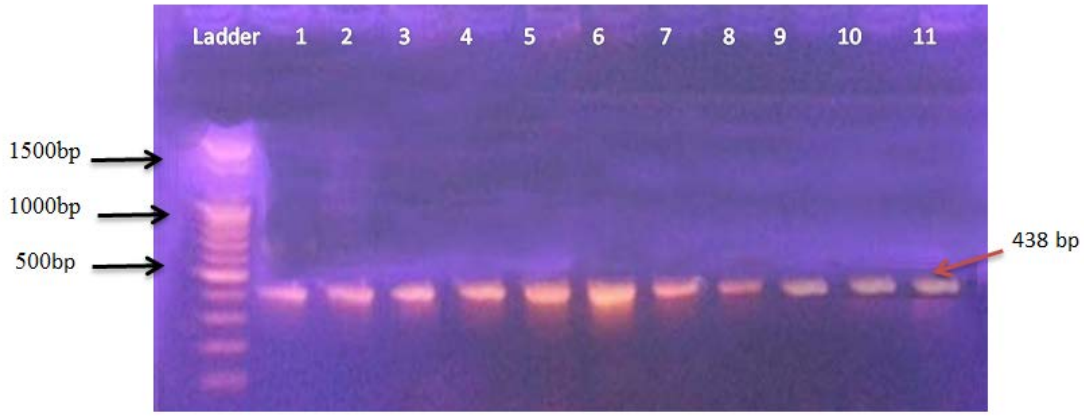

Figure 2: Agarose gel electrophoresis at 80 volt for 30 min. for *uni-1870* gene in *S. aurous*. PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp this positive for this gene with amplicon size438 bp

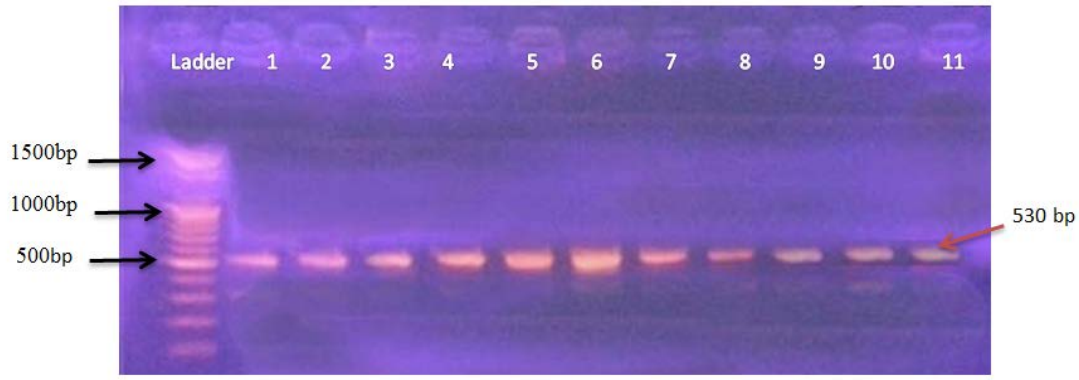

Figure 3: Agarose gel electrophoresis at 80 volt for 30 min. for *Sau* gene in *S. aurous*. PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 2000 bp this positive for this gene with amplicon size530 bp.

These set of genes Used to detect all pathogencited in breast infection ,these primer set ware designed from DNA regions coding for 16S and 235 rRNA respectively and nucleotide sequence data, showed that these set primer are conserved for all pathogen for mastitis.15 (Riffon *et al*;2001) .

Sau gene is also used in this study which is specific gene for detection of *S. aurous* also to confirmation diagnosis for *S. aurous*, out of 100samples,it was observed that only 50 of samples contain this gene with 530 bp fragment as shown in figure (3).

This result also identical to result obtain by culture method which refer that 53 samples belong *to S. aurous* isolates . The difference in the result may be attributed to the amount of DNA used in PCR, and the difference in the copy numbers of the coding regions for the set of primers could explain that also the negative result may due to the gene don't express or non-functional gene .or the gene not found.

Generally ,these genes used study bacterial identification taxonomy and to phylogeny because they are the most communal house keeping genetic markers, and there function over times has not changed ,also there presence in virtually all bacteria often existing a multi gene family or operon.16. In addition the different in the conventional method by culturing of bacteria cell and biochemical tests with molecular detection ,refer to the presence of other species or sub species ,also biochemical based methodologies for identification are not conclusive in many cases ,which may be affected by some environmental laboratory condition .

Molecular Detection of Enterotoxin Genes:
Classical Heamolysin Toxin Genes:
***hla , hlb,hld and hlg* genes:**
Specific primers (*hla,hld,hlb* and *hlg*) were used to amplify the four hemolysin virulence genes in all *S. aureus* isolates. To amplify the genes, 25 ml of reaction mixture was made containing 20 ng of template DNA, 100 ng of primers,160 mM of dNTP mix, 1.25 U Taq polymerase, 1X Taq buffer,and 0.5 mM MgCl2. All 53 isolates were amplified individually for all four genes using the specific primers.

The results of this study was found that 24(54.5%) of *S. aurous* isolates are bored of the *hld* heamolysin coding gene, followed by *hla* heamolysin coding gene which constituted 20(45.4%) of all isolates then *hld* which constituted 16 (36.3%) of all isolate followed by *hlb* which constituted 14(32%) only from all isolates .This done by using PCR technique with specific primers with molecular length (327,856,) bp respectively when compared with allelic ladder as shown in figures (4, 5, 6, 7).

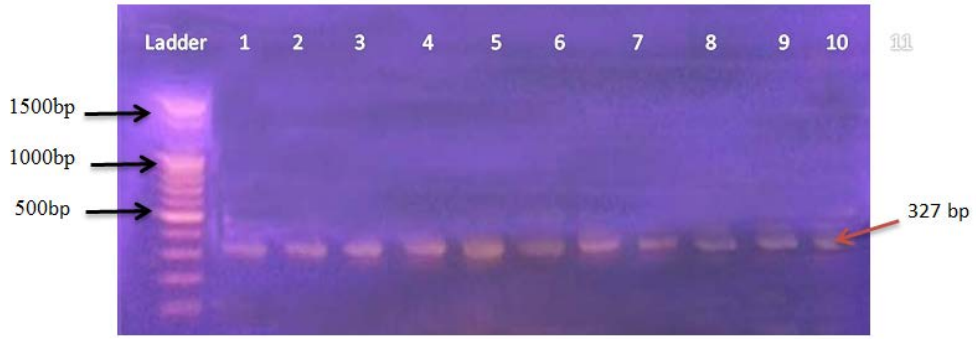

Figure (4) Agarose gel electrophoresis at 80 volt for 30 min. for *hla* gene in *S. aurous*. PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp this positive for this gene with amplicon size327 bp

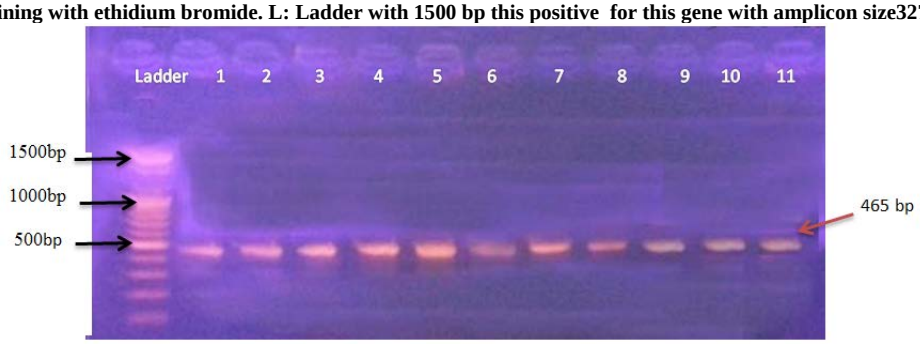

Figure 5: Agarose gel electrophoresis at 80 volt for 30 min. for *hlb* gene in *S. aurous*. PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp this positive for this gene with amplicon size465 bp.

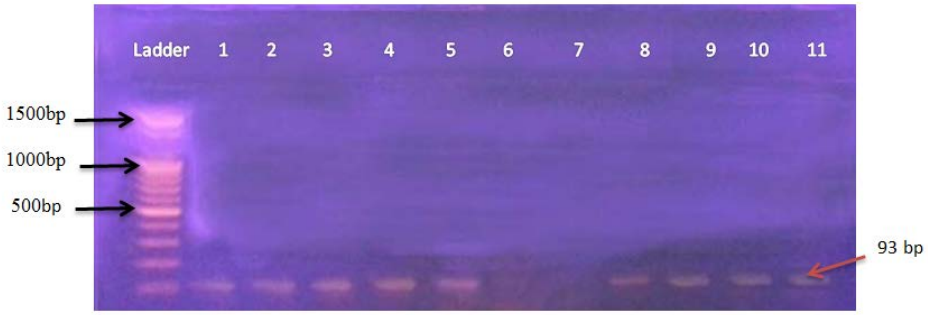

Figure 6: Agarose gel electrophoresis at 80 volt for 30 min. for *hld* gene in *S. aurous*. PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp this positive for this gene with amplicon size93 bp

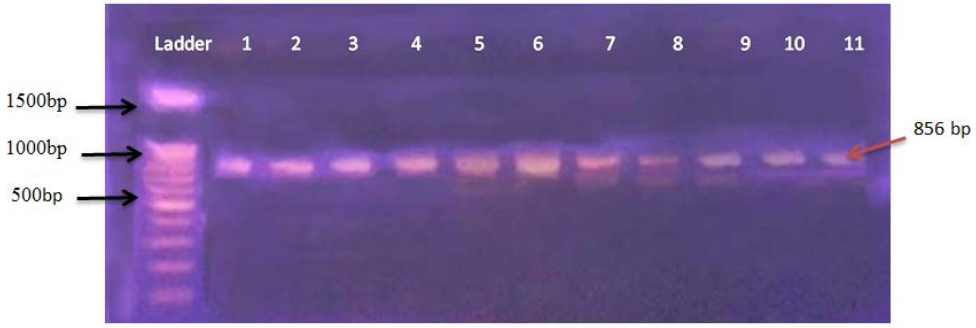

Figure 7: Agarose gel electrophoresis at 80 volt for 30 min. for *hlg* gene in *S. aurous*. PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp this positive for this gene with amplicon size867 bp.

The result is not agree with.17,18,19 . Who said that 100% of *S. aureus* isolates express *hla* agene .

This results also disagree with.20 ,who said that *S. aurous* isolates express *hla* ,*hlb* ,*hld* ,*hlg*. The *S. aureus* and hemolysin was the first described of a family of bacterial pore forming β. barrel toxin ,play on important role in the pathogenesis of Staphylococcal disease ,and it has been found that substitution in amino acid residues may reduce the activity of α-Hemolysin. 21

The large degree of heterogeneity in the prevalence of hemolysin gene can be due to ,1-the presence or absence of a toxin gene , 2-the disruption of toxin –encoding loci, 3-variation in the expression level.22

One study found that α-Hemolysin gene found in 87% of strain .23

While other gene study described a prevelence of α-Hemolysin gene in 30% of isolates ,observed that *hla* and *hla* genes were present in all *Staphylococcus* isolates ,while the *hlb* was found in 15% and ,the research show that the double hemolysin observed in human isolates make the isolates more pathogenic .(24)

Also combination of alpha(327)bp and delta(93)Pb,beta hemolysin was observed in 40%of human strains, the hypothesis that this strain are more pathogenic(.25,26). The role of *hla* gene in *S. aurous* virulence is not well understood. It is only known that hlg ABC locus is associated with strain able to cause human colonization, but no association with a particular infection types.27

Oliverira *et al*; 2013. found that this toxin (β- hemolysin) is produce in large quantities in *S. aureus* strain isolated from mastitis and human skin infection .

β- hemolysin is cytotoxic towards human keratinocytes, Polymorphonuclear leucocyte , monocyte and T- lymphocyte and inhibit interleukin 8 expression by endothelial cell ,and induce biofilm formation in *S. aureus* .28

CONCLUSION

Potentially pathogenic bacteria, often at high levels, were found in breast milk from both healthy donors and women with lactational mastitis. Increasing bacterial counts did not affect the clinical manifestation of mastitis .

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