SEQUENCES AND EXPRESSION OF THE ACTIVE
LYSOSTAPHIN GENE FROM STAPHYLOCOCCUS
SIMULANS ISOLATED OF BOVINE MASTITIS AND ITS
BACTERICIDAL EFFECT ON Staphylococcus Aureus

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ABSTRACT
Sixty milk samples were recovered from cows suffering from clinical mastitis in Basra. Among these 55 (91.66%) gave good growth in blood agar. After microscopical and biochemical tests, 49 isolates have been diagnosed as G+ bacteria staphylococci. Thirty (50%) isolates of S. aureus are coagulase positive staphylococci (CoPS) and 19 (31.66%) isolates were coagulase negative staphylococci (CoNS). Among the recovered isolates, two isolates are S. simulans and only one gave good antimicrobial activity against S. aureus on the basis of inhibition zone. The active lysostaphin gene with determined size and sequences are 738 bp was isolated and then cloned to E. coli. A lysostaphin gene was prepared with terminal Histidine group in order to isolate and purify lysostaphin protein by used special primers. His-tag column was chosen for isolation and purification of protein with in short time. A high fidelity in running, one band of protein in polyacrylamide gel was seen in comparison with standard protein from Sigma which gave more than two bands, after running in polyacrylamide gel, the molecular weight was about 27 KDa. The antibacterial effect of lysostaphin against S. aureus was studied in Vitro which gave good inhibition zone on sold media. The LD50 of lysostaphin was determined and there were no effects of lysostaphin on mice with all concentrations used. The effect of lysostaphin against S. aureus in Vivo was studied by inducible infection in mammary glands (mastitis). The antibacterial activity of lysostaphin showed significant effect. Finally the histological studies of mammary glands showed the significant activities of lysostaphin to inhibit the growth of S. aureus. To eliminate the antibiotics resistant building this study is conducted. The aims of the study: Isolation and identification of Staphylococcus simulans as a source of lysostaphin producer from mastitis cows. Cloning the lysostaphin gene, using expression of this gene as bactericidal effect on S. aureus in plate. Screening the effect of lysostaphin on induced infected mice (locally) with S. aureus.

Key words: Lysostaphin; Staphylococcus simulans; Bovine mastitis

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تحديد تسلسل وتعبير جين اللايسوستافين من الموزعة من إنهاء الضرر البقري ودراسة \textit{Staphylococcus simulans} \\
تأثيره على \\

\textit{Staphylococcus aureus} \\

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الخلاصة

جمعت 60 عينة حليب من أبقار مصاب بالتهاب الضرع السريري في محافظة البصرة، وجد أن 55 عينة بنسبة (91.66% ) أعطت نمو بكتيريا على وسط أكار الدم، وبعد التشخيص المجهرى والكيميوي تم تشخيص عزلة (81.66% ) لجراثيم المكورات العنقودية الموجبة لصفيه كرام، 30 عزلة (50% ) لجراثيم \\
\textit{S. aureus} ، لإختبار التحليل و 19 عزلة (31.66% ) مكورات عنقودية سلبية لإختبار التحليل. حدد منها عزلتان لبكتريا \\
\textit{S. aureus} ، أعطت واحدة منها فعالية تثبيطية لبكتريا ودراسة تأثير البكتريا المتحولة بالجين الفعال على بكتريا الهوس. حجم البالغ 738 زوج قاعدي، كما تم تحديد تتابعه و كلوته إلى بكتريا \\
\textit{E. coli} ودراسة تأثير البكتريا المتحولة بالجين الفعال على بكتريا الهوس. يعد ذلك تحضير جين اللايسوستافين حاوي على مجموعة هستين طرفية لغرض العزل والتنقية بمستعمل عزلة خاصة تم إستنساله، عزل بروتين اللايسوستافين \\
با율ام عيد السكر تلك الذي أظهر كفاءة عالية جداً بالعزل والتنقية بفرات زمنية قصيرة، وقد ظهر ذلك جلياً عند تحليل البروتين على هلام الأكريل أميد، إذ ظهرت حزمة واحدة مقناثة مع البروتين القاسي من شركة اسمها الذي أعطى أكثر من حزمتين عند التحليل، وبعد تحليله على هلام الأكريل أميد تم تحديته تكوينه ونتابعه \\
\textit{S. aureus} وحجم الذي بلغ حوالي 27 كيلو دالتون. تم استعمال البروتين المنفي كمصدر بكتريا لبكتريا وذكذ \\

\textit{S. aureus} بدراسة تأثير الفاكهة لها خارج النظام الحي فأعطى مقداراً واضحاً تثبيط بكتريا \\
\textit{S. aureus} ، لذلك تم تحديد الجرعة نصف القائمة للبروتين المنفي (LD_{50}) وذلك بإصابة 30 فأر، وأشارت النتائج إلى عدم ظهور أي نسبة \\
قتل لللُفرن مع كل التخفيف المستعملة دالفة على سلامة الحيوان المحموق بالبروتين المنفي. كذلك تم دراسة \\
تأثير البروتين المنفي على بكتريا الهوس في داخل النظام الحي وذلك بإحداث إصابات تجريبية موضعية \\
وكذلك بإحداث التهاب ضرع تجريبي، تم معالجة الحيوانات المصابة بالبروتين المنفي، أوضحت \\
النتائج التأثير الكبير لللايسوستافين الذي منع أي تأثر على الحيوان بعد حقفها، كما تم عزل الغذ الدلبية وعمل \\
tقيط تسمجي لمسجية، وأظهر بروتين اللايسوستافين فعالية مميزة واضحة في القضاء على تأثير بكتريا \\
\textit{S. aureus} المشتبه بإحداث الإصابة.
INTRODUCTION

Mastitis is an inflammatory reaction of the mammary gland, usually to microbial infection and its prevalence is alarming (1,2). Mastitis is currently the most economically important disease of dairy cattle. The effects of mastitis go beyond treatment and prevention costs to include issues of animal welfare (3). Mastitis regarded the first common disease which used antibiotics in dairy cattle which is responsible for increasing resistant to antibiotics (4). Mastitis have economic and healthy important which come from gross losses due to decline of quality and quantity of milk production and the death of newborn which is suckle from infected mother (5), in addition to the loses of money due to treatment and veterinarian cure. Most of the mastitis causes are transfer to human which cause many trouble to public health (6). Lysostaphin class III bacteriocins include large peptides ($M_r \geq 25$ KDa) which are generally heat-labile. This class of bacteriocins was further subdivided (7). Lysostaphin was studied in the (1960) and (1970) as a potential therapeutic agent in numerous animal models and in a single human patient. However, it is antimicrobial properties appeared promising. Lysostaphin is an extracellular enzyme, it is produced by (G +) strain of $S$. epidermidis NRRL B-2628 and also designated as $S$. simulans or $S$. staphylolyticus, it was isolated in (1960) by Schindler and Schuhardet (8). Its bacterial activity against staphylococci relies on its capability of clearing the peptidoglycan present in bacterial cell walls. It has activities of three enzymes such as glucosmidase and N-acetyl-muramyl-L-alanine amidase (9). The development of new strategies for the prevention and/or treatment of mastitis should continue to be a high priority among the animal health initiatives, so lysostaphin as a novel therapeutic agent against antibiotic-resistant $S$. aureus infections. Moreover, lysostaphin rapidly lyses actively growing and non-dividing cells (Wu et al., 2003), whereas most antibiotics require actively dividing cells to mediate their action. Hence, due to low toxicity and unique specificity of lysostaphin, it has once again aroused the interest of researchers to investigate the therapeutic values of lysostaphin.

MATERIALS AND METHODS

Samples of milk were collected aseptically from 60 local cattle breeds in Basra, Al-Zubair from 21.12.2009 to 23.3.2010. The udder and especially the teat were cleaned with a textile cloth moistened with distilled distal water (D.W.). Before milking the sample into the vial, a couple of fore strips were milked out to rinse out the normal bacterial flora from the teat canal and orifice (10). Samples were transported immediately to the Lab. in a special box with ice and streaked within (24) hour (hr) (11). To ensure mastitis, the california mastitis test (CMT) was done, the milk and reagent are mixed by slight circular moved in a white container (paddle) in equal quantities, then read the result after (10) second (sec) (12). In the Lab., 10 microlitres ($\mu$l) of milk was streaked on blood agar and incubated at 37°C (18-24 hr). $Staphylococci$ were further identified, the identification was based on colony morphology, microscopy and gram-staining (13). Coagulase negative staphylococci were distinguished from $S$. aureus by some tests such as catalase, oxidase, coagulase, DNase, urease and heamolysin production, the bacterial isolated were stored at -20°C in tryptic soy broth containing 10% glycerol. Used API Staph ID (32) to diagnosis all samples.
Active lysostaphin (active gene) from \textit{S. simulans}

Genomic DNA was isolated from \textit{S. simulans} according to Sambrook and Russel (14) with some modulation. DNA isolated from \textit{S. simulans} used as a tamblet for PCR amplification tables(1,2). The annealing temperature for this PCR 54°C, the primers for this PCR, all this processing is done in Garduno Lab., Dalhousie University, Halifax, NS, Canada:.

Forward primer:
5’ GGT ACC ATG GCT GCA ACA CAT GAA CAT TCA 3’.

Reverse primer:
5’ GAA TTC TCA CTT TAT AGT TCC CCA AAG AAC 3’.

Then run the product in gel electrophoresis, isolated and purified the DNA from gel by QIAquick Gel Purification Kit, then send DNA fragment to sequences, the samples send with primers to sequencer, these primers are:
5’ GGT ACC ATG GCT GCA ACA CAT GAA CAT TCA 3’.
5’ GAA TTC TCA CTT TAT AGT TCC CCA AAG AAC 3’.

Active \textit{lysostaphin} cut according to table (3). \textit{Lysostaphin} gene purified by PCR Purification Kit from Qiagen, ligation the active \textit{lysostaphin} with pBluescript digest by \textit{Eco}RI and \textit{Kpn}I and transformants to rubidium chloride competent cells \textit{E. coli} DH5α. Determination the blue-white color selection for pBluescript and colony PCR reaction to ensure the cloning.

<table>
<thead>
<tr>
<th>Table(1): Amount of PCR reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance</td>
</tr>
<tr>
<td>10X buffer</td>
</tr>
<tr>
<td>1.25mM dNTPs</td>
</tr>
<tr>
<td>Forward primer</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>(P_{fx})</td>
</tr>
<tr>
<td>Enhancer</td>
</tr>
<tr>
<td>Template</td>
</tr>
<tr>
<td>MgSO₄</td>
</tr>
<tr>
<td>ddH₂O</td>
</tr>
<tr>
<td>Total reaction</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table(2): Typical conditions of PCR amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>‘1’</td>
</tr>
</tbody>
</table>
Table(3): The substances and quantity of digestion lysostaphin gene by Kpn I and EcoR I

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity(µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysostaphin gene</td>
<td>20</td>
</tr>
<tr>
<td>10X buffer</td>
<td>5</td>
</tr>
<tr>
<td>BSA</td>
<td>5</td>
</tr>
<tr>
<td>ddH2O</td>
<td>16</td>
</tr>
<tr>
<td>Kpn I</td>
<td>2</td>
</tr>
<tr>
<td>EcoR I</td>
<td>2</td>
</tr>
<tr>
<td>Total reaction</td>
<td>50</td>
</tr>
</tbody>
</table>

Expression of lysostaphin gene in E. coli

Prepare gene with His-tag by PCR (table 1, 2) but with different primers:
Forward primer:
5’ GGT ACC ATG GCT GCA ACA CAT GAA CAT TCA 3’.
Reverse primer:
5’GAATTC TCA ATG ATG ATG ATG ATG ATG CTT TAT AGT TCC CCA AAG AAC 3’.

The annealing temperature for this PCR 53°C. Then run the product in agarose gel electrophoresis, isolated and purified the DNA from agarose gels by QIAquick Gel Purification Kit. After that the purified lysostaphin cut by two restriction enzyme according to table(3). Then purified the lysostaphin gene by PCR Purification Kit from QiaGen. Ligation the active lysostaphin gene with pBluescript digest by EcoR 1 and Kpn 1 and transformants to rubidium chloride competent cells, determination the blue-white color colonies selection for pBluescript and colony PCR reaction to ensure the cloning. Study the antibacterial activity of cloning bacteria against S. aureus in plate and comparison with non cloning bacteria.

Extraction and purification of lysostaphin protein from cloning bacteria by His-tag method

Extraction and purification of lysostaphin protein from cloning bacteria by His-tag column by used HisTrap FF crude columns (GE Healthcare, SE-751 84 Uppssala, Sweden) are designed to direct purification of unclarified cell lysates without centrifugation or filtration in the sample preparation steps. The Bio-Rad Protein Assay from (Bio-Rad laboratories, Life Science Group, U.S. (800) 4BIORAD, California) can also be used with a microplate reader to determined protein concentration. SDS-PAGE analysis of proteins was performed by standard techniques (11), in the same time run standard protein from Sigma to comparative.

Determination of LD50 of lysostaphin protein

LD50 determination of lysostaphin was done according to(15). The LD50 of lysostaphin done with 30 mice (Albino mice, Mus musculus L, Balb/C).Serial concentrations of lysostaphin (1,5,10,20) mg/kg were prepared and each one was given intraperitonealy to 6 mice for each group, which stayed under observation for 5 days and then the dead and survival animals were counted and compared with control group.
Lysostaphin as treatment for *S. aureus* in vivo

Twenty female lactating mice, weight of each mouse 22-30 gm divided into four groups, each group contain 5 mice. *S. aureus* was plated on tryptic soy agar with 5% sheep blood from a frozen stock. Following 24 hr of incubation, three isolated colonies were transferred to 1 ml of tryptic soy broth. The broth culture was incubated on rotating shaker for 16-20 hr at 37°C. Based on preliminary experiments to determine a challenge dose of *S. aureus* that resulted in consistent systemic infection without rapidly killing the mice, the culture of *S. aureus* were diluted to about 1 x 10^7 cfu/mouse in normal saline. The four groups in this study. Group I (negative control): Included 5 female mice which were no inoculated. Group II positive control: Included 5 female mice which inoculated with *S. aureus* to mammary glands subcutaneous (S/C) at a dose of 0.1 ml/gland. The day of challenge was designated as day 1 of the experiment. Group III: Included 5 female mice which inoculated with *S. aureus* to mouse in mammary glands S/C at dose of 0.1 ml/gland. The day of challenge was designated as day 1 of the experiment. After 4 days inoculated with lysostaphin as 5 mg/kg in mammary glands region S/C at dose of 0.1 ml/gland. Group IV: Included 5 female mice which inoculated with *S. aureus* to mouse in mammary glands region S/C at dose of 0.1 ml/gland. The day of challenge was designated as day 1 of the experiment. After 4 days treated with lysostaphin as 10 mg/kg to mouse in mammary glands S/C at dose of 0.1 ml/gland.

After 8 days of first inoculation, collected mammary glands to 10% formalin for histological study by routine haematoxyline and eosin staining method, all samples are isolated and prepared for light microscopic study according to (16). The specimens were fixed overnight at room temperature in Bouin’s fixative. Then the specimens were washed in 70% alcohol plus several drops of ammonium hydroxide for several days until no more yellow color comes out. The tissue samples dehydrated in a graded ethanol series (50%, 70%, 95% and ethanol absolute), then cleared with xylene, two changes 15 min in each. After that the specimens were transferred to melted paraffin for 6-12 hr. Finally the tissue pieces were embedded in paraffin-wax. Serially sections of about 5-10 µm thickness were cut and stained with haematoxyline and eosin. The sections were dehydrated, cleared, mounted in D.P.X. and examined microscopically (17).

RESULTS AND DISCUSSION

All sample had given positive result to CMT, transported immediately to the laboratory. 55 sample (91.66%) grown on blood agar. To distinguished staphylococci colonies from other, gram-staining done for 55 sample which gave good growth on blood agar, 49 (81.66%) isolates are G+ staphylococci. CNS isolates were distinguished from *S. aureus* by some tests such as catalase test, oxidase test, coagulase test, DNase test, urease test and haemolysin production). Recorded 30 (54.54%) isolates of *S. aureus* and 19 (34.54%) isolates are CNS (table 4). All isolates were stored at -20°C in tryptic soy broth containing 10% of glycerol. API Staph ID (32) test used to diagnosis all samples. Two isolates classified as *S. simulans*. The antimicrobial activity of isolates *S. simulans* was investigated against *S. aureus* figure(1). Figure(2) represents the result of total genomic DNA isolation from local isolate of *S. simulans*. 

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Table (4): Catalase, oxidase, coagulase, DNase, urease and heamolysin production of samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Catalase test</th>
<th>Oxidase test</th>
<th>Coagulase test</th>
<th>DNase test</th>
<th>Urease test</th>
<th>Heamolysin production</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CNS</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure (1): Effect of *S. simulans* against *S. aureus*, (A, B) free disc, (C, D) *S. simulans* disc

Figure (2): Total genomic DNA from local isolates of *S. simulans*
Active gene of lysostaphin from S. simulans

Figure(3) showing the PCR product result from *S. simulans* after run the product in the gel. This figure showed the band of product (*lysostaphin* gene) with less than 750 bp. The result appeared in figure(4) showed the sequences and molecular weight of *lysostaphin* gene (738). The antibacterial activity of cloning *E. coli* was investigated against *S. aureus* by a standard disk diffusion assay was shown in figure(5).

![Figure(3): Band of PCR product less than (750) bp](image)

![Figure(4): Sequences of active *lysostaphin* gene](image)
Extraction and purification of lysostaphin protein

Extraction and purification of lysostaphin protein from cloning bacteria by His-tag column table(5), showed the concentration of lysostaphin in the first concentration which (0.125) mg/ml.

Table(5): Protein concentration by measure the absorbance at (595) nm

<table>
<thead>
<tr>
<th>Bio-Rad dye</th>
<th>BSA</th>
<th>Lysostaphin</th>
<th>Lysostaphin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.311</td>
<td>2.688</td>
<td>0.759</td>
<td>0.731</td>
</tr>
<tr>
<td>0.313</td>
<td>1.402</td>
<td>0.45</td>
<td>0.432</td>
</tr>
<tr>
<td>0.311</td>
<td>0.771</td>
<td>0.329</td>
<td>0.323</td>
</tr>
<tr>
<td>0.313</td>
<td>0.481</td>
<td>0.321</td>
<td>0.318</td>
</tr>
<tr>
<td>0.319</td>
<td>0.333</td>
<td>0.313</td>
<td>0.311</td>
</tr>
</tbody>
</table>

Figure(6) showing SDS-PAGE analysis of proteins by standard techniques, the figure showed one band of protein, the molecular weight of this lysostaphin in all lane about (27,000) Dalton while figure(7) showed the SDS-PAGE analysis of standard protein from Sigma, this figure showed more than one band of protein. Figure(8) showed the bactericidal effect of lysostaphin protein against *Staphylococcus aureus* in plate. The sequences of lysostaphin protein was shown in figure (9). LD<sub>50</sub> of lysostaphin was done with 30 mice. Serial concentrations of lysostaphin are inoculated to four groups of mice: (1, 5, 10, 20) mg/kg, the fifth are control. No effect of lysostaphin to mice with all concentrations and no death case after 5 days.
Figure (7): SDS-PAGE analysis of standard lysostaphin protein from Sigma, marker in lane (1), lysostaphin protein in lanes (2,3), (4,5), (6,7), (8,9) with the concentration of (250), (125), (62.5), (31.25) µg/ml respectively.

Figure (6): SDS-PAGE analysis of lysostaphin protein, the marker in lane (1), lysostaphin protein in lanes (2,3), (4,5), (6,7), (8,9) with the concentration of (125), (62.5), (31.25), (15.625) µg/ml, respectively while lane (10) have crude lysostaphin protein before using His-tag column.

Figure (8): The activity of lysostaphin protein against S. aureus.

Figure (9): Sequences of lysostaphin protein by adman degradation method.
Routine haematoxyline and eosin staining method

The result represents in figures (10-17) showed the histological study for mammary glands. Figures (10,11) showed the normal mammary gland of mouse (control negative) while figures (12, 13) showed mammary gland infected by *S. aureus* (control positive) which is a typical case of mastitis, figure(12) showed degeneration of most acini and severe infiltration of inflammation cells, figure(13) showed necrosis, partial degeneration of acini and deposition of collagen fibers while figures(14,15) represented mammary gland infected by *S. aureus* and treated by lysostaphin with (5) mg/kg, normal secretory cells, no inflammatory cells. Figures(16,17) represented mammary gland infected by *S. aureus* and treated by lysostaphin with(10) mg/kg, figure(16) showed a deposition of secretory material and figure(17) normal connective tissue and regeneration of secretory duct.

Figure(10): Normal mammary gland of mouse (control negative). (H and E) (300X)

Figure(11): Normal mammary gland of mouse (control negative). (H and E) (1300 X)

Figure(12): Mammary gland of mouse infected by *S. aureus* (control positive), typical case of mastitis. (A) Degeneration of most acini, (B) Severe infiltration of inflammation cells. (H and E) (300 X)

Figure(13): Mammary gland of mouse infected by *S. aureus* (control positive), typical case of mastitis. (A) Lactating, (B) Necrosis, (C) Partial degeneration of acini, (D) Deposition of collagen fibers. (H and E) (1200 X)
Mastitis is the most costly infectious disease of dairy cattle, the prevalence of mastitis in dairy cattle is relatively high (18,19) Approximately 70% of this cost is associated with a reduction in milk production due to irreversible damage in the mammary tissue (20). Although antibiotics are very useful to treat this infection but do not directly protect the gland from being damaged. Treatment of clinical mastitis are very limited and as result it possible spread of the disease, the antibiotic therapy for mastitis recommended, however an increase in resistant subpopulation, especially the duration which could contribute much to the difficulty of its control and the development of the drug resistance (21). For all that, the purpose of this study is to give new strategy to treat this problem. In this study, 60 sample of milk from clinical case showing concur to (22) in give good result of california mastitis test which estimate the Somatic Cell Counts of a milk sample, particularly as the somatic cell count of milk increases (23,24), the majority of these tests primarily indicate inflammation in the udder (25,26,21).
Result showed clinical mastitis appeared from 55 sample (91.66%) bacterial growth in blood agar which agreement to Mostafa (19), the high percentage of these isolates come from bad defense of the body, high virulence if the bacteria and the effect of environmental predisposing factor (27). *S. aureus* recorded a high ratio of bacterial isolates 50.54% which regarded first causative agent in clinical mastitis (21).

The high percentage of *S. aureus* in clinical mastitis confirm this bacteria endemic in Basra and find this bacteria in teat skin which exposure to environment especially in sitting animal position before and after parturition lead to contaminate the udder in different session (28,29). This study is in agreement with other studies which to perform about mastitis such as (30,31,32). In Iraq Al-Khatib and Al-Bassam (33) recorded that the *S. aureus* is the highest percentage and we are consent to (34,35,36). The high percentage of CoNS (31.66) % appeared in this study is agreement with Morie and Daood (37). The proportion of CoNS are agreement to the most common isolates in milk samples from cows with clinical mastitis, especially mastitis with mild clinical signs (38, 39). It is found variety in biochemical test, lead to variety in bacterial isolates such as CoNS isolates (40), *S. chromogenes*, *S. simulans* and *S. hyicus* are reported most often. Also the result showed a similarity in recorded to CoNS isolated from (9) % of the quarter milk samples in a total of (80) dairy herds and they comprised (35) % of samples positive for bacterial growth(41). According to different studies on(20) conventional and (20) organic dairy farms, the prevalence of CoNS was(14) % on conventional farms and (17) % on organic farms and CoNS were recovered from (38) % and (30) % of milk samples with bacterial growth on conventional and organic farms, respectively(42). Its found in this study CoNS isolates originated from diagnosed clinical mastitis from the field, the predominant CoNS species is usually *S. simulans* which accepted from (43,44). Gianneechini(45) recorded the first percentage of occurrence mastitis are *S. aureus* and (2) isolates to *S. simulans* which agreement with our study. We are to share with opinion of Taponen (46); Thorberg (47) in the most commonly isolated CoNS strains in there study herds, regardless of inflammatory reaction in the udder quarter, *S. simulans* was one of the isolated bacteria and found other CoNS strains. One from two isolates of *S. simulans* are gave effect to *S. aureus* in plate which confirm the ability of *S. simulans* to inhibited *S. aureus* by secretion of enzyme or toxin or any other virulence factor. Figure(4) showed the band of active gene (738) bp (48, 49) have recorded 246 amino acids (aa) in the active lysostaphin protein which mean 738 bp in the active *lysostaphin* gene. Before beginning of purified lysostaphin, we are used primers have His tag, for that the number of bp of these primer are increased by 6 codon, each codon have 3 bp, this mean added 18 bp, for that, it is difficult to amplify any gene from total genomic DNA with primer about 42 bp, the typical primer have 18-26 bp (50), for all that we are did PCR with normal primer (without His tag), then amplify by His tag primer from PCR product to ensure the amplification. Extraction and purification lysostaphin protein from cloning bacteria by His-tag column, showed in figure (6) one band of protein, mean successful in extraction and purification of protein by added 6 x His-tagged to the primers in order to obtain the protein with terminal Histidine group, give many choosing to isolated from other protein mixed with it in the same broth (51). These His tags facilitate selective binding of the expressed protein to a nickel-affinity column. The often tacit assumption is that these tags have no effect on the structure and function of the protein (52).
The result expressed in figure(6) showed the efficiently and high fidelity of His-tag when compared with standard protein from Sigma, the purity of Sigma protein more than 96%, this percent are mention in Sigma Aldrich document but when we are running this standard protein, more then two band appeared, this is mean purified of lysostaphin protein from cloning bacteria by His-tag column are more efficiently and high fidelity than the standard protein. Moreover, by His-tag column, we can purified protein in very fast time and the same column can be used again.

Figure(9) showed the sequences of lysostaphin protein, the sequences have six terminal Histidine and protein are the same sequences of DNA if we are reverses amino acid to DNA code. The molecular weight of this protein in all lane more than (27,000) Dalton which analogous to (53,48,54,7), the result of our study have only one different from these authors, which are found six terminal Histidine because added His tags code in primers, but we are show no any effected of this added in activity. Figure (8) showed the effect of lysostaphin protein against *S. aureus* in plate, the appearance of inhibition zone, lead to no any activity when we added terminal Histidine which showed the safety of adding this terminal amino acid. Lysostaphin activity against staphylococci due to its capability of lysis the peptidoglycan of bacterial cell walls (55,56). The target of the lysostaphin is the pentaglycine cross-bridge of the peptidoglycan (57), *S. aureus* and other staphylococcal species is composed of five glycine (Gly) residues (58, 59). Lysostaphin has the ability to cleave specifically between the third and the fourth Gly residues of the pentaglycine cross-bridge (60,57). The peptidoglycan of staphylococcal species relatively resistant to lysostaphin contains higher amount of serine (Ser) than Gly (61). Determination of LD_{50} of lysostaphin as shown no effect of lysostaphin to mice with all concentrations (1,5,10,20) mg/kg, the results showed no death after (5) days, which means the high safety of this protein when used to eukaryotic system. Naturally *S. aureus* are established in the mammary glands of the animal, it is very difficult to eradicate (62). Lysostaphin has many attractive features for use as an antimicrobial agent: It has activity against non-dividing as well as dividing cells. It is digested by intestinal proteinases, having no influence on the gut microbiota. It has no toxicity. It is relatively stable when conjugated with polyethylene glycol. It maintains its activity in human serum (63,64,65). Moreover, studies demonstrate that lysostaphin retains its bacteriolytic activity in Vivo, without any undesirable immune reaction (66,67). Moreover, lysostaphin rapidly lyses actively growing and non-dividing cells including staphylococci in biofilms (67,68), whereas most antibiotics require actively dividing cells to mediate their action. For all above reasons, the lysostaphin cloning and purification process are of the most critical aspects. Our study are offered great to use this protein against *S. aureus* which give this protein good response in treatment against *S. aureus* especially in mastitis.
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