STUDY OF MOLECULAR COMPOSITION OF VIRULANCE BACTERIA ISOLATED FROM BOVINE MASTITIS WITH BIOFILM PRODUCTION

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ABSTRACT

This study was carried out in order to isolate and diagnosis of common bacteria from bovine mastitis in Baghdad city which have the ability to produce biofilm and detect its molecular composition. Twenty five milk samples were collected from different regions in Baghdad city from udders of cows suffering from clinical and subclinical mastitis.Then cultured on blood agar. Gram staining was done to differentiate between bacteria gram positive which cultured on Mannitol Salt Agar and Nutrient Agar while gram negative bacteria were cultured on MacConkey agar and Eosin Methylene Blue agar. All bacterial isolates were subjected to different biochemical tests, API 20 E System, API Staph System and RapID^{TM}ONE System kit to confirm the diagnosis.Christensen tube method was used to detect the ability of the diagnostic bacterial isolates to produce biofilm. Specific forward and reverse primers were designed according to a program from NCBI-Genbank for *Staphylococcus aureus* (Genbank code: gb |KR265472.1|), for *Escherichia coli* : gb|JQ781567.1| and for *Klebsiella pneumoniae* gb|KT944736.1|, To study the sequence of these genes after amplification of 16srRNA genes by using PCR was appeared because it is sensitive and highly specific assay serve as suitable molecular diagnostic tool for detection and compare these genes sequencing with references strains.Results showed that 8 (32%) out of 25 milk samples were positive for *Staphylococcus* , 6 (24%) out of 25 samples were positive for *Klebsiella pneumonia* . and 11(44%) were positive for *E.coli* . The results showed that 22 (88%) isolates out of 25 milk have the ability to produce biofilm . Genetic identities results showed that *Staphylococcus aureus* and *Klebsiella pneumonia* isolates gave 99% matching and resembling the reference strains while *Escherichia coli* isolates identity showed resemble match of 100% with the reference strain

Keywords: ***staphylococcus aureus, Escherichia coli , Klebsiella pneumoniae*, Biofilm, 16srRNA genes , PCR.**

دراسة التركيب الجزيئي للبكتريا المعزولة من حالات التهاب الضرع المنتجة للغشاء الحيوي

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المستخلص

هدفت هذه الدراسة الى عزل وتشخيص بعض البكتريا الشائعة من حالات التهاب الضرع البقري وتحديد قدرتها على انتاج الغشاء الحيوي والتحري عن التركيب الجزيئي لهذه العزلات .جمعت خمسة وعشرون عينة حليب من اضرع الابقار المصابة بالتهاب الضرع السريري وتحت السريري . من مناطق مختلفة من بغداد وزرعت على وسط اكار الدم وتم عمل ملون كرام لتفريق البكتريا الموجبة لملون كرام اذ زرعت على وسط تخمر سكر المانتول والوسط المغذي بينما البكتريا السالبة لملون كرام زرعت على وسط الماكونكي ووسط صبغة المثيل الازرق تم اخضاع جميع العزلات البكتيرية الى مختلف الاختبارات الكيموحيوية واختبارات API 20 E System و API Staph System RapID^{TM}ONE System kit لتأكيد التشخيص استخدمت طريقة انابيب كريستينسن لتحديد قابلية البكتريا المعزولة والمشخصة لانتاج الغشاء الحيوي و تم تصميم البادي الاولي والعكسي تبعا لبنك الجينات العالمي لبكتريا , *Staphylococcus aureus* (Genbank code: gb |KR265472.1| *Escherichia coli* : gb|JQ781567.1| gb|KT944736.1| *Klebsiella pneumoniae* لدراسة التسلسل الجيني بعد مضاعفة 16srRNA للجين باستخدام تقنية تفاعل البلمرة التسلسلي الذي يعد من التقنيات الحساسة جدا كأداة تشخيص لتحديد ومقارنة التسلسل الجيني مع بنك الجينات العالمي . اظهرت النتائج ان 8 عينة (32%) من اصل 25 عينة حليب كانت موجبة لصفات البكتريا *Staphylococcus aureus* و 6 عينة (24%) من 25 عينة حليب كانت موجبة لصفات بكتريا *Klebsiella pneumoniae* و 11 عينة (44%) كانت تحمل صفات بكتريا *Escherichia coli* بينت النتائج قابلية جميع هذه العزلات على انتاج الغشاء الحيوي بينما اظهرت النتائج ان 22(88%) عزلة من اصل 25عينة حليب كانت لها القدرة على انتاج الغشاء الحيوي اظهرت نتائج التطابق الجيني لعزلات بكتريا *Staphylococcus aureus* و بكتريا *Klebsiella pneumonia* نسبة تطابق 99% مع العينة المسجلة في بنك الجينات العالمي بينما كانت نسبة التطابق 100% بالنسبة لعزلة بكتريا *Escherichia coli*

الكلمات المفتاحية: ***Staphylococcus aureus, Escherichia coli Klebsiella pneumonia* , 16srRNA تفاعل البلمرة التسلسلي .**

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INTRODUCTION

Mastitis is inflammation of the parenchyma of the mammary gland regardless of the cause, mastitis characterized by a range of physical and chemical changes in the milk and pathological changes in the glandular tissue(22). the most important changes in the milk include discoloration, the presence of clots and the presence of large numbers of leukocytes, there is swelling, heat, pain, and odema in the mammary gland in many clinical cases (19). *E.coli* mastitis remains one of the most costly disease in farm animal , and this disease affected many high producing cows in dairy herds and may cause several cases of death per year in most sever cases with economic losses to the dairy industry (6). Recent research on biofilm formation from *E.coli* and *Streptococcus uberis* shed an interesting light on the whole dynamic process of bacterial invasion , adherence ,persistence , evasive strategies of these bacteria and reveal parallels and differences between these bacteria and *Staphylococcus aureus* (8). The search for a successful treatment of biofilm infections that can prevent and eradicate biofilms in the clinical environment is still ongoing (21).From all major pathogens in bovine mastitis biofilm behavior is best studied in *Staphylococcus aureus*, the reason for the preference of *S. aureus* is presumably related to the importance of this species both for bovine mastitis and human (17)**.** The formation of bacterial biofilms of *E.coli* and *Klebsiella pneumonia* in a host in general seems to be, based on current evidence, to a large extent anintra-cellularevent(4). Biofilms are not easily defined as they vary greatly in structure and composition from one environmental niche to another, Microbial biofilms are extremely complex microbial ecosystems consisting of microorganisms attached to a surface and embedded in an organic polymer matrix of microbial origin, As well as microbial components, non-cellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, may also be found in the biofilm matrix (21) Biofilms ,particularly in water systems ,can be highly complex , whilst others such as those on medical devices , may be simpler , and composed of single , cocoid or rod shaped organisms, Given these differences , it does not seem plausible to suggest that a true “biofilm” model can be defined that is applicable to every ecological , industrial and medical situation, Therefore the definition of a biofilm has to be kept general and thus may be redefined as“ microbial cells immobilized in a matrix of extra cellular polymers acting as an independent functioning ecosystem, homeostatically regulated” (20,24). There are a variety of ways in which biofilms can cause health issues in humans ,as well as animals with wounds and mastitis being two common clinical challenges in animals, although research is still compiling evidence on the role of biofilms in veterinary medicine ,much of the information regarding biofilm infection and disease has been extrapolated from human research and applied to the veterinary environment as there are still many gaps in veterinary biofilm research (9).The diseases caused by a particular strain of *E. coli* depend on distribution and expression of many virulence determinants such as adhesion ,biofilm formation, production of haemolysin, enterotoxin, shiga toxin ,endotoxin and capsule formation (13). Bacterial diagnosis by molecular methods have proved beneficial to overcoming some limitations of the conventional biochemical and serological methods and improved sensitivity and rapidity (13, 25), Species-specific PCR assays are available for some bacteria (11), and the polymerase chain reaction was found useful, specific and time saving for identification of bacteria which have the ability to produce biofilm using different oligonucleotide primers (23). Improved gene responsible for produced biofilm detection by PCR, two amplicons based PCR assay were used, targeted *E. coli,Klebsiella pneumonia* and *Staphylococcus aureus* specific 16S ribosomal RNA (rRNA) gene (1, 14). This study aimed to detect the ability of those bacterial isolates from mastitis cases to produce biofilm and identifying its molecular composition.

MATERIALS AND METHODS

Twenty five milk samples were collected from different regions of Baghdad city from udders of cows suffering from clinical and subclinical mastitis. The udder was washed directly with tap water to remove dirt then dry with clean towel, the teat dip in Iodine solution 1:1000 and leave to dry than the teat was dip in 70% alcohol than dry, before sample taken one or two streams of milk discarded. Milk was collected in sterile vial (test tube 10 ml). These samples were transfered to the lab. In cooled container. all these samples were cultured firstly on blood agar , then subjected to gram staining , gram positive bacteria were cultured on mannitol salt agar and nutrient agar while gram negative bacteria were cultured on MacConkey agar and EMB agar.Morphological,cultural and Biochemical tests in addition to API 20 E system and Rapid ^{TM} one system kit were used to complete and confirm diagnosis of these bacterial isolates. A qualitative assessment of biofilm formation was determined by tube method (10). In order to detect the sequence of 16 S rRNA genes of these isolates, Extracted DNA was subjected to the different steps of PCR technique. Table (1) show the components of the PCR and Table (2) show the mixture of the specific interaction for diagnosis gene.Specific primers for 16 S rRNA gene were used to detect the sequence Table(3,4,5),and Table (6) show the optimal conditions used to detect 16 S rRNA.

Table 1. The Components of the PCR

Material	Volume
MgCl	**4 mM**
DATP	**400 µM**
dGTP	**400 µM**
dCTP	**400 µM**
Dttp	**400 µM**
Taq polymerase	**2.25 unit**

Table 2.Mixture of the specific interaction for diagnosis gene

Components	Concentration
Taq PCR PreMix	**5µl**
Forward primer	**10 picomols/µ**
Reverse primer	**10 picomols/µ**
DNA	**1.5µl**
Distill water	**16.5 µl**
Final volume	**25µl**

Table 3.The primers used in the interaction *The specific primer of gene 16s rRNA* of *S.aureus*

Primer	*Sequence*	*Tm (°C)*	*GC (%)*	*Product size*
Forward	**5'-AGAGTTTGATCCTGGCTCAG-'3**	**60.0**	**68.25 %**	**1450 base pair**
Reverse	**5'-GGTTACCTTGTTACGACTT-'3**	**62.00**	**67.59 %**	**1450 base pair**

Table 4.The primers used in the interaction *The specific primer of gene 16s rRNA* of *E.coli*

Primer	*Sequence*	*Tm (°C)*	*GC (%)*	*Product size*
Forward	**5'-GCAGCCTTCGTCTTGGTCAG-'3**	**65.0**	**64.35 %**	**1356 base pair**
Reverse	**5'-AGTTCCGTAGTTATTACAA-'3**	**67.00**	**62.78 %**	**1356 base pair**

Table 5.The primers used in the interaction *The specific primer of gene 16s rRNA* of *Klebsiella pneumonia*

Primer	*Sequence*	*Tm (°C)*	*GC (%)*	*Product size*
Forward	**5'-GCAGCCTTCGTCTTGGTCAG-'3**	**60.0**	**68.25 %**	**1345 base pair**
Reverse	**5'-AGTTCCGTAGTTATTACAA-'3**	**62.00**	**67.59 %**	**1367 base pair**

Table 6.The optimum condition of detection 16s Rrna

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	94°C	3 min.	
2	Denaturation	94°C	1 sec	
3	Annealing	59°C	1sec	35cycle
4	Extension-1	72°C	1sec	
5	Extension -2	72°C	7 min.	

RESULTS AND DESCUSSION

Results of bacterial isolation and diagnosis showed that out of 25 mastatic milk samples were collected from cows suffering from mastitis ,8(32%) were positive to the presence of *S. aureus* this is come in agreement with (16). after culturing on Manitol salt agar and subjected to various Biochemical tests and Rapid ^{TM} One system kit. while 6(24)%,11(44%). of the remmining collected samples were grow on Macconkey agar and EMB agar , the biochemical tests in addition to API20E system confirmed that these isolates belong to *Klebsiella pneumonia* and *E.coli*. -Mohammadi *et al*.(18). found that 36 out of 206 raw milk samples collected from various cow farms in Kermanshah zoon showed positive results for *E.coli* (17.47%) Aseel M and AR .Hassan (5). found that 80 out of 96 milk samples were collected from local market in Baghdad contaminated with *E.coli* (83.9%) and this is disagree with presentresults. On the other hand, Christensen tube method showed that 22(88%) of bacterial isolates from biofilm with the thickness ranged from 0.2 mm to 3 mm. this is in agreement with (16) who showed that 46 of total 56 isolate of *Staphylococcus aureus* produced biofilm (82.14%) while only 10 isolates gave negative results (17.85) , (2). Who showed that 50 out of 54 *E.coli* isolates isolated from milk samples showed the ability to produce biofilm (92.6%) while 4 (7.4%) *E.coli* isolates gave the negative results for it’s biofilm production while (26) demonstrated that 32 of 35 *S. aureus* isolates were produced biofilm (91.42) these results came in agreement with our results percontra (7) found a lower percentage (12%) of biofilm-positive producer strains in 92 bovine strains tested.Genetic identities results showed that *E.coli* isolates matching 100% with the reference *E.coli* strain (c73) in its gene sequence while gene sequencing differ 1% between local isolates of *klebsiella pneumonia* and *Staphylococcus aureus* and the resemble reference strains fig 1,2,3. and these difference in gene sequence lead to difference in Location of Nucleotide Transition and Transversion as showen in table (7,8). The principle of gene expression depend on transformation the storage genetic information in gene as sequence of nucleotids then to protein active in some properties like transcription and translation (27).The gene expression happen duo to the structural gene or refer to as citron which is apiece of DNA (3).In gene expression different bacteria tend to form biofilm which are a layer of exopolysacchrides and gene expression for biofilm formation differ according to the conditions that are inappropriate like starvation of nutrients and decreasing in PH because biofilm formation are important cause for more infection (27).In the study of Ghafoor and Rahm (12) they found that operon pel and operon psl are responsible for gene expression to form biofilm in *pseudomonas aeruginosa* and operon pel consist of seven genes (PA3058 to PA 3064)which are responsible to expression for exopolysacchrides that are rich in glucose that form the matrix of biofilm.Ma. L.(15) found that operon pel arrange biofilm formation duo to gene expression , while operon psl consist of 15 genes (PA 2231 to PA 2245)that encodes to exopolysacchrides rich in mannose and galactose which are more important in development and differentiation of biofilm.

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Score             Expect      Identities          Gaps          Strand
1988 bits(1076)   0.0         1076/1076(100%)     0/1076(0%)    Plus/Plus

Query  1    CTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28   CTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGG  87

Query  61   AGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88   AGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGG  147

Query  121  GGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148  GGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTA  207

Query  181  ACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  ACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAC  267
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Fig1. *Escherichia coli* strain c73 16S ribosomal RNA gene, partial sequenceSequenceID: gb|JQ781567.1|

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Score             Expect      Identities          Gaps          Strand
1827 bits(989)    0.0         1007/1016(99%)      0/1016(0%)    Plus/Plus

Query  1    CTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    CTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG  62

Query  61   GATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGGACC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   GATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGGACC  122

Query  121  TTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  TTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGC  182

Query  181  TCACCTAGGCGACAATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGA  240
            ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
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Fig 2. ***Klebsiella pneumoniae*** **strain Apf-15 16S ribosomal RNA gene, partial sequenceSequence ID: gb|KT944736.1|**

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Score             Expect      Identities          Gaps          Strand
1838 bits(995)    0.0         1015/1025(99%)      0/1025(0%)    Plus/Plus

Query  1    TGCAGTCGAGCGAACGGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGCAGTCGAGCGAACGGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGT  60

Query  61   AACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCG  120

Query  121  GATAATATTTTGAACCGCAGGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGAT  180
            ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GATAATATTTTGAACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGAT  180

Query  181  GGATCCGCGCTGCAGTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAG  240
            |||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAG  240

Query  241  CCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA  300
```

Fig 3. ***Staphylococcus aureus*** **strain TSA-2 16S ribosomal RNA gene, partial sequenceSequence ID: gb|KR265472.1|**

Table 7.differences in location of Nucleotides of *Klebsiella pneumonia*..

sample	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID
Klebsiellapneumoniae	**Transition**	**196**	**A>G**	**3 to 1018**	**gb|KT944736.1|**
	Transition	**412**	**A>G**		
	Transversion	**579**	**T>G**		
	Transversion	**618**	**T>G**		
	Transition	**645**	**A>G**		
	Transition	**740**	**T>A**		
	Transversion	**828**	**C>A**		
	Transversion	**947**	**T>G**		

Table 8.differences in location of Nucleotides of *Staphylococcus aureus*

sample	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID
Staphylococcus aureus	**Transversion**	**140**	**G>T**	**1 to 1025**	gb|KR265472.1|
	Transversion	**195**	**G>T**		
	Transition	**389**	**A>G**		
	Transition	**431**	**C>T**		
	Transition	**478**	**A>G**		
	Transition	**805**	**A>G**		
	Transversion	**852**	**C>A**		
	Transition	**866**	**G>A**		
	Transition	**899**	**A>G**		
	Transversion	**995**	**C>G**		

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