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Virulence factors associated with staphylococci isolated from mastitic Egyptian buffaloes with special reference to the biochemical changes in the serum and milk

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Abstract

A total number of 100 milk samples (50 from clinical mastitis and 50 from apparently healthy) were collected from dairy buffaloes in Giza Governorates, Egypt. Samples were examined for the presence of *Staphylococcus spp*. specially *S.aureus* and their virulent genes and detect some biochemical parameters which affected by mastitis. Results obtained showed that, a total of 34 Staphylococci isolates were recovered from buffaloes with an incidence of 34%. Among the isolates, *S. aureus* is the major pathogen isolated from clinical mastitic milk samples 17(34%), while 4(8%) were recovered from subclinical mastitis. Antibiotic sensitivity pattern revealed that *S.aureus* isolates were high sensitive to Enrofloxacin (90.48%), Ciprofloxacin (85.71%) and Cefotaxime (80.95%), while resist Ampicillin (57.14%) and Amoxicillin (52.38%). Six *S.aureus* isolates were screened for the detection of specific protein (IgG-binding protein) *spa* gene*,* Haemolysine type B (*hlb),* Clumping factor A gene (*clfA***)** and Intracellular Adhesion (*ica*) gene by PCR. PCR amplification revealed that all *S. aureus* isolates were harbored the *clfA* and *spa* genes, while *hlb* and *ica* were detected with an incidence of 50% and 66.67% respectively. On the other hand, serum samples from diseased animals revealed elevated value of total proteins, β1 and γ1-globulins meanwhile albumin and γ2-globulins recorded decreased levels. Analysis of whey milk showed an increased level of total proteins, albumin and immunoglobulin in addition to decreased value of α- lactoalbumin and β-lactoglobulin. While sodium level increased meanwhile the values of potassium, calcium and phosphorus were decreased.

The study revealed that, *S.aureus* is an important bacterial pathogen causing buffalo mastitis and it has many virulence factors that causing inflammatory response in the mammary gland tissues. Using PCR in our study not only sensitive and rapid technique but also can detect the presence of some virulent genes as *clfA*, *spa*, *hlb* and *ica* genes which increase pathogenicity of *S.aureus*. Biochemical parameters can be used as important indicators as complementary to bacteriological examination for mastitis detective and can be aid in treatment regimen improvement.

Key words: *Buffalo, Mastitis, S. aureus, Virulence gene, PCR, chemical alterations.*

Introduction

Bovine mastitis is one of the major social and economically significant diseases, which reduce milk yield and consequently causes loss of income to dairy breeders, also with treatment costs. Variety of microorganisms are incriminated in bovine mastitis among these microorganisms are pathogenic staphylococci **(Heikkilä *et al.*, 2018).**

Staphylococcus aureus is an important bacterial pathogen causing bovine mastitis, but little is known about the virulence factor and the

inflammatory responses in the mammary infection **(Fang *et al.,* 2019).** The interaction of the microorganism with the host is strongly dependent on its cell surface properties especially the presence of the capsular polysaccharide containing layer, which appear to play an important role in virulence. *S.aureus* is able to produce capsular polysaccharide in vivo or under defined culture condition **(Tollersurd *et al*., 2000).** Staphylococci are recognized worldwide as a pathogen causing many serious diseases in humans and animals, and are one of the most important etiological agents of clinical and subclinical bovine mastitis **(Pumipuntu *et al.,* 2019)**. In Egypt, it was confirmed that *S.aureus* is considered as the predominant factor among mastitis causing pathogens (**Elhaig and Selim, 2015)**

S. aureus has been described as a major pathogen responsible for this disease among more than 137 different microorganisms associated with the etiology of mastitis. One of the typical features that distinguishes the more pathogenic Staphylococcus strains from the less pathogenic is the ability to produce free coagulase and bound coagulase (clumping factor), by analyzed isolates (**Cabral *et al*., 2008).** Strains that produce coagulase (coagulase-positive staphylococci; CPS) are simply called *S.aureus*. *S.intermedius* is also coagulase-positive, as are some strains of *S. hyicus* (**Bierowie *et al.,* 2019).**

Ability of *S. aureus* to cause various infections and intoxication, results from the production of different virulence factors **(Harper *et al*., 2018).** The capsule production increases microbial virulence of bacteria becoming resistant to phagocytosis. Finally, the α- and β-hemolysins are the most important virulent factors in the pathogenesis of bovine mastitis. They are pore-forming exotoxins that induce pro-inflammatory changes in mammalian cells, inactivate the immune system by their direct cytotoxic effect, and degrade tissues, providing bacteria with nutrients and facilitating spreading to new sites **(Bownik and Swicki, 2008).**

Protein A, a surface protein of *S. aureus* binds to the IgG molecules by their Fc portion and inhibits phagocytosis of bacteria and thus contributes to the development of the disease. It is encoded by *spa* gene which is considered as one of the important virulence factors in development and severity of mastitis **(Sharma *et al.,* 2000 and Akineden *et al.,* 2001).**

On the other hand, chemical and physical changes of milk besides the pathological changes of udder tissues are associated with mastitis **(Hussain *et al.,* 2012).** Either subclinical or clinical mastitis affects the milk composition and its manufacturing properties **(Pyorala, 2003).** Some minerals are important for body growth, somatic cell count reduction and increasing milk production **(Hameed *et al.,* 2010).** Moreover, protein electrophoresis is a useful technique for diagnosis of some diseases and is indicator for the inflammation presence. Also, it can provide information about acute or chronic inflammation helping in the treatment regimen and the prognosis **(Cray and Tatum, 1998).**

The aim of the present study is mainly directed to characterize *Staphylococcus spp.* specially *S.aureus* strains isolated from the milk of buffaloes with mastitis and its virulence and antibiotic profiles as well as the biochemical changes in serum and milk of these animals.

Materials and Methods

Sample collection

1- A total of 100 mastitic milk samples were collected from 40 buffaloes from different private buffalo farms in Giza.

Out of these samples 50 were taken from 15 clinically mastitic buffaloes according to clinical observation and 50 milk samples from 25 apparently healthy buffaloes and 10 normal samples were taken as control for the chemical examination. Apparently normal milk samples were subjected to White Side Test (WST**)** to detect the incidence of subclinical mastitis according to **APHA (1978)**.

2- Blood samples were collected from jugular vein and sera were obtained from clinically mastitic animals and the heathy one for biochemical analysis.

Isolation and identification of *Staphylococcus* species: (Quinn *et al.*, 2002).
Milk samples were incubated aerobically at 37 °C for 24h then centrifuged at 3000 r.p.m. for 20 min. The cream and supernatant were discarded. A loopful from the sediment was streaked onto the surface of blood agar, mannitol salt agar and paired barker agar.

The inoculated plates were incubated at 37C for 24-48h and examined for the bacterial growth. Suspected colonies were sub-cultured, purified and preserved in semisolid nutrient agar for further identification. Colonies of staphylococci were selected on the base of their strong catalase positive reaction. Further identification was carried out using the following tests: Coagulase test, mannitol fermentation and salt tolerance using mannitol salt agar, hemolytic activity in 5% sheep blood agar and fermentation of sugars viz. sucrose, maltose. Urease, acetonin, oxidase, nitrate, Deoxyribonuclease (DNAase) tests also applied.

Antimicrobial susceptibility testing: All *S.aureus* isolates were tested by the agar disc diffusion method according to the **Finegold and Martin (1982)** for detection of resistance to 9 different antimicrobial agents **(**Enrofloxacin (EN), Ciprofloxacin (CIP), Gentamicin (GEN), Rifampicin (RIF), Amoxicillin (AMX), Chloramphenicol (CHL), Cefotaxime (CTX), Ampicillin (AMP) and Tetracycline (TET).

Detection of some virulent factors of *S. aureus*
1-Detection of Capsular poly saccharide by using Serum soft agar technique (Tollersurd *et al*., 2000): All isolates were grown in 18ml of mod. Staphylococcus 110 broth at 37C for 18h, about 100µl were transferred in 2ml volume of mod.110 broth and incubated at 37C for 24h of which one loopful was used to inoculate serum soft agar (SSA). The colony morphology was recorded as diffuse or compact after incubation at 37Cfor 24h.

2-Detection of some virulent genes in isolated *S. aureus* by PCR: Six *S.aureus* isolates were analyzed for four virulent genes including, *clfA*, *hlb* (gene encoding β haemolysin), *icaA* **and** *spa* gene (X-region of protein A).
DNA extraction: DNA extraction from six *Staph.aureus* isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.
Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany)** are listed in table (1).
PCR amplification. Primers in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied bio-system 2720 thermal cycler.

Analysis of the PCR Products
The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot 100 bp ad 100 bp plus ladders (Qiagen, gmbh, Germany) and Generuler 100 bp ladder (Fermentas, Thermo) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences (5-´3)	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
hlb	CAATAGTGCCAAAGC CGAAT TCCAGCACCACAACG AGAAT	496	94˚C 5 min.	94˚C 30 sec.	53˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Fei *et al*., (2011)**
icaA	CCT AAC TAA CGA AAG GTA G AAG ATA TAG CGA TAA GTG C	1315	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 1.2 min.	72˚C 12 min.	**Ciftci *et al*., (2009)**
spa	TCA ACA AAG AAC AAC AAA ATG C GCT TTC GGT GCT TGA GAT TC	226	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Wada *et al*., (2010)**
clfA	GCAAAATCCAG-CACAACAGGAAACGA CTTGATCTCCAGCCA-TAATTGGTGG	638	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Mason *et al., (*2001)**

Preparation of whey milk

1-Rennin solution preparation: Five ml of rennin was dissolved in 270 ml of saline

2-Centrifugation of milk sample at 3000 rpm for 10 m to remove cells and cream, then one ml of rennin solution was added to 10 ml of defatted milk. After 30 m of incubation at 37 C, the milk sample was centrifuged at 10000 rpm for 20 m and then the supernatant (milk whey) was separated **(Frost and Tina, 1988).**

Serum and whey milk electrophoretic pattern

Total protein and its electrophoretic pattern of serum and whey milk samples were carried out according to **Davis (1964)** and **Sonnen Wirth and Jaret (1980),** respectively and then calculated according to SynGene S. No. 17292*14518 sme*mpcs.

Whey milk mineral analysis

Whey milk samples sodium and potassium were determined according to **Schoenfeld and Lewellen (1964).** Then calcium, phosphorus and magnesium were determined according to **Kramer and Tisdall (1982)**, **Fiske and Subbarow (1982) and Taylor (1959)** respectively.

Statistical analysis

Obtained data were interpreted using student's t -test according to **Petrie and Watson (1999)**

Results and Discussion

Table (2). Prevalence of *Staphylococcal spp*. isolated from mastitic buffaloes.

Staphylococcal spp.	**Clinical mastitic milk samples (50)**		**Sub-clinical mastitic milk samples (50)**		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**
S.aureus	17	34	4	8	21	21
S.epidermidis	2	4	7	14	9	9
S.intermedius	3	6	1	2	4	4
Total	22	44	12	24	34	34

% calculated according to the total NO. of milk samples.

It is clear from the table (2) that prevalence of staphylococcus spp. were 34% and *S.aureus* was isolated from mastitic milk with an incidence of 34% for clinical mastitic milk and 8% from sub-clinical mastitic milk. While in subclinical mastitis *S.epidermidis* isolated with an incidence of 14%. Nearly similar results reported by **Ali *et al.* (2011),** who found that the percentage of bacterial growth obtained from mastitic buffalo's milk samples were staphylococcus spp. (28.32).

Table (3). Growth pattern of *S.aureus* strains isolated from clinical and subclinical mastitic milk in S.S.A.

Source of isolates	Total NO. of isolates	Growth pattern			
		Diffuse growth		Compact growth	
		No.	%	No.	%
Clinical mastitis	17	11	64.7	6	35.3
Sub-clinical mastitis	4	1	25	3	75
Total	21	12	57.14	9	42.86

% calculated according to the total NO. of *S.aureus* strains isolated.

As shown in table (3), the majority of *S.aureus* isolated recovered from clinical mastitis (64.7%) grew as diffuse colonies in SSA, whereas almost the same percent of isolates obtained from subclinical mastitis (75%) grew as compact colonies. **Aguilar *et al.* (2001)** stated that the majority of *S.aureus* strains involved in mastitis is surrounded by this layer.

Table (4). Antibiotic sensitivity of (21) *S.aureus* isolated from mastitic milk of buffaloes

Antibiotic agents	Potency	Degree of sensitivity			
		S	%	R	%
Enrofloxacin (EN)	20µg	19	90.48	2	9.52
Ciprofloxacin (CIP)	5 µg	18	85.71	3	14.29
Gentamicin (GEN)	10 µg	15	71.43	6	28.57
Rifampicin (RIF)	30 µg	16	76.19	5	23.81
Amoxicillin (AMX)	10µg	10	47.62	11	52.38
Chloramphenicol (CHL)	10 µg	16	76.19	5	23.81
Cefotaxime (CTX)	30µg	17	80.95	4	19.05
Ampicillin (AMP)	30 µg	9	42.86	12	57.14
Tetracycline (TET)	30 µg	14	66.67	7	33.33

% calculated according to total NO. of *S.aureus* isolated from mastitic milk samples of buffaloes.

As sensitivity test is important for selecting the appropriate antimicrobial agent for treating mastitis, results obtained in table (4) revealed that most of isolates were sensitive to Enrofloxacin (**90.48**%), Ciprofloxacin (**85.71%)** and Cefotaxime **(80.95%)**, while resist to Ampicillin **(57.14**%) and Amoxicillin (**52.38%)**. These results were consistent with a previous report from Poland, where 68.9% of *S.aureus* strains were resistant to Ampicillin and Amoxicillin **(Malinowski *et al.,* 2002).** Also **Oliveira *et al.* (2011)** reported that, the high resistance of *S. aureus* strains isolated from mastitic buffalo to ampicillin. The antimicrobial sensitivity of staphylococci isolated from bovine mastitis revealed maximum sensitivity to Enrofloxacin recorded by **Bhatt *et al.* (2017).** Also Antibiotic sensitive pattern of **Mahendra (2018)** revealed that the high sensitivity of *S.aureus* isolates towards Enrofloxacin.

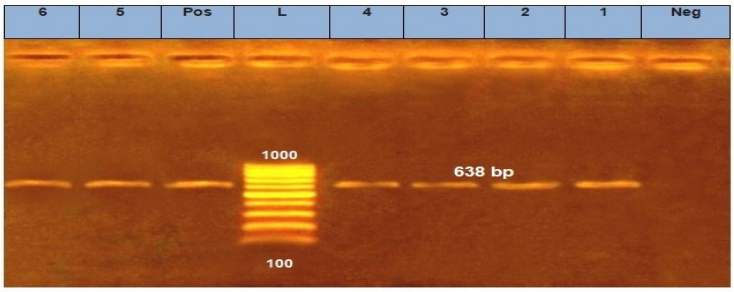

Fig. (1): Agarose gel electrophoresis of Clumping factor A gene (*clfA* gene) of *S.aureus* PCR products (638bp).
L- DNA ladder. Pos- Positive control. Neg- Negative control.
All 6 *S.aureus* isolates harbored the *clfA* gene amplification (638 bp)

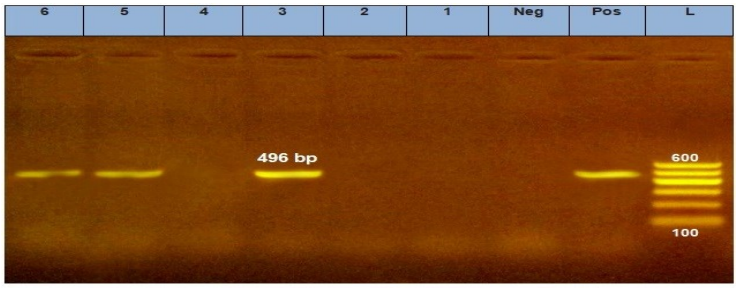

Fig. (2): Agarose gel electrophoresis of *hlb* gene (gene encoding β haemolysin) of *S.aureus* PCR products (496bp).
L- DNA ladder. Pos- Positive control. Neg- Negative control.
Lanes 3,5 and 6 showing *hlb* gene amplification (496 bp)

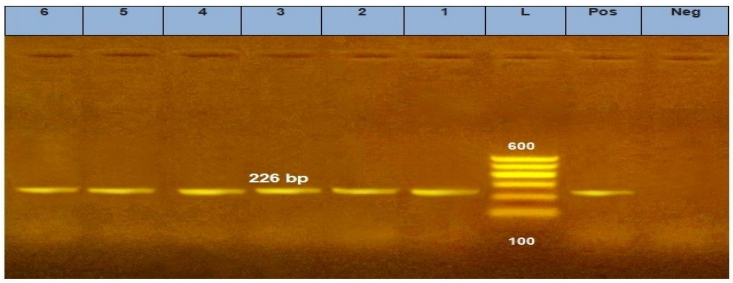

Fig. (3): Agarose gel electrophoresis of specific protein (IgG-binding protein) *spa* gene of *S.aureus* PCR products (226bp).
L- DNA ladder. Pos- Positive control. Neg- Negative control.
All 6 *S.aureus* isolates showing *spa* gene amplification (226 bp)

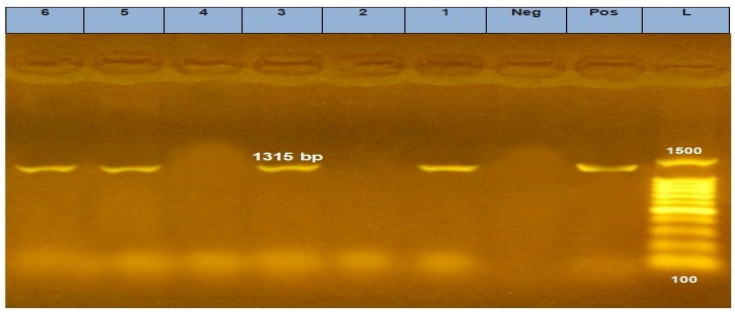

Fig. (4): Agarose gel electrophoresis of Intracellular Adhesion (*ica*) gene of *S.aureus* PCR products (1315bp).
L- DNA ladder. Pos- Positive control. Neg- Negative control.
Lanes 1,3,5 and 6 showing *icaA* gene amplification (1315 bp)

Epidemiologic studies indicate that *S. aureus* strains agents of mastitis produce a group of virulence factors and it is believed that there is a relationship between the severity of mastitis and the virulence factors produced by *S. aureus* (**Akineden *et al.*, 2001).**

Using PCR in our study not only sensitive and rapid technique but also can detect even a few numbers of pathogenic bacteria which failed to revealed by culture method. In the present study, molecular surveillance carried out in six *S.aureus* strains to screen the presence of four virulence determinants encoding gens. *S. aureus* isolates are harboring genes coding for clumping factor (*clfA*), has a greater capability to adhere to extracellular matrix proteins, essential for colonization and the establishment of infections, where *clf*A bind the C-terminus of plasma fibrinogen gamma chain, which participates in mediating fibrinogen-platelet interaction and fibrin cross-linking, resulting in thrombus formation **(Cremonesi *et al*., 2013).** The distribution of virulent genes differed among the examined strains; in fig (1): *clf*A gene was present in all of the strains examined. These results were in complete agreement with **Giada *et al*. (2017)** who detected *clf*A in all isolates from Italian dairy cows with mastitis.

Fig. (2): Revealed that *hlb* gene was recovered from 3(50%) of *S.aureus* isolates. This result was nearly similar to **Elsayed *et al* (2015)**, who determine the virulence factors of *S.aureus* isolated from clinical and subclinical mastitic cattle and buffaloes, and found that, the existence rate of *hlb* was 43.75%. Higher results obtained by **Memon, *et al.* (2013)**, where *hlb* was present in 70% of *S.aureus* isolated from subclinical mastitis. While **Ikawaty *et al* (2010)** reported the presence of *hlb* in all strains of *S. aureus* isolated from bovine mastitis in Netherland. While lower results obtained by **Ahmed *et al.*, (2016)**, who examined 150 buffalo for subclinical mastitis and *S. aureus* was isolated from 14.5% and *hlb* virulence gene was not found in any isolate. The β-toxin is a haemolysin, which targets lipid-rich membranes. It causes lysis of erythrocytes and mononuclear cells, and also induces a strong inflammatory response. The majority of human isolates of *S.aureus* do not express ß-toxin, but it is frequently found among strains responsible for bovine mastitis **(Marshall *et al*., 2000).**

Fig (3): PCR amplification revealed that all *S. aureus* isolates were harbored the genes encoding the immunoglobulin G binding region of protein A. Protein A, a surface protein of *S. aureus* binds to the IgG molecules by their Fc portion and inhibits phagocytosis of bacteria and thus contributes to the development of the disease. It is encoded by *spa* gene which is considered as one of the important virulence factors in development and severity of mastitis **(Akineden *et al.*, 2001).** Our results agreed with those obtained by **Abd El-Tawab *et al.* (2016)** and **Enany *et al.* (2013**) and nearly similar to **Choudhary *et al.* (2018)**, who determine virulence associated protein-A (*spa* gene) in 93.33% of *S. aureus* isolated from cattle with clinical mastitis.

Fig. (4): Revealed that *icaA* gene was detected in4 isolates (66,67%). These results were nearly similar to. **Khoramrooz *et al* (2016)** who determined the prevalence rate of *ica*A in *S.aureus* isolated from mastitic cows was 56.25.

In regard to serum protein and its fractions (table 5). The results showed a significant increase in serum total proteins of mastitic animals meanwhile a significant decrease in albumin level. At the same table proteins fractions β1, γ1 showed a significant increase accompanied with a significant decrease in γ2 of mastitic animal with a non-significant increase α1 while β2 had a non-significant decrease. The result came in accordance with the result of **(Tóthová *et al*., 2017)**. Also decreased serum albumin level of mastitic cow was observed by **(Risvanli *et al.*, 1999),** it could be attributed to its role as a negative acute phase protein

(Gruys *et al.,* 1994). Increase of serum total proteins in clinical and subclinical mastitis recorded previously by **(Kovac *et al.,* 2011)** due to the animal response to the inflammation and as a result of serum globulins increment. The observed increment of β1, γ1-globulins may be attributed to the host inflammatory response activation due to the mammary tissue infection **(Tóthová *et al.,* 2017).** Moreover **(Korhonen *et al.,* 2000)** stated that cow serum immunoglobulins increased with the increment of udder infections.

Table (5): Mean values (±SE) of serum total proteins and its fraction of healthy and mastitic animals

Parameters (g/dl)	Total proteins	albumin	α1-globulins	α2-globulins	β1-globulins	β2-globulins	γ1-globulins	γ2-globulins
Healthy animals	7.692± 0.048	1.965± 0.052	0.558 ± 0.027	0.428± 0.014	1.190± 0.073	0.642± 0.037	2.124± 0.071	0.786± 0.055
Mastitic animals	7.940± 0.085**	1.698± 0.068**	0.625± 0.039	0.427± 0.068	1.39± 0.077*	0.534± 0.059	2.60± 0.060***	0.667± 0.035*

* Significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$

Theoretically, during inflammation all mammary secretion changes could be used for measuring the mastitis effects **(Batavani *et al.,* 2007).** Alpha-hemolysin is one of staphylococcal pore-forming toxin activates overproduction of inflammatory mediators enhancing inflammation (**Bhakdi *et al.,* 1989).** Table (6) showed those whey milk proteins and its fractions, the observed data revealed that the total proteins had a significant increase in masititic milk comparing with the heathy one. Protein fractions recorded a significant decrease of β-lactoglobulin and α- lactoalbumin. Meanwhile albumin and immunoglobulins were significantly increased. This result came in accordance with the result of **(Coulon *et al*., 2002 and Batavani *et al.,* 2007).** β- Lactoglobulin and α- lactoalbumin are synthesized in the mammary gland **(Batavani *et al.,* 2007)** and consequently their decrease may be attributed to its synthesis decline in the infected mammary tissue. Besides that, the inflammatory damage of mammary gland with the blood milk barrier destruction limit the protein transfer from interstitial fluid to the milk **(Ishikawa *et al.,* 1982).** Owing to the increase of albumin and Immune-globulin which is mainly derived from the blood **(Ishikawa *et al.,* 1982)** their increment is due to the destruction of blood-milk permeability barrier. Besides, Immunoglobulins of milk whey are also produced in the udder then pass to the milk, its synthesis is increased in inflamed mammary tissue **(Batavani *et al.,* 2007)** as it may reduce the mastitis severity through preventing bacterial adherence to the epithelial membranes, stopping its multiplication and neutralize its toxins **(Persson, 1992)** consequently its level increase. The recorded total protein increment is a result of the increase of albumin and immunoglobulins.

Table (6). Mean values (±SE) of whey milk protein and its fraction of healthy and mastitic animals

Parameters (g/dl)	Total proteins	β-lactoglobulin	α- lactoalbumin	albumin	Immune-globulin	Other protein
Healthy animals	1.32± 0.02	0.45± 0.02	0.28± 0.01	0.14± 0.01	0.36± 0.01	0.09± 0.01
Mastitic animals	1.40± 0.02*	0.38± 0.01**	0.25± 0.01*	0.21± 0.02**	0.47± 0.01***	0.08± 0.01

* Significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$

Table (7) in this study revealed that potassium, calcium and phosphorus level of mastitic whey milk were significantly decreased in comparing with the healthy one. Meanwhile sodium level was significant increased. On the other hand, magnesium level recorded a non-significant decrease. The recorded data came in accordance to the results of **(Batavani *et al.,* 2007, Bruckmaier *et al.,* 2004 and Coulon *et al.,* 2002).** Changes in the ionic environment are noticeably in mastitis as infection of intramammary leads to breakdown of the ductal and secretory epithelium, open the tight junctions between the secretory cells so the permeability of blood capillaries increases. Sodium which is high in extracellular fluid transfers to the alveolus lumen and consequently potassium decreases in order to sustain osmolarity **(Batavani *et al.,* 2007).** In regard to Calcium and Phosphorus, **Raji *et al.,* (2012)** stated that interring of the microorganisms to the mammary gland secretary cells cause infection and cellular damage, then its normal functions and mechanisms are impaired. Moreover, casein micelle (casein is the main carrier of calcium) synthesis in Golgi apparatus declines as a result of tissue damage **(Harding 1996)** lead to impairment of calcium transport. Indeed, reduction of Citrates and phosphates (calcium carriers) in case of mastitis was recorded causing reduction of the milk calcium content **(Raji *et al.,* 2012).**

In concern to phosphorus, its reduction may be due to the decrease of milk casein content as a result of its synthesis decline as previously noted during mastitis and the most of the milk phosphate is associated with casein micelles **(Raji *et al.,* 2012).** Moreover, decline of phosphate production through damaged epithelial cells is recorded **(Jensen, 1995).** In addition to reduced phosphate production, its flow to the milk is changed due to the injury of secretory cell membrane and over this the permeability of cell membrane is changed leading to alteration of osmotic pressure and blood-milk barrier in mastitis **(Jones and Bailey, 1998)** result in moving phosphates from milk to the extracellular space or the blood stream **(Raji *et al.,* 2012)** result in decline in milk phosphorus content.

Table (7), Mean values (±SE) of whey milk mineral of healthy and mastitic animals

Parameters (mg/dl)	Potassium	Calcium	Phosphorus	Sodium	Magnesium
Healthy animals	167.00± 1.84	40.32± 0.60	45.97± 0.88	44.29± 0.71	8.60± 0.13
Mastitic Animals	150.305± 0.77***	34.605± 0.47***	38.165± 0.54***	62.07± 0.94***	8.41± 0.15

*** significant at $p < 0.001$

Conclusion

S.aureus is an important bacterial pathogen causing buffalo mastitis and it has many virulence factors that causing inflammatory response in the mammary gland tissues. Using PCR in our study not only sensitive and rapid technique but also can detect the presence of some virulent genes as *clfA*, *spa*, *hlb* and *ica* genes which increase pathogenicity of *S.aureus*. Biochemical parameters can be used as important indicators as complementary to bacteriological examination for mastitis detective and can be aid in treatment regimen improvement.

References

Abd El-Tawab, A.A.; Ahmad, M.; Amaar, M.; Abd El-Hakeem S.; Fatma, I. El Hofy and Heba, S.S.S. (2016). Molecular characterization for some virulence and antibiotic resistance genes of *Staphylococcus aureus* isolated from dairy cattle's subclinical mastitis in EL- Sharkia Governorate. Benha veterinary medical journal, Vol. 30 (1): 219-230

Aguilar, B.; Amorena, B. and Iturralde, M. (2001). Effect of slime on adherence of *Staphylococcus aureus* isolated from bovine and ovine mastitis. Vet. Microbiol. 78: 183–191.

Ahmed, H.F.; Fehlhaber, K.; Gafer, J.A.; Ibrahim, S.A. and El-Magd, M.A. (2016). Genotypes and virulence factors of *Staphylococcus aureus* isolated from bovine subclinical mastitis. Global Veterinaria 17 (5): 476-481.

Akineden, Ö.; Annemüller, C.; Hassan, A.A.; Lämmler, C.; Wolter W. and Zschöck M. (2001). Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clin Diagn Lab Immunol. 8(5): 959-964.

Ali, M.A.; Ahmad, M.D.; Muhammad, K. and Anjum, A.A. (2011). Prevalence of sub clinical mastitis in dairy buffaloes of Punjab. J. Ani. Plant Sci. 21(3): 477-480.

APHA (American Public Health Association) (1978). Standard Methods for the Examination of Dairy Products (13th ed.) APHA, New York.

Bhat, A.M.; Soodan, J.S.; Singh, R.; Dhobi, I.A.; Hussain, T.; Dar, M.Y. and Mir, M. (2017). Incidence of bovine clinical mastitis in Jammu region and antibiogram of isolated pathogens, Veterinary World, 10(8): 984-989.

Batavani, R.A.; Asri, S. and Naebzadeh, H. (2007). The effect of subclinical mastitis on milk composition in dairy cows. Iran J Vet Res, 8: 205-211.

Bhakdi, S.; Muhly, M.; Korom, S. and Hugo, F. (1989). Release of interleukin-1 beta associated with potent cytocidal action of staphylococcal alpha-toxin on human monocytes. Infect Immun 57: 351

Bierowie, K.; Korzeniowska-Kowal, A.; Wzorek, A.; Rypuła, K. and A. Gamian, A. (2019). Prevalence of Staphylococcus Species Colonization in Healthy and Sick Cats. BioMed Research International. Volume 2019, 1-10.

Bownik, A. and Siwicki, A. (2008). Effects of staphylococcal hemolysins on the immune system of vertebrates. Cent. Eur. J. Immunol., 33(2): 87-90.

Bruckmaier, R.M.; Ontsouka, C.E. and Blum, J.W. (2004). Fractionized milk composition in dairy cows with subclinical mastitis. Vet. Med. Czech., 49: 283-290.

Cabral da Silva, S.O.; Mdenezes, B.E. and Vasconcelos Paida Brito, M.A. (2008). Identication of coagulase-negative staphylococci from bovine mastitis using RFLP-PCR of the *groEL* gene. Vet Microbiol, 130: 134–140.

Choudhary, S.; Diwakar, T.B. and Kataria, A.K. (2018). Molecular typing of virulence associated gene (*spa*) of *S. aureus* isolated from cattle clinical mastitis. Journal of Entomology and Zoology Studies; 6(1): 1057-1060.

Ciftci, A.; Findik, A.; Onuk, A. and Savasan, S. (2009). Detection of methicillin resistance and slime factor production of *Staphylococcus aureus* in bovine mastitis. Brazilian Journal of Microbiology, 40: 254-261.

Coulon, J.B.; Gasqui, P.; Barnouin, J.; Ollier, A.; Pradel, P. and Pomies, D. (2002). Effect of mastitis and related-germ on milk yield and composition during naturally-occurring udder infections in dairy cows. Anim. Res., 51: 383-393.

Cray, C. and Tatum, L.M. (1998). Applications of protein electrophoresis in avian diagnostics. J. Avian. Med. Surg.,12: 4-10.

Cremonesi, P.; Zottola, T.; Locatelli, C.; Pollera, C.; Castiglioni, B.; Scaccabarozzi, L. and Moroni, P. (2013). Identification of virulence factors in 16S-23S rRNA intergenic spacer genotyped *Staphylococcus aureus* isolated from water buffaloes and small ruminants. J. Dairy Sci. 2013; 96: 7666–7674.

Davis, B. (1964). Disk electrophoresis. II Method and application to human serum protein. Ann. N.Y. Acad. Sci., 121: 404-427.

Elhaig, M.M. and Selim, A. (2015). Molecular and bacteriological investigation of subclinical mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae* in domestic bovids from Ismailia, Egypt. Trop. Anim. Health Prod., 47: 271-276.

Elsayed, M.S.; El-Bagoury, A.M. and Dawoud, M.A. (2015). Phenotypic and genotypic detection of virulence factors of *Staphylococcus aureus* isolated from clinical and subclinical mastitis in cattle and water buffaloes from different farms of Sadat City in Egypt, Veterinary World 8(8): 1051-1058.

Enany, M.E.; Younes, S.; AL-gammal, A.M.; Salem, M. and El-Dieb, H.A. (2013). Phenotypic and genotypic characterization of *Staphylococcus aureus* isolated from clinical and subclinical bovine mastitis. SCVMJ, XVIII, (1) 139-147.

Fang, R.; Jingchun C.; Tengteng C.;, Haiyong G.; Hisaya K.; Ono, C.P.; Masashi, O.; Akio, N. and Dong-Liang, Hu (2019). Staphylococcal enterotoxin C is an important virulence factor for mastitis. Toxins, 11, 141: 1-13.

Fei, W.; Hongjun, Y.; Hong-bin, H.; Changfa, W.; Yundong, G.; Qifeng, Z.; Wiaohong, W. and Yanjun, Z. (2011). Study on the hemolysin phenotype and the genetype distribution of *Staphylococcus aureus* caused bovine mastitis in Shandong dairy farms. Intern J Appl Res Vet Med., Vol. 9, No. 4, 2011.

Fingold, S.M. and Martin, W.T. (1982). Diagnostic M icrobiology.6th Ed. C. V. Moby Co St Louis Toronto. London.

Fiske, C.H. and Subbarow, Y. (1982). Determination of inorganic phosphour in serum and urine. In: Fundamentals of Clinical Chemistry. Ed. Tietz, N. W., W. B. Saunders Company, London.

Frost, A.J. and Tina, M. (1988). The effect of vaccination with a cell wall extract of *Staphylococcus aureus* on the inflammation of bovine mammary gland.j. Vet. Med, (35): 688 – 694.

Giada, M.; Stefano B.; Giulietta, M.; Ralf, E.; Stefan, M.; Mario, L. and Renata, P. (2017). Virulence Genes of *S. aureus* from Dairy Cow Mastitis and Contagiousness Risk. Toxins (Basel). 2017 Jun; 9(6): 195. 1-12

Gruys, E.; Obwolo, M.J. and Toussaint, M.J.M. (1994). Diagnostic significance of the major acute phase proteins in veterinary clinical chemistry: a review. Vet Bull, 64: 1009-1018.

Hameed, A.; Anjum, F.M.; Zahoor, T. and Jami, A. (2010). Consequence of oxytocin injections on minerals concentration in Sahiwal cow milk. Pak J Agric Sci, 47: 147-152.

Harding, F. (1996). Milk quality, Aspen Publishers. 1-38.

Harmon, R.J. (1994). Physiology of mastitis and factors affecting somatic cell counts. J. Dairy Sci., 77: 2103-2112.

Harper, L.; Balasubramanian, D.; Ohneck, E.A.; Sause, W.E.; Chapman, J.; Mejia-Sosa, B.; Lhakhang, T.; Heguy, A.; Tsirigos, A.; Ueberheide, B.; Boyd, J.M.; Lun, D.S. and Torres, V.J. (2018). *Staphylococcus aureus* responds to the central metabolite pyruvate to regulate virulence. mBio. Jan 23; 9(1):1-17

Heikkilä, A.M.; Liski, E.; Pyörälä, S. and Taponen, S. (2018). Pathogen-specific pro-

duction losses in bovine mastitis. J Dairy Sci. Oct; 101(10): 9493-9504.

Hussain, R; Javed, M.T. and Khan, A. (2012). Changes in Some Biochemical Parameters and Somatic Cell Counts in the Milk of Buffalo and Cattle Suffering from Mastitis. Pak. Vet. J., 32(3): 418-421.

Ikawaty, R.; Brouwer, E.C.; Van Duijkeren, E.; Mevius, D.; Verhoef J. and Fluit, **A.C. (2010).** Virulence factors of genotyped bovine mastitis *Staphylococcus aureus* Isolates in The Netherlands. International Journal of Dairy Science Volume 5 (2): 60-70.

Ishikawa, H.; Shimizu, T.; Hirano, H.; Saito, N. and Nakano, I. (1982). Protein composition of whey from subclinical mastitis and effect of treatment with levamisole. J. DairySci.,65(4): 653-658.

Jensen, R.G. (1995). Handbook of milk composition, Academic press. California. U.S.A. 577-622.

Jones, G.M. and Bailey, T.L. (1998). Understanding the basics of mastitis, Virginia Polytechnic Institute and State University.

Khoramrooz, S.S.; Mansouri, F.; Marashifard, M.; Malek, H. S.A.; Akbarian Chenarestane-Olia F.; Ganavehei, B.; Gharibpour, F.; Shahbazi, A.; Mirzaii, M. and Darban-Sarokhalil, D. (2016). Detection of biofilm related genes, classical enterotoxin genes and *agr* typing among *Staphylococcus aureus* isolated from bovine with subclinical mastitis in southwest of Iran. Microb Pathog. Aug; 97: 45-51.

Korhonen, H.; Marnila, P. and Gill, H.S. (2000). Milk immunoglobulins and complement factors. Br J Nutr, 84:75-80.

Kováč, G.; Tóthová, C.; Nagy, O. and Seidel, H. (2011). Milk amyloid A and selected serum proteins in cows suffering from mastitis. Acta Vet Brno, 80:3-9.

Kramer, B. and Tisdall, F.F. (1982). Determination of serum calcium by oxalate precipitation and redox titration. In: Fundamentals of Clinical Chemistry. Ed. Tietz, N. W., W. B. Saunders Company, London.

Mahendra, M.Y. (2018). Prevalence of *Staphylococcus aureus* in Lactating Cows with Subclinical Mastitis and their Antibiogram in Organized Dairy Farm, Maharashtra, India . Int. J. Curr. Microbiol. App. Sci,7(3): 3674-3680.

Malinowski, E.; Klossowska, A.; Kaczmarowski, M.; Lassa, H. and Kuzma K. (2002). Antimicrobial susceptibility of staphylococci isolated from affected with mastitis cows. Bull. Vet. Inst. Pulawy 46: 289-294.

Marshall, M.J. and Bohach, G.A. (2000). Characterization of *Staphylococcus aureus* betatoxin induced leukotoxicity. J. Nat. Toxins., 9 (20): 125-138.

Mason, W.J.; Blevins, J.S.; Beenken, K.; Wibowo, N.; Ojha, N. And Smeltzer, M.S. (2001). Multiplex PCR protocol for the diagnosis of staphylococcal infection. Journal of Clinical Microbiology, Vol. 39 (9): 3332–3338.

Memon, J.; Yang, Y.; Kashif, J.; Yaqoob, M.; Buriro, R.; Soomro, J.; Liping, W. and Hongjie, F. (2013). Genotypes, virulence factors and antimicrobial resistance genes of *Staphylococcus aureus* isolated in bovine subclinical mastitis from Eastern.

Oliveira, L.P.; De Soares Barros, L.S.; Silva, V.C. and Cirqueira, M.G. (2011). Study of *Staphylococcus aureus* in raw and pasteurized milk consumed in the Reconcavo area of the State of Bahia, Brazil. J. Food Process Technol. 2:128.

Persson, K. (1992). Studies on inflammation in the bovine teat with regard to its role in the defense against udder infections. Dissertation, Uppsala, Sweden, 11-17.

Petrie, A. and Watson, P. (1999). "Statistics for Veterinary and animal science 1st Ed.pp.90-99, The Blackwell science Ltd, United Kingdom.

Pumipuntu, N.; Tunyong, W.; Chantratita, N.; Diraphat, P.; Pumirat, P.; Sookrung, N.; Chaicumpa, W. and Indrawattana N. (2019). Staphylococcus spp. associated with subclinical bovine mastitis in central and northeast provinces of Thailand. PeerJ 7:e65871-21 PeerJ 7: e6587:1-23

Pyorala, S. (2003). Indicators of inflammation in the diagnosis of mastitis. Veterinary Re-

search, 34(5): p. 565-578.

Quinn, P.J.; Markey, B.K.; Carter, M.E.; Donelly, W.J.C and Leonard, F.C. (2002). Veterinary Microbiology and Microbiological Diseases. 1st Iowa State University Press Blackwell Science.

Raji, S.; Ezzatpanah, H. and Givianrad, M.H. (2012). The effect of different somatic cell levels on calcium and phosphorus contents of milk. J. FBT, IAU, 2, 1-8.

Rişvanli, A.; Türköz, Y.; Kalkan, C. and Çetin, H. (1999). An investigation on the serum levels of biochemical variables in the cows with clinical mastitis. Firat Üniv Sağlik Bil Derg, 13: 131-134.

Schoenfeld, R.G. and Lewellen, C.J. (1964). A Colorimetric Method for Determination of Serum Chloride. Clinical chemistry, 10, (6): 533-539.

Sharma, N.K.; Rees-Catherine, E.D. and Dodd-Christine, E.R. (2000). Development of a Single-Reaction Multiplex PCR Toxin Typing Assay for *Staphylococcus aureus* Strains. Appl Environ Microbiol.; 66(4): 1347-53.

Sonnen Wirth, A. and Jareet, L. (1980). "Garduals Clinical Laboratory Methods and Diagnosis. "Vol.1, 8th Ed., Mosby.

Taylor, T.G. (1959). Colorimetric determination of magnesium. J. Agri. Sci. 52, 207.

Tollersrud, T.; Kenny, K.; Caugant, D.A. and Lund, A. (2000). Characterization of isolates of *Staphylococcus aureus* from acute, chronic and subclinical mastitis in cows in Norway. APMIS 108:565-572

Tóthová, C.; Mudroň, P. and Nagy, O. (2017). The electrophoretic pattern of serum proteins in dairy cows with infl ammatory diseases. Acta Veterinaria-Beograd, 67 (2), 178-190

Wada, M.; Lkhagvadorj, E.; Bian, L.; Wang, C.; Chiba, Y.; Nagata, S.; Shimizu, T.; Yamashiro, Y.; Asahara, T. and Nomoto, K. (2010). Quantitative reverse transcription-PCR assay for the rapid detection of methicillin-resistant *Staphylococcus aureus*. Journal of Applied Microbiology 108: 779–788.