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IMMUNO-HISTOMORPHOMETRIC STUDIES AND THE EFFECT OF SOME ANTIBIOTIC ALTERNATIVES ON SHE-CAMEL STAPHYLOCOCCUS MASTITIS

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ABSTRACT

Bacteriological examination of 50 she camel's udder tissue samples collected from different slaughter houses revealed that *Staphylococcus* spp. were the most common isolated bacteria (90 %) as single and mixed infection followed by environmental *Streptococcus* spp. (40%) then *E.coli* (26%) in the form of mixed infection. Most of the isolated *S.aureus* and coagulase negative staphylococci (CNS) were resistant to 14 commercially available antibiotic discs. By using 4 antimicrobial peptides (AMPs) as alternatives to antibiotics; included lysozyme (Lz), lactoferrin (Lf), serum amyloid A (SAA) and haptoglobin (Hp) the results revealed only Lz and Lf have antibacterial activities for both *S.aureus* and CNS isolated from the udder tissues while SAA and Hp did not show antibacterial effects for the tested strains. By using combinations of the different antibiotics and the four AMPs we noticed significant synergistic effect for all of them on the tested strains. Analysis of protein profile of the infected udder tissue samples with both *S.aureus* and CNS using SDS-PAGE used to evaluate the immunological status of the naturally infected she camel mammary tissue samples with *S.aureus* or CNS in comparison to bacteriologically negative mammary tissue samples and the results cleared that the concentrations of Lz, Lf, Hp and mSAA were significantly higher in tissue samples infected with both *S.aureus* and CNS when compared with the non-infected tissue samples. While the concentration of Lf showed a significant rise in tissue samples infected with CNS when compared with tissue samples infected with *S. aureus*. UPGMA clustering dendrogram analysis was applied to compare the protein fingerprints of the isolated *Staphylococcus* spp. and illustrated that there were no or weak similarities in the protein fingerprints of the different isolated *S.aureus* and CNS strains ranging from (0 – 0.36). In the present study, histopathological changes in singly isolated *S.aureus* mastitic tissue sections revealed grossly, varied degrees of congestion with oedema in 19 (38 %) of the mammary tissue samples which represented 7 tissue samples of acute diffuse mastitis (14%) only 2 of them showed suppuration and 12 cases (24%) subacute interstitial mastitis and the majority of tissue samples 26 (52%) showed fibrosis and paleness. On the other hand, microscopically exhibited severe histological changes which varied from epithelial degeneration, alveolar atrophy and necrosis, in the acute and sub-acute suppurative inflammatory response marked by neutrophil infiltration, congestion, to increased stromal tissue (fibrosis) and presence of non-suppurative inflammation marked by lymphocytic infiltration in alveoli with disappearance of the alveolar lumen, through fibrosis to the complete destruction of the parenchyma in chronic mastitis which was the most majority noticed type of mastitis was chronic non suppurative mastitis. Histochemicallly, interstitial, perialveolar, intra and inter acinar fibrosis were confirmed with Masson's Trichrome stain as greenish blue fibres. The activity of alkaline phosphatase in tissue sections of non mastitic animals showed high secretory activity on the outer boundary of alveolar secretary cells. While, tissue sections taken from *S. aureus* singly infected mastitic animals showed weak alkaline phosphatase activity (AP), on the outer membrane and weak protein expression by protein loci (mercury–bromophenol blue) comparing to the non-mastitic once. On the other hand histomorphometrically, tissue sections from singly infected S. *aureus* showed ($P<0.05$) significant decrease in alveolar diameter, number of alveoli and alveolar cell population whereas the interstitial connective tissues showed ($P<0.05$) significant increase compared to the non mastitic tissue. We concluded that, Staphylococci were the major bacteria causing mastitis in she camels and showed great differences in their protein profiles, consequently caused significant increase in the inflammatory responses of the udder tissues represented by the increase of Lz, Lf, SAA and Hp concentrations in the tissues. Staphylococci as single infection enough for inducing sever degenerative changes and damage that reflecting impaired activity as well as printed on the different histopathological, histochemical and morphometric changes in she camel's mammary parenchyma. Furthermore, this study offers opportunities for development of treatment strategies that may eliminate or even reduce mammary tissue damage caused by Staphylococcal mastitis with or without the use of antibiotics and/or anti-inflammatory molecules like Lz, Lf, SAA and Hp.

Key words: She camel; mastitis; *Staphylococcus* spp; antibiotic/antibiotic alternatives (Lz, Lf, SAA, Hp) sensitivity; immunology; histopathology; histochemistry; histomorphometrical changes.

INTRODUCTION

The camel (*Camelus dromedaries*) is the most dominant and widely distributed animal in Africa and Asia. It makes an important contribution to human survival and utilization in dry and arid land (Husein *et*

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al., 2013). Also, they are considered as wealth investment and insurance against natural disasters. It is a multipurpose animal kept for milk and meat production as well as transportation. Milk of camel is one of the main components of the diet of the nomads and is consumed in its raw or naturally processed (soured) form as it could meet a big part of the daily needs of humans from nutrients (Al-Otaibi and El-Demerdash, 2013).

Udder infection was considered as one of the main constraints for camel rearing. The major pathogens isolated from udder tissues of she camels were *S.aureus*, *S.agalactiea*, *S.uberis*, *E.coli*, *Klebsiella*, *Micrococcus* spp., *Corynebacterium* and *A.pyogens* (Abdelgadir, 2014; Iyer *et al*., 2014 and Abeer *et al.*, 2016). Despite advances in diagnosis and management practices aimed at reducing the incidence of ruminant mastitis associated with the contagious pathogens, *S.aureus* is one of the major contagious causative agents of clinical and subclinical mastitis that tend to become chronic and difficult to be eradicated by conventional antimicrobial therapies worldwide causing significant economic loss to the dairy industry (Sears and McCarthy, 2003; Pereira *et al*., 2011 and Tiwari *et al*., 2013). Once the intra-mammary infection is established, the organism adheres to epithelial cell receptors for bacterial adhesions resulting in the production of virulence factors and intracellular uptake of the small colony variants of *S. aureus*. Damage to the mammary gland epithelial lining is initiated by ulceration and occlusion of lactiferous ducts, alveoli and infiltration of inflammatory cells in the parenchyma. Mammary tissue damage is further compounded by various toxins and extracellular enzymes produced by *S.aureus* (Zhao and Lacasse, 2008; Middleton, 2013 and Foster *et al*., 2014).

Antimicrobial peptides (AMPs) exhibit a broad range of activities against Gram-negative and Gram-positive bacteria, fungi, viruses, and parasites. To date, 2645 AMPs from various sources have been listed in "The AMPs Database" (Wang *et al*., 2009), a database dedicated to natural AMPs. This rich source of antimicrobial agents has aroused growing interest, especially in the light of the decreasing effectiveness of antibiotics not only against severe infections, but also in treating common infectious diseases. Resistance to antibiotics has become a threat to global public health and is driving novel research into the development of new antimicrobial agents (Laxminarayan *et al*., 2013).

Recently camel milk was used in some parts of the world to cure certain diseases (Attia *et al*., 2001) such as treatment of food allergies, Crohn's disease, autism and diabetes (Shabo and Yagil, 2005), as camel milk is rich with several proteins that are well known of their innate immunity activities. The most important of these proteins are Lf, Lz and Hp (Alluwaimi *et al*., 2017). The antibacterial and antiviral activities of the Lf were widely recoded as it exerts its effect by depriving pathogen utilization of iron by depletion. Also, Hp acts as an antioxidant, has antibacterial activity. Lysozyme has antibacterial properties against gram-positive and gram-negative bacteria (El-Wassell, 2000; Masschalck and Michiels, 2003 and Agamy, 2009). Moreover, the estimation of MAA concentration in milk is a more useful diagnostic tool to monitor the udder health in dairy cattle (Hany *et al*., 2018).

Innovation is needed not only for the development of new antibiotics but also for combination therapies. By targeting different mechanisms of resistance simultaneously, combination therapy might help slow the emergence of resistance (Gill *et al*., 2015). So, combinations of milk AMPs with a greater number of antimicrobials have to be tested, as they provide new directions to control pathogens growth. Further, systematic evaluations on *in-vivo* models, selecting different pathogens, pathologies and administration routes should be welcomed. Another important issue is the cell-penetrating potential of AMPs as vectors for intracellular targets (Durzýnska *et al*., 2015).

In this study and for previous causes special care must be applied for camel's mammary gland tissues to produce clean milk, also an attempt was made to use natural antibacterial proteins as Lz, Lf, Hp and SAA that can be extracted from milk to be used as alternatives to antibiotics to avoid many problems, such as antibiotics resistance and residues, hypersensitivity, direct toxicity, antibiotic- induced immune-suppression and super-infections as camel milk is used as therapeutic agent for treatment of many human dangerous diseases.

MATERIALS AND METHODS

A- Mammary gland tissue samples collection: Fifty tissue samples from she camel's mammary glands were randomly collected from adult slaughtered animals, from Cairo and Giza abattoirs and were visually examined for gross lesions. Then each tissue sample was divided into three parts, one part was put in a small polyethylene bag in an ice box under aseptic conditions for bacteriological examination. The second part was immersed in 10% neutral formalin solution for histopathological evaluation. The third part used for immunological examination.

B- Microbiological examinations:

1- Bacteriological examination: - Mammary gland tissues were cultured on blood agar media, Mannitol salt agar, Edward's medium, MacConkey's agar plates and brain heart infusion agar media then incubated at 37°C for 24–48 hrs. Suspect colonies were examined for colony morphology, Gram stain

characteristics and motility. Gram negative bacilli and Gram-positive cocci were further subjected to IMVIC tests, TSI, urease hydrolysis, catalase, oxidase and coagulase tests as well as other standard biochemical tests (Koneman *et al*., 2005 and Quinne *et al*., 2011) to identify the isolates.

2- Antibacterial bio-gram assay against the isolated *Staphylococcus* spp.:-
It was applied according to the National Committee for Clinical Laboratory Standards (NCCLS, 2008) using disk diffusion technique on Mueller Hinton agar. It was applied following 3 steps:-

a- Antibiotic susceptibility test:- Randomly selected singly isolated strains of *S.aureus* and CNS were subjected to 14 commercially available antibiotics discs [ciprofloxacin; CIP (5µg), norfloxacin; NOR (5µg), levofloxacin; LEV(5µg), Enrofloxacin; ENR (5µg), ofloxacin; OFX (5µg), amoxicillin-clavulanic acid; AMC (30µg), amoxicillin; AMX (25 µg), ampicillin; AM (10µg), penicillin; P (10 U), tetracycline; TE (30µg), florofenicol: FFC(30µg), gentamycin; CN (10µg), streptomycin; S (10µg) and cefoquinom; CEQ (30µg)] and the inhibition zones were recorded as sensitive, intermediate susceptibility and resistant according to the NCCLS recommendations.

b- Using natural alternative antimicrobial agents alone:- Lysozyme, lactoferrin, serum amyloid A and haptoglobin were used as natural antibacterial agents against the isolated *Staphylococcus* spp. using disk diffusion method by saturating clean discs with 100µl of 10 mg/ml Lz once, 100µl of 10 mg/ml Lf alone, 100µl of SAA alone and 100µl of Hp (Sunredbio Co., Shanghai, China).

c- Combination between commercial antibiotics and natural alternative antimicrobial agents:- The selected strains of *S.aureus* and CNS were subjected to the previously mentioned antibiotic discs saturated with 5µl/disc of each of Lz, Lf, SAA and Hp with the same concentrations mentioned in step (b). The results were recorded by measuring the inhibition zones and scored as sensitive, intermediate susceptibility and resistant according to the NCCLS recommendation

C-Immunological studies:
1- Preparation of *S. aureus* strains for PAGE: Bacterial isolates grew in BHI broth, were collected by centrifugation at 10,000g for 10 minutes, washed with physiological saline, diluted by physiological saline to 1.5 ml/v. Cell suspension was sonicated for 3 minutes (at level 4), then centrifuged at 11,000 g for 3 minutes. Supernatant obtained was taken and stored at 0-4 ºC for further analysis according to Tuasikal *et al*. (2012).

2- Analysis of protein profile of the tested udder tissue samples using SDS-PAGE: Protein was purified from camel's udder tissue samples according to Dignam (1990) and compared with standard bovine Lz, Lf, SAA and Hp for determining the concentration of these immunological bioactive parameters in the udder tissues.

3- Polyacrylamide gel electrophoresis: Coomassie blue staining analysis of proteins was carried out by standard protocols (Laemmli, 1970). The selected tissue samples with mastitis where submitted to SDS-PAGE and their protein patterns were compared with a database of normalized protein fingerprints derived from normal tissue samples.

4- Computer-aided analysis of the gels: Images of the gels were captured using a sharp JX-330 flat-bed scanner and image analysis of the protein profiles was performed using Amersham Pharmacia Biotech Image master 2-D Elite software.

D- Histopathological examinations:
1- Histopathological procedures: The udder was incised quickly and mammary tissue parenchyma were immediately fixed in 10 % buffered formalin for routine histopathological examination. The fixed specimens were trimmed, washed, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. The embedded samples were sectioned at 3-5 µm thickness, stained with H & E stain according to Suvarna *et al.* (2013).

2- Histochemical procedures: Masson's Trichrome stain was used for connective tissue proliferation. All these procedures were applied as previously described by, Suvarna *et al.* (2013), in addition to cytoplasmic protrusions. For demonstration of AP activity and density of protein staining (mercury–bromophenol blue) in mammary epithelial cells were determined according to El-Sayed *et al.* (2009) was applied then examined using a light microscope.

3- Histomorphometric analysis: It was applied on The paraffin-embedded mammary tissues that stained with H& E for measurement of mammary alveolar diameter, number of alveoli and alveolar epithelial cell population as well as inter lobular connective tissue thickness in non-mastitic and mastitic she camel mammary gland using a light microscope (BX50, Olympus, Tokyo, Japan) equipped with a digital camera and software program (Image Pro6, Tokyo, Japan) that performed according to Hussain *et al.* (2012c). The following parameters were estimated among 5 different sections from mastitic she-camel from which isolated *S.aureus* as single infection and compared to non mastitic one. The average numbers of alveolus, alveolar cell population cells (µ2) as well as alveolar diameter and the interlobular connective tissue thickness in (µ) were calculated for each case,

in randomly detected five microscopic fields according to EL-Sayed *et al*. (2013); i.e. the total counted sections are twenty.

* Number of alveoli and the alveolar epithelial cell population in (μ2): The sections were viewed by the light microscope at 40x then the fields were examined at 400x for manually counting of the alveolar number and the number of cells per alveolus using a computer.

* Alveolar diameter in (μ): they were measured at x100. A straight line was drawn vertically from basement membrane to basement membrane at margins of the alveoli. The line runs from the outermost boundary across the lumen to the opposite end of the alveoli. These figures were automatically generated with the aid of the software.

* Interlobular connective tissue thickness in (μ): A straight line was drawn at two opposite highest of the interlobular connective tissue. The measurement was done at x100.

E- Statistical analysis: The obtained results and morphometric data were subjected to analysis of variance according to Sendecor and Cochran (1982). Values were expressed as mean ± SE. Statistical comparisons between the means of different experimental groups were made with completely randomized one-way ANOVA ″Student_ Newman_ Keuls test ″by COSTAT program version one. A probability ″P″ value of <0.05 was assumed for statistical significance.

RESULTS

Table (1) showed bacteriological examination of the collected 50 she camel's udder tissue samples and revealed that *Staphylococcus* spp. were the most common isolated bacteria (90 %) followed by Environmental *Streptococcus* spp. (40%) then *E.coli* (26%). Isolated *Staphylococcus* spp. were found either as single or mixed infection. *S.aureus* was found with a percentage of (10%) as single infection and (20%) as mixed infection while CNS were represented (24%) and (36%) as single and mixed infection, respectively. Environmental *Streptococcus* spp. and *E.coli* were found as mixed infection only with *S.aureus* and/or CNS with percentages of 40% and 24%, respectively. The overall infected udder tissue samples were 45 out of 50 (90%) while only 5/50 (10) tissues samples showed no bacterial isolation on the used media.

Table 1: Bacteriological results of the examined mammary tissue samples.

Bacterial isolation	*Staphylococcus* spp.				Environmental *Streptococcus* spp.		*E.coli*		Bacteriologically negative samples	
	S.aureus		CNS							
	No.	%	No.	%	No.	%	No.	%	No.	%
Single infection	5	10	12	24	—	—	—	—	5	10
Mixed infection	10	20	18	36	20	40	13	26		
Total	15	30	30	60	20	40	13	26	5	10

% were calculated according to total number of examined sample (n=50)

As mastitis caused by *S.aureus* has been documented to be one of the most important udder infections in dairy animals. So, this study gave special attention to *Staphylococcus* spp. especially for the highly antibiotic resistant strains.

S.aureus and CNS strains that were isolated as single infection were selected, prepared after biochemical identification and used for studying the effect of antibiotics and their natural substitutes (Lz, Lf, SAA and Hp) on the previous strains. Antimicrobial susceptibility of *S.aureus* and CNS were divided into 3 steps:-

First one, antibiotic discs were used alone on the selected *S.aureus* and CNS strains. This step showed high resistance to antibiotic discs as showed in fig. 3A and fig. 4A, respectively.

In the second step, alternatives to antibiotic (Lz, Lf, SAA and Hp) were used alone. Table (2) and fig. (1, 2) explained that Lz, Lf, SAA and Hp were used alone as antibacterial substances. Lysozyme and Lf showed antibacterial effect on *S.aureus* as the inhibition zones were 18 mm and 13 mm respectively, while in CNS were 20 mm and 16 mm respectively, but *Staphylococcus* spp. either *S.aureus* or CNS showed high resistance to SAA and Hp when used alone as antibacterial substances.

Table 2: Using of alternatives for antibiotics against *S.aureus* and CNS isolated strains.

Bacteria	Average of inhibitory zones of the used alternatives (mm)			
	Lz	Lf	SAA	Hp
S.aureus	18	13	R	R
CNS	20	16	R	R

R= resistant

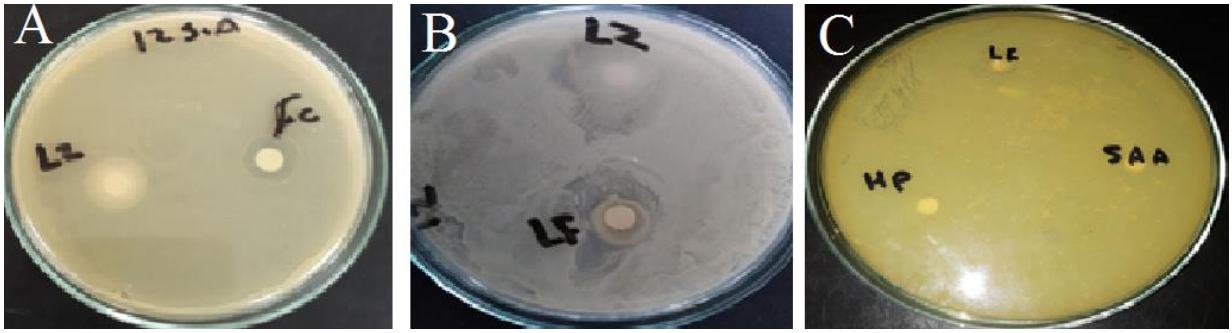

Fig. 1: Showed different antimicrobial effects of Lz, Lf, SAA and Hp alone on different strains of *S.aureus*.

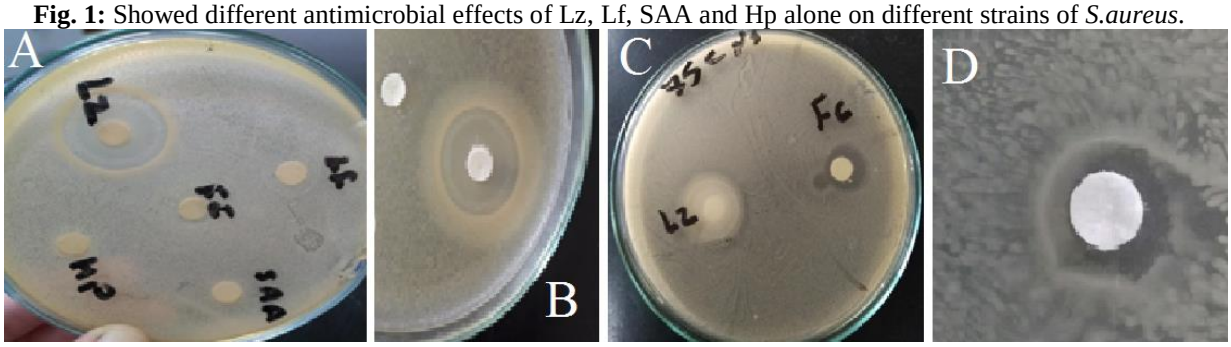

Fig. 2: Showed different antimicrobial effects of Lz, Lf, Hp, SAA alone on different strains of CNS.

In the third step, antibiotic discs were used in combination with the other alternatives to detect the synergistic effect between both of them for *S.aureus* and CNS. Table (3) and fig. (3) showed significant synergistic antibacterial effect of Lz then Lf followed by SAA and Hp, respectively, with antibiotics against *S.aureus* while *S.aureus* showed high resistance to antibiotics when used alone. Correlation coefficient showed as difference in the letters between antibiotic when used alone and when saturated with any of other alternatives.

Table 3: Detection of the synergistic effect of the combination between antibiotics and their alternatives on highly resistant isolated *S.aureus* strains.

Antibiotic	Diameter of inhibition Zones (mm) of antibiotics alone or combined with other alternatives.				
	Antibiotics alone	Antibiotics +Lz	Antibiotics +Lf	Antibiotics +SAA	Antibiotics +Hp
AMX	6^{c}	12^{a}	10^{a}	8^{b}	8^{b}
AMC	6^{b}	15^{a}	18^{a}	12^{a}	13^{a}
AM	6^{a}	6^{a}	6^{a}	6^{a}	6^{a}
TE	6^{c}	15^{a}	13^{a}	8^{b}	10^{b}
P	13.33^{b}	18.66^{a}	17^{a}	18^{a}	15^{b}
FFC	20^{b}	31.66^{a}	30.33^{a}	29^{a}	30^{a}
LEV	12^{c}	18^{a}	16.66^{a}	14.33^{b}	12^{c}
NOR	6^{b}	8^{a}	6^{b}	6^{b}	6^{b}
CIP	6^{c}	12^{a}	10^{b}	8^{b}	6^{c}
ENR	12.33^{b}	17.66^{a}	16.33^{a}	15^{a}	14.33^{a}
OFX	8^{c}	15.66^{a}	15^{a}	12^{b}	12.33^{b}
CN	14.66^{c}	22.66^{a}	21.33^{a}	20^{a}	18^{b}
CEQ	6^{c}	12^{a}	8^{b}	6^{c}	6^{c}
S	6^{c}	18^{a}	17^{a}	8^{b}	10^{b}

The difference in the letters between antibiotic only and with other alternatives were significance ($p < 0.05$)

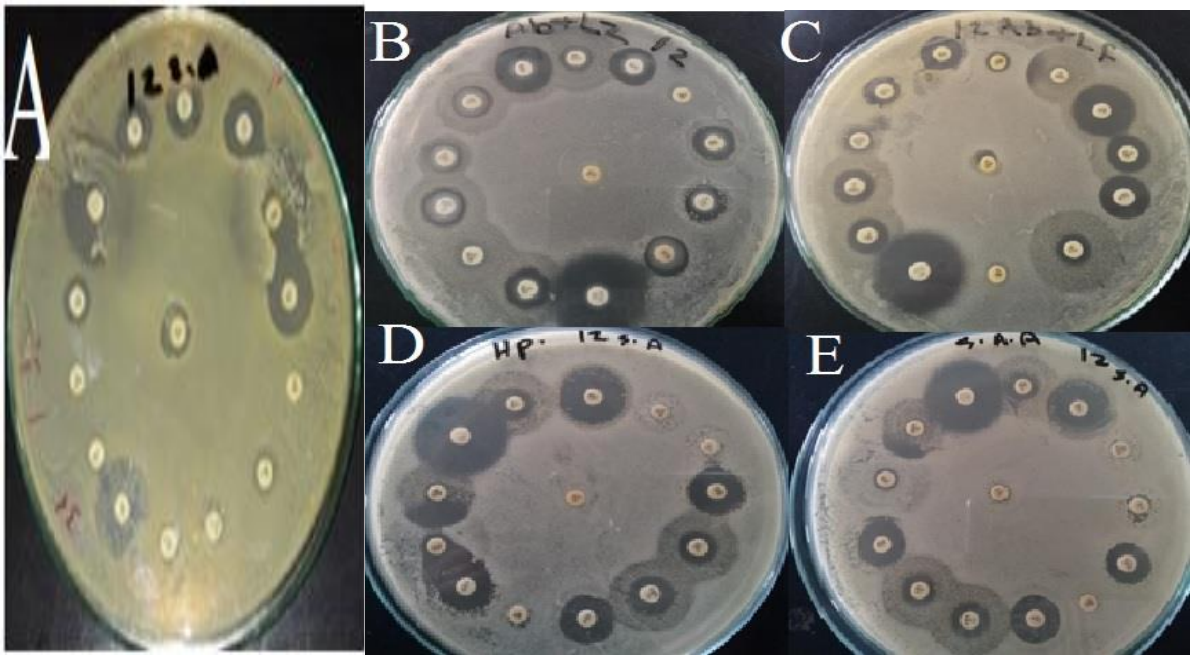

Fig. 3: Showed the antimicrobial effect of antibiotics alone (A) and saturated with 5 µl of each of Lz, Lf (10mg/ml), Hp and SAA on *S.aureus* strain (B, C, D and E, respectively).

Table (4) and Fig. (4) showed significant synergistic antibacterial effect of the combination of Lz then Lf followed by SAA and Hp respectively, with antibiotics against CNS while CNS showed high resistance to antibiotics when they were used alone. Correlation coefficient showed difference in the letters revealed synergistic effect between the combinations of antibiotics with the other alternatives than that of antibiotic only.

Table 4: Detection of the synergistic effect of the combination between antibiotics and their alternatives on isolated CNS strains.

Antibiotic	Diameter of inhibition zones (mm) of antibiotics alone or combined with other alternatives.				
	Antibiotic alone	Antibiotics +Lz	Antibiotics +Lf	Antibiotics +SAA	Antibiotics +Hp
AMX	11.33^{c}	31^{a}	30^{a}	27^{b}	26.66^{b}
AMC	20.33^{c}	35^{a}	33.33^{a}	30.66^{a}	29.33^{b}
AM	6^{c}	14.66^{a}	11.8^{a}	7^{c}	8^{b}
TE	10^{b}	17^{a}	15.33^{ab}	13.66^{ab}	14.33^{ab}
P	21.33^{b}	30^{a}	30^{a}	25^{ab}	24^{ab}
FFC	29^{b}	35^{a}	35^{a}	33^{a}	31^{b}
LEV	25.66^{b}	30^{a}	28^{ab}	27^{ab}	26^{ab}
NOR	27^{b}	33^{a}	31^{a}	27^{b}	28^{b}
CIP	24^{b}	33^{a}	32^{a}	28^{a}	27^{ab}
ENR	22.33^{c}	29^{a}	28^{a}	25^{b}	26^{b}
OFX	24^{b}	30^{a}	28^{a}	26^{b}	25^{b}
CN	11^{b}	15^{a}	14^{a}	13^{a}	12^{ab}
CEQ	12^{b}	16^{a}	15^{a}	15^{a}	15^{a}
S	6^{a}	6^{a}	6^{a}	6^{a}	6^{a}

The difference in the letters between antibiotic only and with other alternatives were significance ($p < 0.05$)

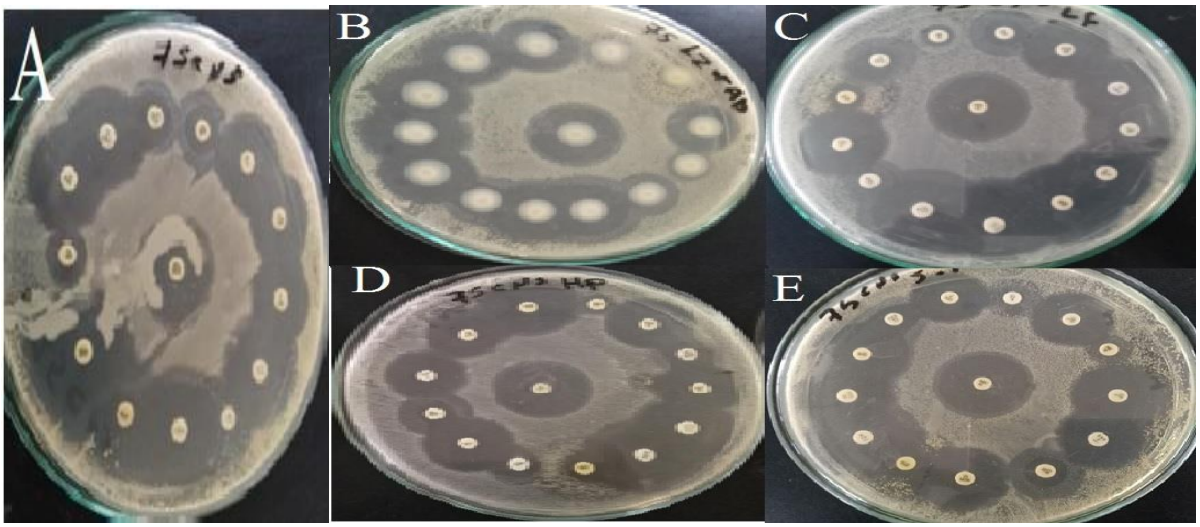

Fig. 4: Showed the antimicrobial effect of antibiotics alone (A) and saturated with Lz, Lf, Hp and SAA on CNS strain (B, C, D and E, respectively).

Different immunological bioactive parameters included Lz, Lf, Hp and mSAA (mammary SAA) were determined to evaluate the immunological status of the naturally infected she camel mammary tissue samples with *S.aureus* or CNS in comparison to bacteriologically negative mammary tissue samples. Table (5) and Fig. (5) showed that the concentrations of the Lz, Hp and SAA were significantly higher in tissue samples infected with both *S.aureus* and CNS when compared with non-infected tissue samples. While the concentration of Lf showed a significant rise in tissue samples infected with CNS when compared with tissue samples infected with *S. aureus*.

Table 5: Correlation between some immunological parameters in the mammary gland tissue and the isolated bacteria.

Isolated bacteria	Average concentration of different immunological bioactive parameters			
	Lz	Lf	SAA	Hp
S. aureus	20.32 ± 1.2***	12.33 ± 1.4**	19.83 ± 1.4***	18.66 ± 2.67***
CNS	19.39±2.3***	19.7 ± 1.1***	13.78 ± 1.55**	14.45 ± 2.2**
Bacteriologically negative tissue samples	8.92 ± 1.9	8.67 ± 1.3	9.77 ± 2.3	9.35 ±1.98

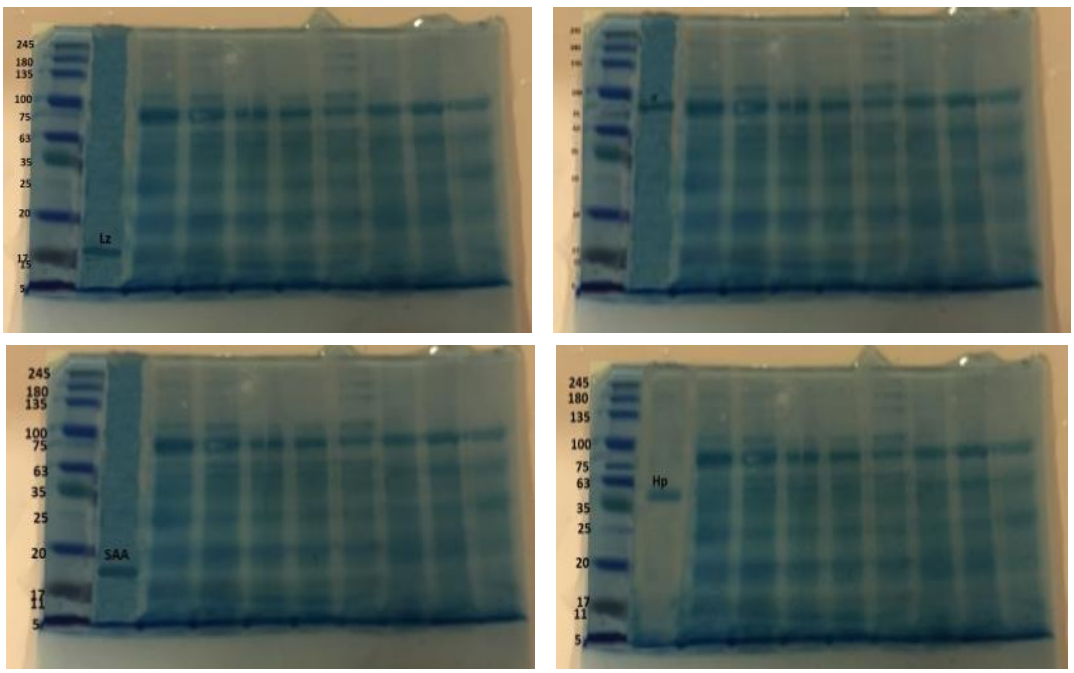

Fig. 5: Showed Lysozyme mol.wt. (14.33 KD), Lf mol.wt. (81.14KD), mSAA mol.wt. (17.02 KD) and Hp mol.wt. (35.02KD).

We used the UPGMA clustering dendrogram analysis to compare the protein fingerprints of the isolated bacteria (Fig. 6&7). The present dendrogram illustrated that there were no or weak similarities in the protein fingerprints of the different isolated *S. aureus* and CNS strains ranging from 0 to 0.36, i.e approximately from 0 to 36% when converted to percent values as shown in table (6).

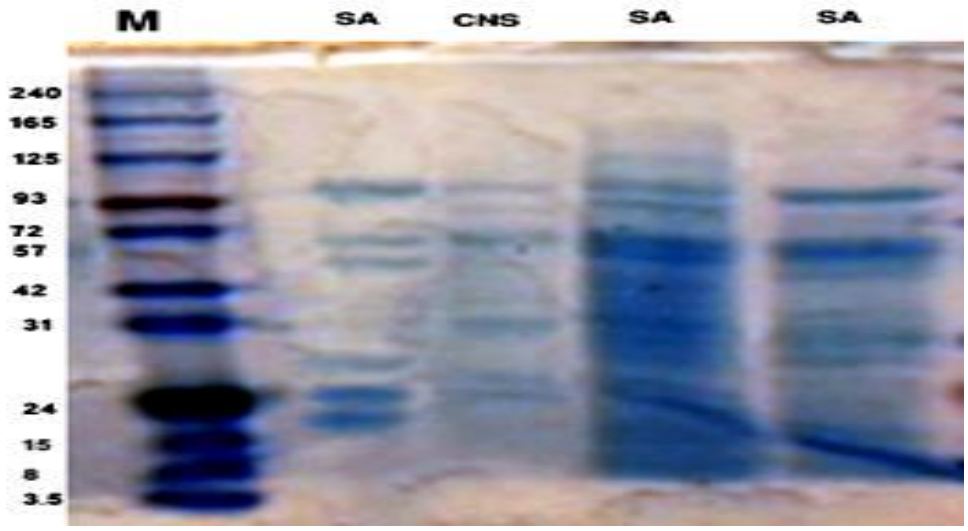

Fig. 6: Electrophoretic whole cell protein profiles of different isolated bacteria Lane 1: Molecular size marker; Lane 2: *S.aureus*., Lane 3: CNS, Lane 4:*S.aureus*, Lane 5: *S.aureus*.

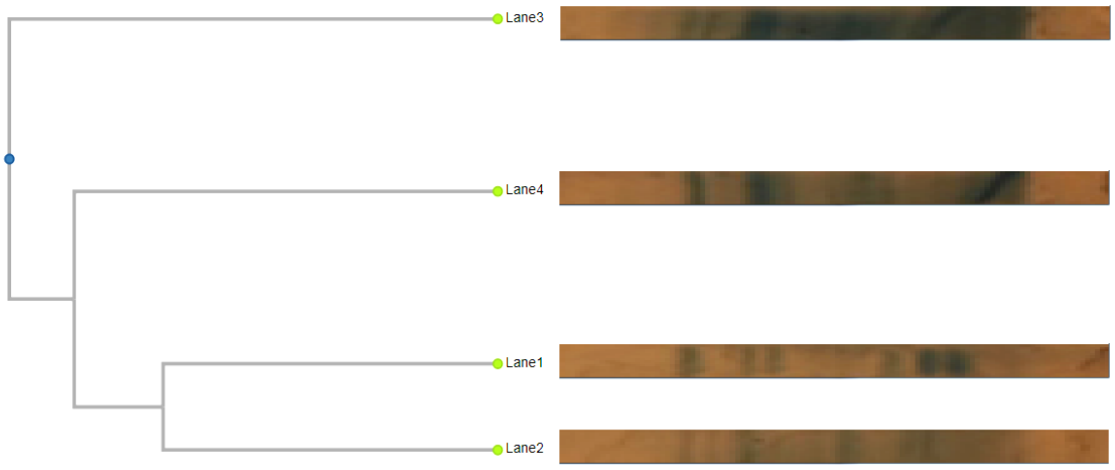

Fig. 7: Dendrogram analysis of the bacterial isolates protein bands, Lane1: *S.aureus*, Lane 2: CNS, Lane 3: *S.aureus*, Lane 4: CNS.

Table 6: The similarities between the protein fingerprints of different isolated *S.aureus* and CNS strains.

Isolated strains	*S.aureus*	CNS	*S.aureus*	CNS
S.aureus	1	0.36	0	0.18
CNS	0.36	1	0.2	0.2
S.aureus	0	0.2	1	0
CNS	0.18	0.2	0	1

Table 7: Types of mastitis of examined she camel mammary tissues depending on histopathological examination in relation to the isolated bacteria.

Type of mastiis	Isolated bacteria	No.	%
1-Acute diffuse mastitis	*S.aureus* (1), CNS (1), *S.aureus*+*Streptococcus* spp. (2), CNS +*Streptococcus* spp. (2), *S.aureus*+CNS (1)	7	14
2-Subacute interstitial mastitis	*S.aureus* (1), CNS (5), *S.aureus*+ *Streptococcus* spp. (3), *S.aureus*+ *E.coli*+ CNS (2), *S.aureus*+ *Streptococcus* spp. + CNS (1).	12	24
3- Chronic interstitial mastitis	*S.aureus*(3), CNS(6), *S.aureus*+*E.coli*(1), CNS+ *Streptococcus* spp.+*E.coli*(2), CNS+*Streptococcus* spp.(6), *E.coli*+ *Streptococcus* spp.(4), CNS+ *E.coli* (4).	26	52
Total		45	90

*Incidence was calculated according to the total no. of 50 mammary tissues of the examined she camels.

In the present work, all the positively cultured collected tissue sections were evaluated for histopathological alterations. Singly isolated S.*aureus* tissue sections revealed grossly, varied degrees of congestion and edema in some cases whereas fibrosis and paleness were noticed in the other most cases. Histopathologically, acute diffuse mastitis was detected in 14% of the cases and the isolated organisms were *S.aureus* (1), CNS (1), *S.aureus* + *Streptococcus* spp. (2), CNS +*Streptococcus* spp. (2), *S.aureus*+ CNS (1), only 2 of them showed suppuration. In addition 12 cases (24%) showed subacute interstitial mastitis and the isolates were *S.aureus* (1), CNS (5), *S.aureus*+ *Streptococcus* spp. (3), *S.aureus*+ *E.coli*+ CNS (2), *S.aureus*+ *Streptococcus* spp.+ CNS (1). The majority of tissue samples 26 (52%) showed fibrosis and paleness and the isolates were *S. aureus* (3), *CNS* (6), *S.aureus*+ *E.coli* (1), CNS+ *Streptococcus* spp.+ *E.coli* (2), CNS+ *Streptococcus* spp. (6), *E.coli*+ *Streptococcus* spp. (4), CNS+ *E.coli* (4).

Microscopically, seven (14%) of tissue samples showed acute diffuse mastitis which characterized by sever vacuolar degeneration, desquamation and coagulative necrosis of the alveolar epithelium within the lumen forming eosinophilic exudates as well as, suppurative inflammatory response marked by neutrophil and macrophages infiltration with congestion (Fig.8). The interstitial tissues appeared oedematous and infiltrated with inflammatory cells mainly neutrophils and macrophages (Fig. 9). Wherever, the non-suppurative types owned few neutrophil infiltrations with lymphocytes and macrophage in the parenchyma without noticed coagulative necrosis and *S.aureus* colonies in side alveolar lumen of damaged alveoli **(**Fig.10). Whereas 11 tissue samples (24%) of subacute interstitial mastitis showed mild vacuolization, desquamation of the alveolar epithelium with inflammatory cells infiltrations and vascular congestion. The interstitial tissues showed few neutrophils and mononuclear cellular infiltrations as well as mild oedema and vascular congestion (Fig.11). On the other side 26 (52%) of tissue samples revealed chronic non suppurative interstitial mastitis that characterized by variable inflammatory changes ranging from the disappearance of the alveolar lumen, through fibrosis to the complete destruction of the parenchyma and interstitial, perialveolar, intra and inter alveolar fibrosis (Fig.12). The mammary lobules were atrophied or replaced completely by fibrous tissue accompanied with mononuclear cells infiltration mainly lymphocytes, macrophages and histiocytes (Fig.13). Prominent corpora amylacea were observed. Hyperplasia of epithelial lining of lactiferous ducts was noticed and associated with sub-epithelial mononuclear cell aggregations as well as lymphocytic exocytosis. Moreover, most of ducts were dilated with wall fibrous thickening and desquamation of the lining epithelium. Hypertrophy of some blood vessels wall with round cellular infiltration was seen (Fig.14). Increase of the interstitial, perialveolar, intra and inter acinar fibrosis were confirmed with Masson's Trichrome stain as greenish blue fibres (Fig. 15, 16, 17).

The activity of alkaline phosphatase in tissue sections of non mastitic animals were high secretory activity on the outer boundary of alveolar secretary cells. However, tissue sections taken from singly isolated S.*aureus* showed weak AP activity, on the outer membrane (Fig.18&19).

Also, the thick-walled mammary alveoli with larger cells in normal non mastitic mammary tissues showed the greatest density of protein when evaluated by mercury–bromophenol blue stain while weak protein density detected in singly isolated *S.aureus* tissue sections comparing to the non-mastitis (Fig.20&21).

Table 8: Morphometric analysis of twenty counted sections from slaughtered she camel mammary parenchyma in non mastitic and mastitic samples (acute, subacute and chronic) caused by *S.aureus* as single infection.

Parameter	Types of mastitis			
	Non mastitic tissue	Acute diffuse mastitic tissue	Subacute interstitial mastitic tissue	Chronic interstitial mastitic tissue
Number of alveoli (µ2)	9.576±0.7113 [a]	6.912±0.6707[b]	8.074±0.5463[ab]	6.240±0.8159[b]
Alveolar diameter (µ)	7.922±1.518 [a]	5.011±1.474[b]	3.636±1.801[c]	0.627±0.0733[d]
Alveolar cell population (µ2)	74.60±4.474 [a]	49.59±4.106[b]	18.18±2.020[c]	13.57±0.9911[d]
Interstitial connective tissue (µ)	160.7±24.77 [a]	178.5±20.91[b]	261.7±22.29[c]	293.8±7.091[d]

Means within the same row are bearing different letter superscripts (a, b, c & d) differ significantly (P≤0.05).

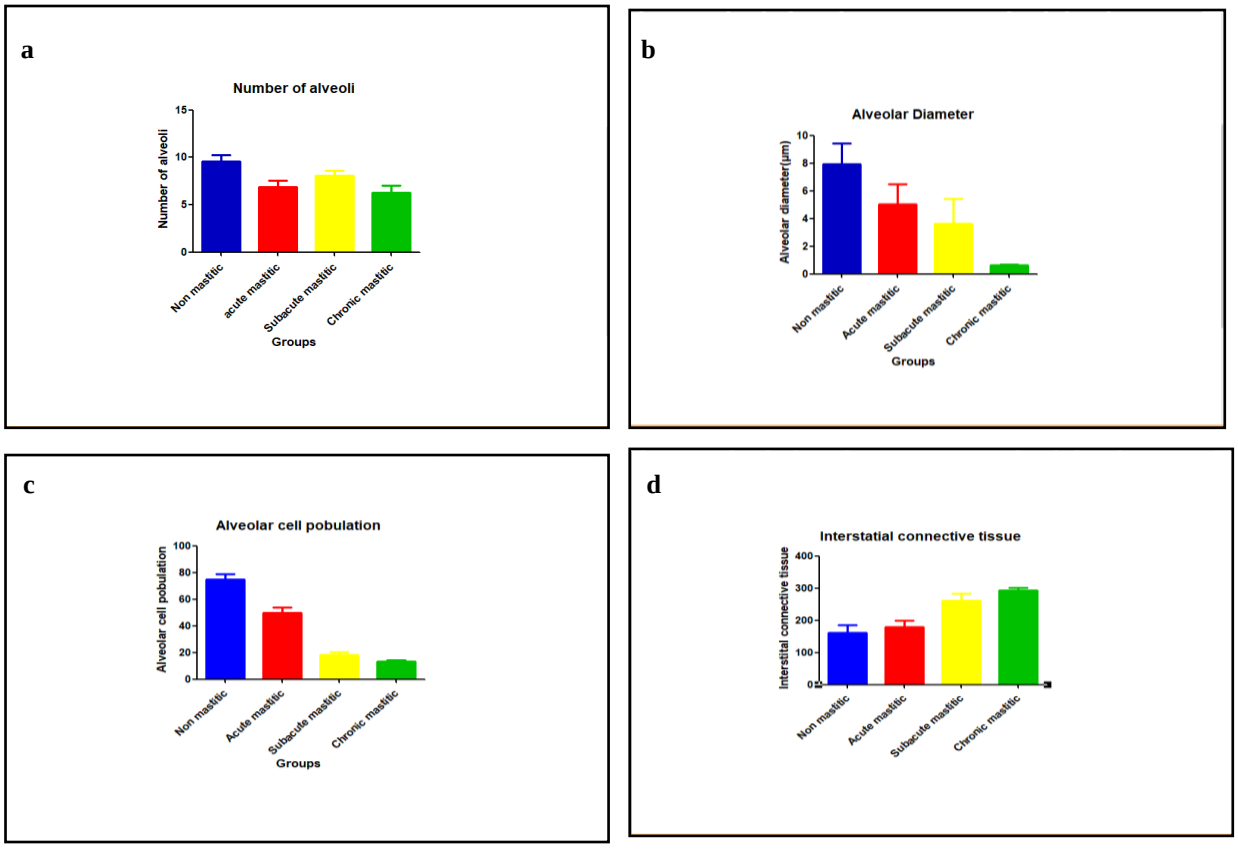

Fig. charts (I; a, b, c and d): Showed morphometric analysis of alveolar number, diameter and cell population as well as interstitial connective tissue of different types of mastitis caused by *S.aureus* infection.

Regarding to the morphometric evaluation in the present study, as showed in Fig. charts (I; a, b, c and d) mammary parenchyma of she camel from 5 different tissue sections from which recovered *S.aureus* as single infection, it was noticed that there were significant decreases (P≤0.05) in alveolar number, diameter and cell population per field, while the interstitial connective tissue showed significant increases (P≤0.05) in each of acute diffuse mastitis, subacute interstitial mastitis and chronic interstitial mastitis when compared to the non mastitic once.

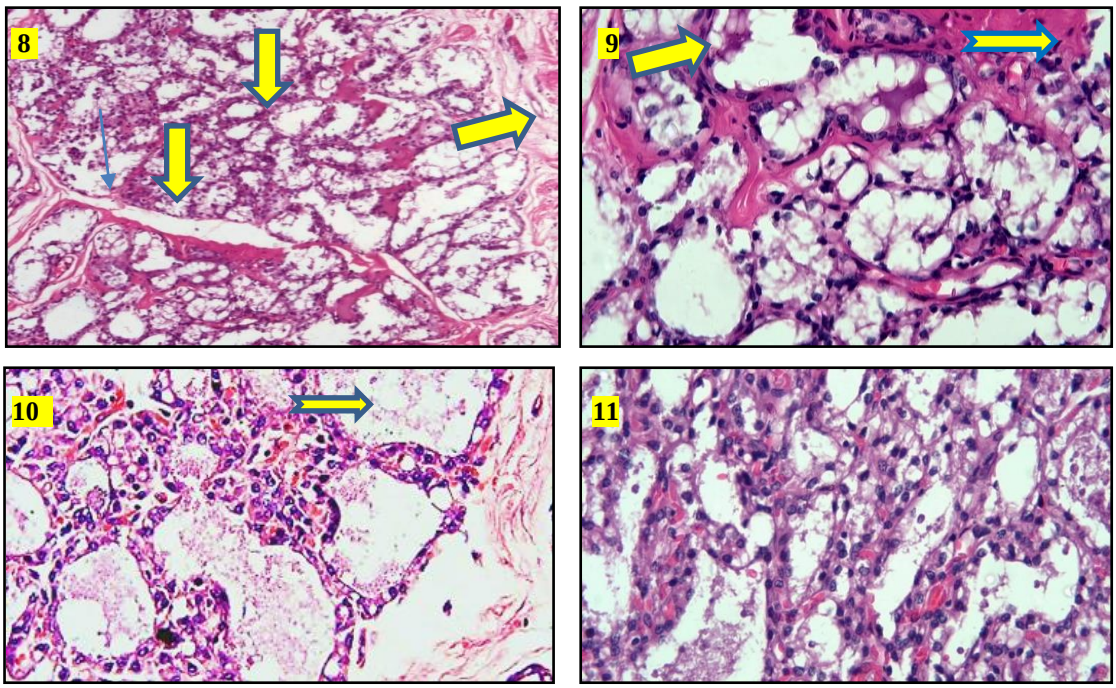

Fig. 8: She camel singly infected *S.aureus* mammary gland showing acute suppurative mastitis, characterized by atrophy and damaged alveoli as well as marked suppuration (yellow arrow) and vascular congestion (H&E, X100).
Fig. 9: She camel singly infected *S.aureus* mammary gland showing acute suppurative mastitis with marked suppuration (yellow arrow) of damaged alveoli with neutrophils and macrophages infiltration as well as necrosis and desquamated of alveolar epithelium (H&E, X400).
Fig. 10: She camel singly infected *S.aureus* mammary gland showing acute non suppurative mastitis with *S.aureus* colonies (yellow arrow) in side the alveolar lumen of damaged alveoli (H&E, X400).
Fig. 11: She camel singly infected *S.aureus* mammary gland showing subacute mastitis vascular congestion and mononuclear cell infiltrations H&E, X40).

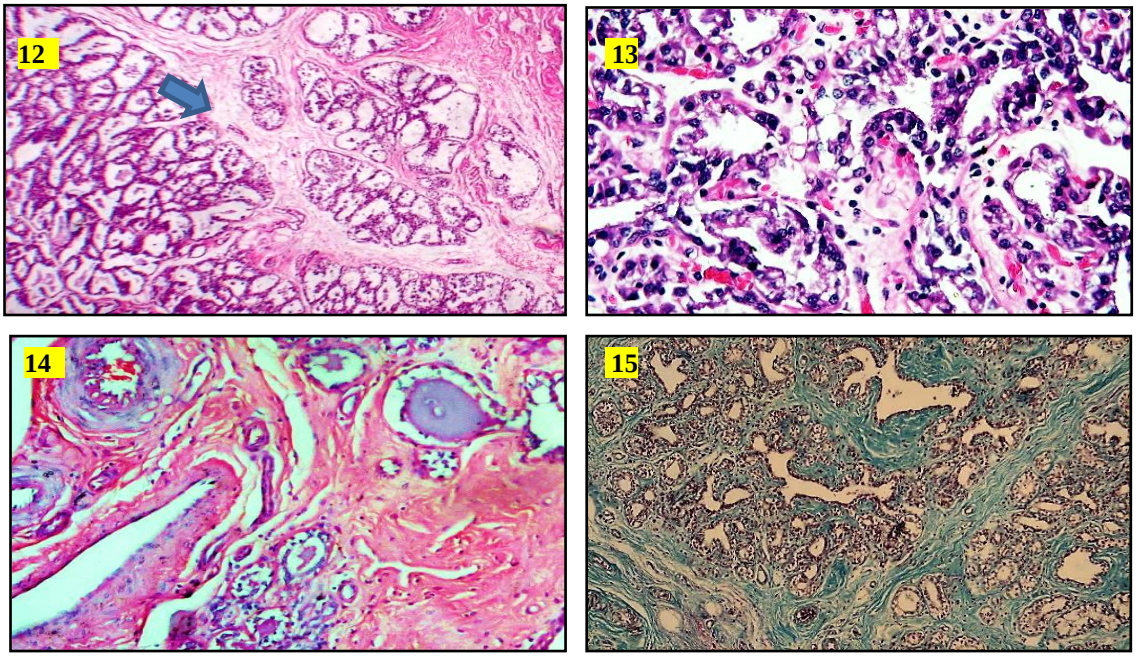

Fig. 12: She camel mammary gland showing chronic interstitial non suppurative mastitis with atrophied lobules (blue arrow), diffuse interstitial fibrous connective proliferation and mononuclear cell infiltrations H&E, X40).
Fig. 13: She camel singly infected *S.aureus* mammary gland showing high power of the previous one (H&E, X100).
Fig. 14: She camel singly infected *S.aureus* mammary gland showing chronic interstitial non suppurative mastitis with fibrous connective proliferation, atrophied lobules(green arrow), hypertrophied vascular wall (blue arrow) and hypertrophied ducal epithelium as well as corpora amylasia (black arrow) (H&E, X100).
Fig. 15: She camel singly infected *S.aureus* mammary gland showing chronic interstitial non suppurative mastitis, fibrous connective proliferation (Masson's Trichrome stain, X40).

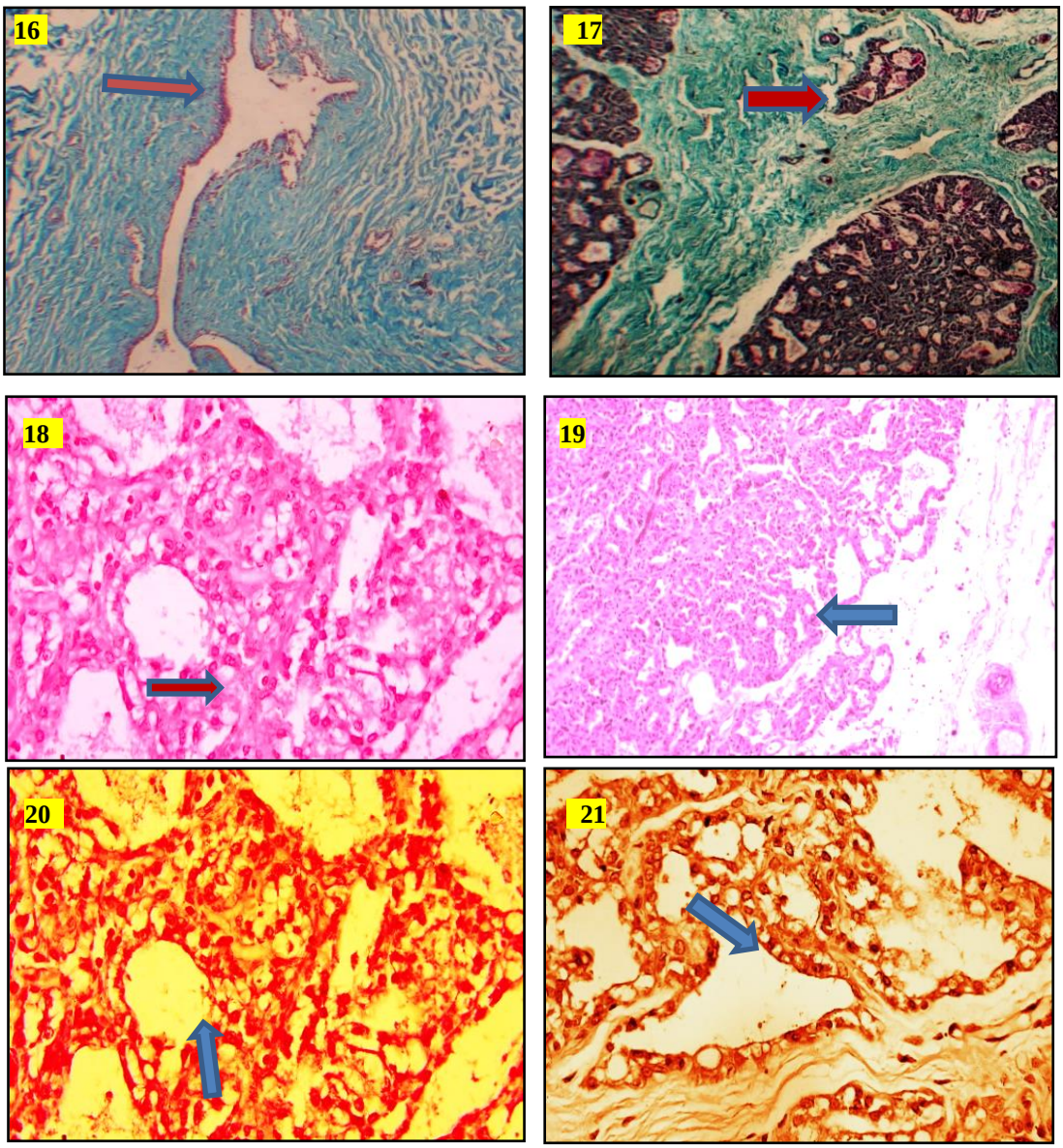

Fig. 16: She camel singly infected *S.aureus* mammary gland showing chronic interstitial non suppurative mastitis, fibrous connective proliferation and ducal epithelium (red arrow) (Masson's Trichrome stain, X100).
Fig. 17: She camel singly infected *S.aureus* mammary gland showing chronic interstitial non suppurative mastitis, fibrous connective proliferation with atrophied lobules (red arrow) (Masson's Trichrome stain, X40)
Fig. 18: Section of mammary tissue from non mastitic she camel showing high level of alkaline phosphatase activity (blue arrow) (alkaline phosphatase X100).
Fig. 19: Section of mammary tissue from singly infected *S.aureus* mastitic she camel showing no to weak alkaline phosphatase activity (blue arrow) (alkaline phosphatase X100).
Fig. 20: Section of mammary tissue from non mastitic she camel showing high protein staining (blue arrow) (mercury–bromophenol blue X100).
Fig. 21: Section of mammary tissue from singly infected *S.aureus* mastitic she camel showing activity of showing weak protein staining (blue arrow) (mercury–bromophenol blue solution X100).

DISCUSSION

Mammary gland infections especially in she-camel lead to produce infected milk which is unacceptable as camel's milk have many therapeutics uses especially in Egypt, so in this study we tried to find alternative treatment to mammary gland pathogens with special references to *Staphylococcus* spp. because it is difficult to be treated as it can be embedded deeply in mammary gland tissues (Foster *et al*., 2014). In recent years, antibiotics resistance and its impact on human health have drawn much attention worldwide. Antibiotics residues in milk pose health hazards to consumers and of high economic importance because such milk unfit for processing and subsequent consumption (Salama *et al*., 2013). Moreover, the antibiotic therapy has many complications, such as hypersensitivity, direct

toxicity, antibiotic-induced immunosuppression and super-infections. This is highlighting the need for new strategies for non-antibiotic therapy through the use of alternative novel antibacterial substances as Lz, Lf, SAA and Hp those used in this study.

In the present study, bacteriological examinations of she camel mammary gland tissues revealed that pathogens mainly caused mammary tissue inflammation were *Staphylococcus* spp. (90%) in the form of single and or mixed infection with environmental *Streptococcus* spp. and *E.coli*. A lower percentage (42.6%) of mixed infection of *S.aureus* and *S.agalactiae* of female camel mammary gland tissues was detected by Hegazy *et al*. (2004). Another study done in UAE at 2013 showed that the main pathogens causing mastitis were *Staphylococcus* (41.67%) and *Streptococcus* spp. (21.67%), *Enterobacter* spp. (15%) and other bacteria with lower percents were isolated (Al-Juboori *et al.*, 2013). Meanwhile another study was done in Sudan at 2013 showed that pathogens isolated were mainly *Staphylococcus* spp. (80.30%) (Alamin *et al.*, 2013). Woubit *et al.* (2001) reported that the major mastitis pathogens isolated included species of *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Corynebacterium* and *Bacillus* and *A.pyogenes*, *E.coli* and *P.haemolytica*.

Bacteriological results revealed high percentage of *Staphylococcus* spp.; this high frequency of *S.aureus* in mammary gland infections could be due to ability of *S.aureus* to stay and survive in keratin layers of teat canal of animals (Qayyum *et al.*, 2016 and Aqib *et al*., 2017).

In this study an *in-vitro* trail was performed to use naturally bioactive immunological agents suggested to have antibacterial activities as safe alternatives to traditionally used antibiotic for treatment of mastitis. Lysozyme and Lf when used alone it was noticed that, they had antibacterial activity on *S.aureus* and CNS that came in agreement with Amany *et al*. (2005); Conesa *et al*. (2008); Mona *et al*. (2010); Gizachew *et al.* (2014) and Abrhaleya and Leta (2018) they reported that Lz and Lf exerted antibacterial, antiviral, antifungal and anti-parasitic activity.

Rabab (2009) studied the antimicrobial action of Lf against different microorganisms. She recorded that Lf had a pronounced effect on the growth of on some pathogenic bacteria as *S.aureus*, *S.pyogenes*, *E.coli*, and *E.aerogenes* and none of them was able to resist the bacteriostatic effect of Lf that came in accordance with our study which showed growth inhibition of *Staphylococcus* spp. either *S.aureus* or CNS when used Lf alone with relatively higher inhibitory zone in case of CNS (16 versus 13mm). Recently Niaz *et al*. (2017) reported that isolated Lf from camel milk exhibited promising antibacterial activity against *E.coli* and *S.aureus* and the concentration of Lf 4 mg/ml showed the best results against both the pathogens.

Al-Majali *et al*. (2007) studied the antibacterial effects of camel Lf against some selected isolated bacteria from subclinical mastitic milk and found that, all tested bacterial isolates were resistant to the camel Lf except *S.aureus* (20 isolates), *S.agalactia* (2 isolates), and 12 isolates Streptococci other than *S.agalactiae*. Abrhaleya and Leta (2018) stated that lactoferrin had potent antimicrobial and anti-inflammatory properties, including bacterial inhibition for *S.aureus* and *E.coli*. Also Rabab (2009**)** said that the maximum growth inhibition of Lf was recorded with *E.coli* than any other strain and explained that may due to Lf is a glycoprotein which is able to bind two metal binding cations, preferably ferric ions at specific binding sites. Therefore, it competes with other bacteria for iron present in the media. Since *E.coli* is the highest strain for iron requirement in the growth, therefore it was the most affected by Lf. Previously, Kappeler *et al.* (1999) reported the inhibition effect of Lf against mastitic isolates of *S.aureus* but there was no effect against *S.agalactiae* and *S.uberis*. Meanwhile it was found that antimicrobial activity of Lf may be different in Gram-negative and Gram-positive bacteria due to the differences in the cell membrane structure. At the same time bacterial isolates (either Gram-positive or Gram-negative) were inhibited by *in-vitro* addition of Lf which can act either as a bacteriostatic and/or bactericidal agent. This difference in the activity may explain the wide range of Lf action. The presence of Lf binding proteins or Lf receptors on the surface of the microorganisms may partially explain the resistance of these isolates to Lf (Farnaud *et al*., 2004).

Lysozyme is among the minor milk proteins that attracted attention recently due to its potent antimicrobial activity against a wide range of microorganisms. Lysozyme has been shown to have antimicrobial activities towards bacteria, fungi, protozoan and viruses (Benkerroum, 2008 and Shakir *et al.*, 2013). The current results revealed that Lz had strong antibacterial effect on *S.aureus* and CNS that was cleared with the large inhibitory zones (18 versus 20 mm, respectively) when it was used alone. The antibacterial activity of lysozyme is essentially directed towards gram-positive bacteria, as their target cell-wall component (peptidoglycan) is freely accessible to the enzyme, contrary to that of gram-negative bacteria, which is shielded by the lipopolysaccharidic (LPS) layer of the outer membrane. These results agreed with Gul *et al*. (2015) who said that, Lysosyme is a protective enzyme, has antibacterial activity against gram-positive.

With using SAA and Hp alone as antibacterial agents in this study no significant inhibitory effect on the growth of *Staphylococcus* spp. under study was noticed and showed complete resistance to both them. This may be attributed to these strains need higher concentrations/or amount of SAA and Hp as the used strains showed very high antibiotic resistance. This was concluded by De Buck *et al*. (2016) who observed that the effects of SAA were dose-dependent and, specific *in-vivo* biological effects of SAA really were dependent on the actual concentrations, whether in serum or in specific local microenvironments. On the other hand, Wanda (1997) reported that Hp has bacteriostatic effects by binding free haemoglobin, thus depriving bacteria from iron required for their growth and provide a novel microbicidal mechanism that may explain the mode of action of Hp *in-vivo* while *in-vitro* showed no antibacterial effect when used alone that may support our results.

In explanation of the mode of action of Hp as antibacterial agent, Davies *et al*. (2015**)** reported that, Hp's mechanism of action is sequestering iron, therefore, reducing the iron available for bacteria. Importantly, iron is crucial for bacterial growth, with a deficiency causing bacterial growth inhibition (Cherayil, 2011). Although iron is essential for most bacteria, the levels required and uptake mechanisms vary considerably between microorganisms. Generally, Gram-negative bacteria recognize iron sources via an outer membrane receptor. The iron is then transported into the cell by an ATP-binding cassette transporter within the inner membrane (Krewulak and Voge, 2008). Because Gram-positive bacteria lack an outer membrane, iron uptake mechanisms differ from that of the Gram-negative bacteria. The differences in iron uptake mechanisms are the likely reason Hp only demonstrated antibacterial properties against the Gram-negative bacteria examined.

Contrary to the present result that SAA hadn't antibacterial inhibitory action against *Staphylococcus* spp. under study; a study described a direct antibacterial activity of the SAA protein (Molenaar *et al*., 2009). This direct activity could be also participating in the reduction of the bacterial translocation observed *in-vitro* assay. SAA exerts antibacterial and antiviral activities by functioning as an opsonin for bacteria and by interfering with virus infection of host cells (Bucka *et al*., 2016). Chandrabala *et al*. (2006) demonstrated that SAA binds to a range of Gram negative bacteria including *E.coli*, *K.pneumoniae*, *Sh.flexneri*, *V.cholerae*, and *P.aeruginosa* through outer membrane protein A (OmpA) family members but not Gram-positive organisms such as *S.pneumoniae* and *S.aureus* which agreed with our results and may explain why SAA had no antibacterial effect on Staphylococcus spp. when used alone in the current work. Otherwise, Verginia *et al*. (2017) stated that SAA is one of the acute phase proteins (APPs) which has many highly linked genes (SAA1, SAA2, SAA3 and SAA4). Mammary SAA-3 had antibacterial activity (Schneider, 2015) and activates the involution via increasing cytokines related to innate immunity in cattle infected with *S. aureus* (Kalmus *et al*., 2013 and Domenech *et al*., 2014).

Antimicrobial peptides (AMPs) represent a vast array of molecules produced by virtually all living organisms as natural barriers against infection. Among AMP sources, an interesting class regards the food-derived bioactive agents including whey protein Lf **(**Bruni *et al*., 2016). In studying the antibacterial susceptibility of *S.aureus* and CNS isolated from the examined mammary gland tissues to antibiotics and/or their alternatives, the result revealed that, by using antibiotics alone bacteria under study showed multidrug resistance to the most used antibiotics especially *S.aureus*. Meanwhile synergistic effects were clearly observed by using mixtures of antibiotics and their alternatives as Lz, Lf, SAA and Hp. This is a novel technique for improve antibacterial effect of antibiotics or their alternatives as can be considered first step trying to use natural antibacterial substances. Bruni *et al*. (2016) explained the synergistic effects as the loss of inner membrane integrity may promote the uptake of other agents, for example antibiotics or other antibacterial peptides, leading to synergy with conventional antibiotics. In agreement with our results Lacasse *et al*. (2008) recommend that inframammary treatment with Lf was not satisfactory for overcoming beta-lactam resistant *S.aureus* infection. However, Lf co-administered with penicillin G increased the cure rate (from 12.5% to 33%), reducing beta-lactamase activity in resistant *S.aureus* strains. The strong activity against mastitis pathogens of AMP and Lf in particular, has spurred interest in their potential application to the control of udder infections. In an *in-vivo* trial, Kawai *et al*. (2003) tested an infusion of Lf in cows affected by subclinical mastitis, caused by various bacteria, including *E.coli* and Staphylococci. Their results showed a significant reduction of bacteria in the mammary tissue already on day one after infusion and eradication of the disease after14 days.

Very little information for using SAA and Hp to improve the action of antibiotics especially for microorganisms that have multidrug resistance as *S. aureus* so this study may open the doors for more researches in these aspects to improve the control measures of mastitis and udder health in she camels or bovines.

Since the problem of mastitis is compounded by the incidence of subclinical mastitis which is a form of

the disease where signs of inflammation (systemically and locally) are absent, Akerstedt *et al.* (2007). Consequently, an inability to readily recognise and diagnose animals with mastitis occurred, leading to a delay in treatment and control of infections thus allowing a possible spread to other uninfected quarters, (Funmilola *et al*., 2015). Thus, tests for indicators of inflammation can be used to screen quarters for intra-mammary inflammation (Pyörälä, 2003). In this investigation, we proposed that the major acute phase reactants (e.g. Lz, Lf, SAA and Hp) may be synthesized by the mammary gland tissue, and this came in agreement with previous studies of Close *et al.* (1997); Hsiang (2009); Berg *et al.* (2011) and Jiang *et al.* (2015). One of the aims of the on-going work was to study the effect of the isolated microorganisms on the levels (concentrations) of Lf, Lz, Hp and SAA in she-camel udder tissue samples that were affected with mastitis, and the results in (Table 5 and Fig.5) showed that the concentrations of the Lz, Hp and SAA were significantly higher in tissue samples infected with both *S.aureus* and CNS when compared with the non-infected tissue samples. While the concentration of Lf showed a significant rise in tissue samples infected with CNS when compared with tissue samples infected with *S. aureus*. These findings came in agreement with Grönlund *et al.* (2003), who found that concentrations of Hp and SAA increased rapidly in both serum and milk during the acute phase of mastitis and a significant rise in milk concentrations of SAA were also found during chronic subclinical mastitis. They added that serum concentrations of SAA also tended to be higher during the chronic phase than pre-infection, and that increases in milk Hp and SAA were specific for the infected udder quarters. They concluded that, measurement of SAA in milk samples could be a useful tool in diagnosing mastitis. Similarly, Annamaria *et al.* (2016) found that, one in four milk samples where *S. aureus* was isolated had much lower Lf concentrations than the other three samples where Corynebacterium spp. and CNS.

Meanwhile, Bera *et al.* (2005) and Takahiro (2010**)** mentioned that Staphylococcus species belong to one of the few bacterial genera that are completely lysozyme resistant.

The characterisation of pathogenic Staphylococci especially isolates that showed resistance to antibiotics is important in combating of diseases caused by them. This was of a particular interest in the present study. It was desirable to characterise *S.aureus* isolates specially isolates that showed resistance for antibiotics. So, we used the one-dimensional SDS-PAGE in studying protein profile of the isolates that showed resistance to antibiotics. The results revealed protein profiles containing (6-12 or more) discrete bands with molecular weights of (16.95- 76.48) KDa. We compared the protein fingerprints of the protein bands of these isolates using the UPGMA clustering dendrogram analysis (Fig. 7 and Table 6). The results of the present dendrogram analysis illustrated that there were very weak or no similarities in protein profiles of the protein bands of the isolates ranging from 0 to 0.36; i.e approximately from 0 to 36% when converted to per cent values. This may be attributed to the different localities from which the samples were collected that may cause great difference in the protein profile of the isolates under this study. On the other hand, Fitzgerald *et al.* (2001) and Fábio *et al.* (2006) referred these weak similarities between the *S.aureus* strains to the extensive variation in gene content which may be caused by a change in the host environment, they also added that comparative genomic analysis of *S.aureus* strains revealed a high degree of inter-strain variation. The epidemic of toxic shock syndrome that occurred in the 1970s was caused by a change in the host environment, rather than rapid geographic dissemination of a new hyper-virulent strain. DNA microarray analysis of large samples of clinically characterized strains provides broad insights into evolution, pathogenesis, and disease emergence.

Infectious Mastitis that caused by the bacterial harmful toxins that release in the udder and inducing lesions may vary from increased milk leukocytes counts with no gross changes in milk to increased vascular permeability or develop fibrosis or severe toxemia (Yousaf *et al*., 2010 and Ibrahim *et al.*, 2011). In the present study, noticed histopathological changes in *S. aureus* isolated as single infection tissue sections revealed grossly, varied degrees of congestion and edema only two of them showed suppuration while most of the other cases showed fibrosis and paleness. On the other hand, microscopical examination exhibited variable histopathological changes which ranged from epithelial degeneration, congestion, coagulative necrosis and neutrophil infiltration, in the acute diffuse mastitis and sub-acute interstitial mastitis. On the other side alveolar atrophy, fibrosis with lymphocytic and histiocytic infiltration in chronic interstitial mastitis was the vast majority. Some cases showed disappearance of the alveolar lumen, through fibrosis to the complete destruction of the parenchyma. Similar observations were recorded in she camel by Hungerford (1989); Bakeer *et al.* (1994) and Abdurahman (1996).

Acute diffuse mastitis was attributed to *E.coli* infection. The inflammation mostly involved one quarter and caused destruction of the lining of the ducts, edema of interstitial tissues and infiltration of the acini with serous and inflammatory cells mainly neutrophils and macrophages. Similar findings were observed in she camel acute *E.coli* infection by

Bakeer *et al*. (1994). It was also isolated from peracute mastitis in camels by Kapur *et al.* (1982); Quandal and Qudan (1984); Khan and Khan (2006) and Iyer *et al*. (2014). Marked leukocyte infiltration during mastitis was detrimental to the developing mammary parenchymal tissue which ultimately leading to milk loss (Nickerson, 2009 and Piepers *et al.*, 2009). Whereas Barbour *et al.* (1985) and Karmy (1990) reported that, Streptococcus was a major cause of chronic mastitis in she camels. Those findings were in line with other previous studies (Kheira and Abdellatif, 2014 and Zeleke, 2016) in Ethiopia. According to Hussai *et al.* (2012c) the tissue sections from mastitic animals revealed mild, moderate or severe atrophy of alveoli with cellular exudate in the lumen of the alveoli. The existence of acute and chronic inflammation in mammary parenchymal tissues was confirmed and fibrous tissue proliferation was seen in the mammary gland.

Moreover, lesions of the mammary tissue reduce the number and activity of epithelial cells and therefore contributes to lower milk production with increasing proportions of lymphocytes and macrophages was reported by Zhao and Lacasse (2008), multiple inflammatory cell types and structural damage in the *S.aureus* group, similar results observed in previous research-α, IL-6 and IL-1β play important roles in the inflammatory response. TNF-α is an early cytokine, which plays a critical role in the cascade of other pro-inflammatory cytokines and inflammatory mediators. IL-1β which is considered to be a gatekeeper of inflammation plays an essential part in the early inflammatory response. Pathological changes occurred in udder tissue could be due to severe tissue damage caused by different mastitis pathogens.

Alkaline phosphatase, a membrane-associated glycoprotein enzyme, increases hydrolysis of phosphates and is located mainly on the outer cellular membranes of tissues having vigorous transport processes (Murray and Ewen, 1992). Milk protein synthesis in mammary tissue is a complex mechanism under the influence of local and systemic hormones along with some other factors those affect milk yield (Khaliq and Rahman, 2010 and Hussain *et al*., 2010). The weak AP activity and protein density that observed in this work in singly isolated *S.aureus* mastitic tissue sections were observed previously by Hassan (2004); Silanikove (2008); El-Sayed *et al*. (2009); Hussain *et al*. (2012c) and Hussain *et al*. (2013) which attributed to the degenerative changes of mammary epithelium with connective tissue proliferation and impaired activity of endoplasmic reticulum that induced by microbial agents. These degenerated mammary cells encompassing the active cellular protein was substituted by the spread of connective tissue under the bacterial toxins effect which resulted in poor biosynthetic capacity of udder and decreases cellular differentiation. As well as may be related with deactivation of this enzyme owing to negative regulatory process of mammary gland, also could be due to impaired milk secretory mechanism (El-Sayed *et al*., 2009; Hussain *et al*., 2012c and Hussain *et al*., 2013).

Regarding to the Morphometric investigations of tissue sections from singly infected S. *aureus* comparable to non mastitic showed significant decrease ($P \leq 0.05$) in alveolar diameter, number of alveoli and alveolar cell population whereas the interstitial connective tissues showed significant increase ($P \leq 0.05$). These results indicated severe tissue degenerative changes and damage due to different mastitis pathogens. However, the decrease number of alveoli, luminal area and less number of alveolar secretary cells has been determined in advance stage of lactation (Akers *et al.*, 2006). In addition, the decreased number of alveolar secretory cell could be due to cell death induced by milk accumulation in alveoli (Singh *et al.*, 2005). The number of secretory cells per alveolus was the best indicator of mammary gland lactogenic activity (El-Sayed *et al*., 2009; EL-Sayed *et al.*, 2013 and Hussain *et al.*, 2013).

CONCLUSION

We concluded that, Staphylococci were the major bacteria causing mastitis in she camels and showed great differences in their protein profiles, consequently caused significant increase in the inflammatory responses of the udder tissues represented by the increase of Lz, Lf, SAA and Hp concentrations in the tissues. Staphylococci as single infection enough for inducing sever degenerative changes and damage that reflecting impaired activity as well as printed on the different histopathological, histochemical and morphometric changes in she camels mammary parenchyma. Furthermore, this study offers opportunities for development of treatment strategies that may eliminate or even reduce mammary tissue damage caused by Staphylococcal mastitis with or without the use of antibiotics and/or anti-inflammatory molecules like Lz, Lf, SAA and Hp.

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دراسات هستومورفومتريه ومناعيه علي الميكروب العنقودى المسبب لإلتهاب الضرع في إناث الجمال ودراسة تأثير بعض بدائل المضادات الحيويه على الميكروب العنقودى المعزول

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تم الفحص البكتيريولوجي لعدد ٥٠ عينة من أنسجة الضروع للنوق (إيناث الجمال) المذبوحه فى المجازر وكشفت النتائج أن المكورات العنقودية كانت البكتيريا المعزولة الأكثر شيوعًا بنسبة ٩٠٪ كعدوى مفردة ومختلطة تليها المكورات السبحيه البيئية بنسبة٤٠٪ ثم الايشريشيا كولاى بنسبة ٢٦٪ في شكل عدوى مختلطة لكلاهما مع المكورات العنقوديه. كانت معظم المكورات العنقودية المعزولة سواءاً المكورات العنقودية الذهبيه أو المكورات العنقودية الغير الذهبيه (السالبه لإختبار التجلط لبلازما الأرانب) مقاومة ل١٤ نوع من المضادات الحيوية المتوفرة تجاريا. وبإستخدام ٤ أنواع مختلفه من ببتيدات مضاده للميكروبات (AMPs) كبدائل للمضادات الحيوية ؛ شملت الليسوزيم واللاكتوفيرين والسيرم أميلويد (أ) والهابتوجلوبين أظهرت النتائج أن الليسوزيم واللاكتوفيرين لديهم أنشطة مضادة لكل من عترات المكورات العنقودية الذهبيه أوالمكورات العنقودية الغير الذهبيه المعزولة من أنسجة الضرع. بينما لم يظهرالسيرم أميلويد (أ) والهابتوجلوبين أى تأثيرات مضادة للسلالات المختبرة. وبإستخدام خليط من المضادات الحيوية المختلفة والأربعة بدائل الأخرى للمضادات الحيويه قد لوحظ وجود تآزر كبيرفي التأثير المضاد لجميعهم على السلالات التى تم إختبارها. أيضا تم تحليل الملف البروتينى لأنسجة ضروع النوق المصابة طبيعياً بالمكورات العنقودية الذهبيه والمكورات العنقودية الغير الذهبيه بإستخدام تقنية الفصل الكهربى (SDS-PAGE) وذلك لتقييم الحالة المناعية لعينات نسيج الضرع المصابة ومقارنتها مع عينات الأنسجة التى لم يتم عزل بكتيريا منها وقد أظهرت النتائج أن تركيزات كل من الليسوزيم واللاكتوفيرين والسيرم أميلويد (أ) والهابتوجلوبين أعلى بكثير في عينات أنسجة الضروع المصابة بالمقارنة مع عينات الأنسجة الغير مصابة. بينما أظهر تركيز اللاكتوفيرين إرتفاع معنوي أعلى في عينات الأنسجة المصابة بـالمكورات العنقودية الغير الذهبيه بالمقارنة مع عينات الأنسجة المصابة بالمكورات العنقودية الذهبيه. تم تطبيق تحليل UPGMA clustering dendrogram لمقارنة بصمات بروتين المكورات العنقودية المعزولة سواءاً كانت المكورات العنقودية الذهبيه أوالمكورات العنقودية الغيرالذهبيه وقد أوضحت النتائج أنه لم يكن هناك أى أوجه تشابه أوضعيفة جداً إن وجدت في بصمات البروتين في السلالات المختلفة لكل منهم وتراوحت أوجه التشابه مابين الصفر و ٠,٣٦. وكان نتيجة الفحص الهستوباثولوجى للعينات الموجبة العزل إستنتاج ثلاث أنواع من إلتهاب الضرع للعينات المعزول منها ١٩ حالة (٣٨٪) تميزت ظاهريا بإحتقان وتورم بدرجات متفاوتة مكونة من ٧ حالات (١٤٪) منها عبارة عن إلتهاب حاد منتشر إثنين فقط منها أظهرت إلتهاب حاد صديدى وهستوباثولوجيا تبين وجود العديد من النيوتروفيل والماكروفاج داخل الحويصلات اللبنية وفى النسيج البينى مع وجود الميكروب العنقودى داخل الحويصلات اللبنية وتميزت هستوباثولوجيا بإحتقان ووجود عدد قليل من النيوتروفيل بين الحويصلات و فى النسيج البينى والغالبية كانت للإلتهاب المزمن البينى المنتشر وكان نسبته ٥٢٪ تميز بزيادة تكوين النسيج الليفى مما يضغط على بعض الحويصلات والفصوص فتصبح ضامرة مع إنتشار للخلايا الإلتهابية أحادية الخلية مثل الماكروفاج واليمفوسيت والهستيوسيت مع تضخم لجدار الأوعية الدموية وقناة اللبن وقد تم إظهار النسيج الليفى هستوكيميائيا بصبغة المسون تراى كروم. أيضا هستوكيميائيا تم فحص النشاط الخلوى للحويصلات للألكالين فوسفاتيز والبروتين فى الأنسجة المعزول منها ميكروب المكور العنقودى بصوره منفرده فقط وقورنت بالأنسجة سالبة العزل فوجد ضعف فى نشاط الخلايا أو ندرة اللأكالين فوسفاتيز والبروتيين فى العينات المصابة. كما تم إجراء الفحص الهيستومورفوميترى للعينات المعزول منها ميكروب المكور العنقودى فقط وبمقارنتها بالأخرى سالبة العزل فقد تبين نقص فى عدد الحويصلات وعدد خلاياها ونصف قطرها وزيادة النسيج الليفى ومساحته بطرق متفاوتة حسب نوع الإلتهاب وقد إستنتج من هذه الدراسة أن ميكروب المكور العنقودى له تأثير مدمر متفاوت على أنسجة الضرع مما يؤثر على الخصائص والنشاط الإنزيمى والتخليقى للخلايا كما يؤثر فى التركيب والخصائص الهستومورفومترية. وقد خلصت هذه الدراسة إلى أن المكورات العنقودية هى البكتيريا الرئيسية التى تسبب إلتهاب الضرع فى النوق وأظهرت البكتيريا إختلافات كبيرة فى البصمات البروتينية لها مما أدى إلى زيادة ملحوظة فى الإستجابات المناعية والإلتهابية لأنسجة الضروع المختلفة ممثلة فى زيادة تركيزات الليسوزيم واللاكتوفيرين والسيرم أميلويد (أ) والهابتوجلوبين. أيضا المكورات العنقودية كعدوى منفردة تكفي لإحداث تغييرات تنكسية خطيرة وأضرار تعكس نشاطًا ضعيفًا وعكسياً على الكفاءه الإنتاجيه للضرع بالإضافة إلى تأثيرها على التغيرات الهستوباثولوجيه والهستوكيميائيه والمورفولوجية المختلفة فى أنسجة ضروع النوق. علاوة على ذلك، هذه الدراسة قد توفر فرصًا لتطوير إستراتيجيات جديده للعلاج التى قد تؤدي إلى السيطرة على أو حتى تقليل الأضرار فى الأنسجة الثديية الناجمة عن إلتهاب الضرع المسبب بعدوى المكورات العنقوديه بأنواعها المختلفه بدون إستخدام المضادات الحيوية أو مزجها مع بدائل طبيعيه أمنه مضادة لهذه البكتيريا خاصة الأنواع الشديدة المقاومه للمضادات الحيويه هذه البدائل مثل الليسوزيم واللاكتوفيرين والسيرم أميلويد (أ) والهابتوجلوبين.