Some Bacteriological and Biochemical Studies on Subclinical Mastitis in Buffaloes

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Abstract: *A* total of (180) positive california mastitis test (CMT) out of (240)buffalo milk samples collected from (60) cases of subclinically mastitic dairy Egyption buffaloes were examined bacteriologically for the detection of the causative microbial agent including Mycoplasma species.Comparison of different diagnostic procedures including culture techniques, CMT, polymerase chain reaction(PCR), evaluate the role of milk serum enzymes (Alkaline Phosphatase (ALP), Lactate Dehydroginase (LDH), Gamma Glutamyle Transferase (GGT) and Aspartate Aminotransferase (AST)) as well as electrophoretic pattern in whey milk were studed.The result of this study revealed that *Staphylococcus aureus* was the most common bacteria followed by *Escherichia coli* then *Streptococcus agalactiae*, while *Mycoplasma bovis* was the most detected mycoplasma species. All the isolates were sensitive to Streptomycin, Amikacin, Gentamycin and Ofloxacin. On the other hand, negative isolates were sensitive to Colistin and Ciprofloxacin. All isolates were resistant to Ampicillin and Tetracycline. Milk serum biochemical studies revealed that GGT, LDH and ALP activities were increased significantly in subclinically mastitic buffalo's whey milk. However, the concentration of AST was insignificant compared to its concentration in healthy buffalo's whey milk. The electrophoretic pattern of whey milk protein recorded five fractions. Mastitis caused a significant increase in non-casein protein concentration of whey milk. Also, the protein content of serum albumin and immunoglobulin in whey milk increased in mastitic buffaloes compared with healthy ones. Alternatively, the major whey proteins (beta-globulin and alpha globulin) were significantly decreased. Based on the current study, it could be concluded that despite of California mastitis test (CMT) is still the gold standard for SCM diagnosis, the changes in the whey milk enzymes' activities that associated with the presence of pathogenic bacteria and mycoplasma species in subclinically mastitic buffaloes are associated with SCM.Therefore, such milk enzymatic changes could be used as indicators for SCM (especially in early lactation and dry periods where CMT could not be detected it in such periods**.**

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1. Introduction

Buffalo and cattle are mostly reared for milk production the mastitis decreases the milk production and sometimes causes bloody milk alone or mixed with mucus may cause milk unfit for consumption.It is one of the most important reasons for termination of lactation and unwanted culling of dairy buffalo **(McDowell *et al.,* 1995).**

Mastitis namely, clinical and subclinical, is an economically damaging disease of the dairy industry, which causes physical, chemical and bacteriological alteration in the milk and blood along with morphopathological changes in the mammary gland **(Guha *et al.,* 2012).** The causes of mastitis involve a complex relationship of three major factors, that is, host resistance, bacterial agents and the environmental factors **(Gera and Guha, 2011).**

The dominant bacterial isolates in a revious study of subclinical mastitis were Staphylococcus species (62.9%), Streptococcus species (15.5%) and Escherichia coli species (12.4**%) (Yuan *et al.,* 2012)**. It is concluded that major causes of buffalo's subclinical mastitis are teat skin opportunistic bacteria (Mehdi **and Jamal, 2012).**

In Egypt, Mycoplasma spp.were isolated from mastitic milk of cows and buffaloes by several researchers, (**El-Ebeedy *et al.,* 1985 and Eissa, 1986**) their studies reported that severe outbreak of *Mycoplasma bovis* mastitis in herds of Friesiancows. Mycoplasma bovis is the most important aetiological cause of Mycoplasmosis in cattle in Europe.

Despite susceptibility to mastitis is low in buffaloes when compared to cattle (**Saleh, 2005).**

Subclinically infected cows are intermittent shedders of organisms and may cycle through low and high shedding patterns during lactation period. However, milk culture may yield no bacteria from truly subclinically infected glands due to the presence of very low numbers of pathogens when samples are collected **(Cai *et al.,* 2003).**

As a common perception, the inflammation of udder strikingly augments the somatic cell count

(SCC) in milk, leading to low-grade processing characteristics and reduced acceptance of dairy products due to changes in components and properties of raw milk. Indeed, rise in the leucocyte number in milk and in the mammary gland, as a response to the assaulting pathogens or to their metabolites leads to an increase in SCC (**Atasever, 2012**). For this reason, the most reliable index next to bacterial culture examination, subclinical mastitis (SCM) detection is by somatic cell count (SCC) (**Guha *et al.*, 2012).** Apositive diagnosis of mastitis should **f**ulfill two criteria, appositive bacteriological test and an inflammatory cellular change **(Katsoulos *et al.*, 2010).** Cell counts are used for the latter purpose.The invasion of polymorphonuclear leukocytes and macrophages is one of the essential body defence against clinical and subclinical mastitis.During the inflammatory process,these cells and damaged cells of the udder tissues secret enzymes such as lactate dehydrogenase or lysosomal enzymes(**Oliszewski *et al.*, 2002**). Also, the inflammatory products tend to damage udder parenchyma which results in a release of intra-cellular enzyme activity **(Babaei *et al.*, 2007).** The albumin, total protein and globulin are altered in mastitis milk than in normal one **(Seleim *et al.*, 2002**).

It has been previously shown that milk samples could serve as substrate for the amplification of specific DNA sequences using PCR **(Lipkin, 1993).** These PCR methods offer the option of identification of bacteria within hours. PCR can also improve the level of detection because of its ability to detect low numbers of organisms.

The aim of this study is to evaluate the biochemical analysis of some whey milk constituents of mastitic buffaloes compared to healthy ones to be used as diagnostic indicators for SCM(especially in early lactation and dry periods that could not be detected by CMT).These biochemical studies were carried out in parallel with bacteriological, mycoplasmal and biomolecular studies**.**

2. Materials and method

Animals:

The current work was carried out in 60 animals collected from some private farms of dairy buffaloes (Elsharkia and Giza) governorates during the period of 2011-2012.

Milk Samples:

A total of 240 milk samples (50ml each) collected aseptically in sterile mcCarteney bottles from 60 buffaloes of which 180 were positive to CMT and used in this study **(Blood and Henderson**, **1986).** Each sample was divided into four parts, each part put in a sterile McCartney bottles. One was incubated at 24 h for bacteriological examination,the second part for detection of biochemical analysis,the third part for mycoplasmal examination and the last part for PCR testing.

California Mastitis test:

California mastitis test (CMT) was carried out according to (Schalm **and Noorlander, 1957).** According to the changes of colour and grade of gel formation,its results were interpreted as negative, trace, 1+, 2+, and 3+, as described by **(Schalm *et al.*, 1971).**

Bacteriological Examination:

Milk samples were incubated aerobically at 37ºC for 24 hrs then centrifuged at 3000 rpm for 20 minutes, the supernatant fluid was discarded and a sterile loopfull from the sediment was streaked onto the surface of mannitol salt agar, blood agar, MacConkey agar and Edwards media, The plates were incubated at 37ºC for 24-48 hrs, then examined for bacterial growth, the growing surface colonies were purified, picked up and identified according to (**Finegold and Baron**. **1986).** The organisms identified biochemically by biochemical tests using the API Staph and Strep systems accordingly**.** Furthermore, the isolates were serologically identified by polymerase chain reaction.

Mycoplasmal Examination:

Milk samples were cultured by inoculation on broth media then plated on PPLO agar media (**Sabry and Ahmed, 1975)** and incubated at 37°C for 3-7 days. Genus determination **(Freundt *et al.*, 1973),** biochemical characterization of the isolated purified strains was carried out,**(Erno. and Stipkovits, 1973).** The film and spot formation according to **(Fabricant. and Freundt,1967).** The isolates were serologically identified by growth inhibition **(Clyde, 1964)**, and polymerase chain reaction **(Sambrook *et al.*, 1989)**

Antibiogram technique:

Antibiotic sensitivity test for all isolated bacteria and mycoplasma was done using standard disc technique according to **(Boone and Castenholz, 2001)**.

Preparation of DNA for PCR: using the Dneasy Tissue kit from Qiagen Cat No 51104.

PCR primers: PCR primers were supplied by Sigma USA.

The Sequence of bacteria Primers were the following:-

Universal primer for bacteria:

Uni 678:5'- GGA ATT CCA TGT GTA GC-3'.

Uni 888:5'- GAG TGC TTA ATG CGT TAG CT-3'

Staph primer

Sau 3275'- GGA CGA CAT TAG ACG AAT CA -3'

Sau 1645'- CGG GCA CCT ATT TTC TAT CT-3'

Ecoli primer

Eco 223 E. coli5'- ATC AAC CGA GAT TCC CCC AGT-3'

Eco 455E. Coli 5'-TCA CTA TCG GTC AGT CAG GAG-3'

16S gene of Mycoplasma from milk buffalo:

65 muniv F 5'- AGA CTC CTA CGG GAG GCA GCA-3'

16Smuniv R 5-ACT AGC GAT TCC GAC TTC ATG-3'

Mycoplasma Vsp A specific gene for *M.bovis* was used (Alberto *et al.*, 2006).

MYBF: 5'- CTT GGA TCA GTG GCT TCA TTA GC -3'

MYBREV: 5'-GTC ATC ATG CGG AAT TCT TGG GT -3'

PCR amplification for bacteria:

PCR was performed in Bio-Rad thermal cycler. All reactions were carried out in a final volume of 50 µl.

A pre-PCR step at 94°C for 2 min was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 45 s, annealing (56°C in Uni, 64 ºC in Eco and Sau) for 1 min, and extension at 72°C for 2 min. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. The PCR products were stored in the thermocycler at 4°C until they were collected. The PCR-amplified product was analyzed by electrophoresis on a 1.7% agarose gel stained with 0.5 ug of ethidium bromide/ml. Gels were visualized under UV illumination and photographed (**Samboork *et al.*, 1989**).

The PCR amplification protocol used for ruminant mycoplasma and VSP was as follow:

Pre-PCR step for 5 minute at 94 °C, then 35 cycles of denaturation for 45 sec at 94 °C, annealing for 1 min. at 60 °C, and extension for 1.5 min at 72 °C, a final extension step at 72°C held for 5 min.. The PCR-amplified product was analyzed by electrophoresis on 1.5 % agarose gel then DNA was visualized usingUV fluorescence after ethidium bromide staining and then photographed.

Biochemical examination:

Defatted milk samples (whey milk) were used for enzyme assays,they were prepared from the milk according to the technique of(**Kumar and Mikolajcik 1972).**The enzymes activities of GGT were determined according to**(Persijn and van der Slik, 1976)**, LDH**(Caud and Wroblewski 1985),** AST **(Reitman and Frankel 1957)** and ALP **(Belfield** and **Goldberg 1971)**.The milk serum total protein was determined as described by (**Sonnen Wirth and Jaret, 1980).** The milk Serum protein electrophoresis was carried out according to **(Davis, 1964*)***.The obtained data were statistically evaluated using student's-t- test according to **(Snedecor and Cochran., 1982).**

3. Results

1-Microbiological and Molecular studies:

In the present study, it was found that 180 milk samples out of 240 samples were positive for California mastitis (CMT). **Table (1**) shows the incidence of bacterial and mycoplasmal pathogens isolated from positive CMT milk samples. The biochemical characterization and serological identification for all isolated strains,showed that the incidence of. coagulase negative staph(23.3%), coagulas positive staph (7.8%), *Strept. agalactia* (4.4%), *Strept. dysagalactia* (5.6%), streptuberis (3.3%), enteroccocus species(8.95%),*E.coli* (13.9%), Klebsiella species (2.2%) and the bacteriologically negative samples (12%). While the incidence of Mycoplasma in the studied dairy buffaloes after the biochemical characterization and serological identification of species, 30 strains were digitonin sensitive, this means that they belonged to Genus Mycoplasma. However, 24 strains (13%) were glucose negative, arginine negative and film &spot positive and identified as *Mycoplasma bovis.* On the other hand two strains (1.1 %) were seriologically identified as *M.bovirihinis,* they were glucose positive, arginine negative, film& spot negative.Moreover,,four strains(2.2%) were were seriologically identified as *Mycoplasma arginini,*they were arginine positive, glucose and film & spot negative. Another, 4 tested strains (2.2%) were digitonin negative, they were identified as Acholeplasma by the current biochemical.

Table (2): shows the antibiogram of the isolated organisms of milk samples from subclinically mastitic buffaloes, it was clear that all the isolates were resistant to Ampicillin and Tetracycline and sensitive to Streptomycin, Amikacin, Gentamycin and Ofloxacin. Nevertheless, gram negative isolates were sensitive to Colistin and Ciprofloxacin.

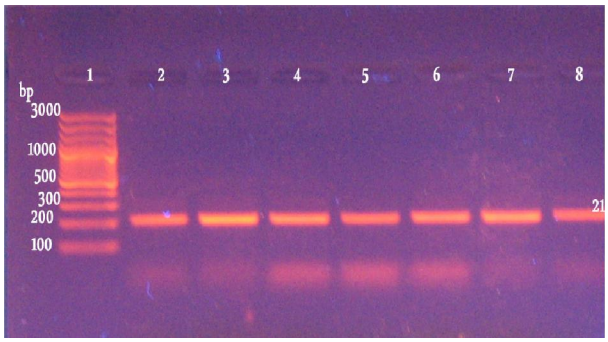
Identification by polymerase chain reaction (PCR).

Bacterial primer amplification:

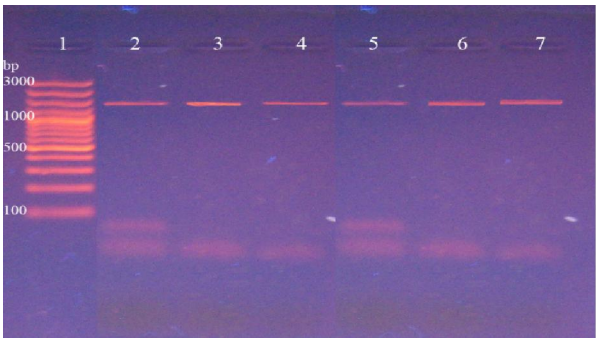
Universal primer pairs Uni 678 plus Uni 888 a product of 210 bp was observed (Fig1)while Sau 327 plus Sau 1645 was given a product at 1,318 bp(Fig2) and Eco 223 plus Eco 455 was given a product at 232 bp (Fig3).

Mycoplasmal primer amplification:

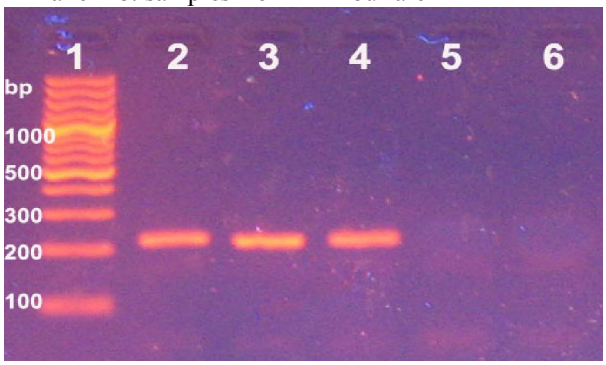
The sequence of 16S gene was amplified a product at 1000 base pair (Fig4), while a specific gene of M. bovis amplified a product at 342 bp(Fig5).



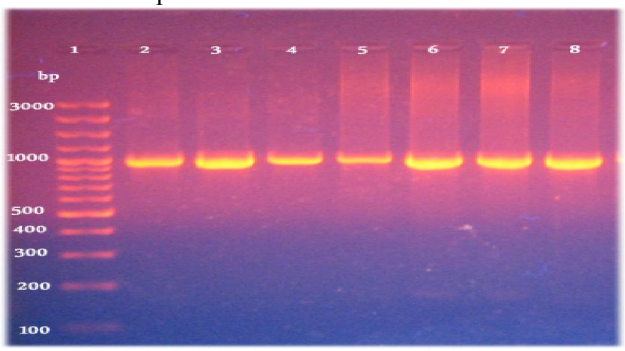
Fig(1):PCR amplification of purified bacterial DNA by using Universal primer
Lane (1):100 bp DNA Ladder
Lane 2-8: samples from milk buffalo



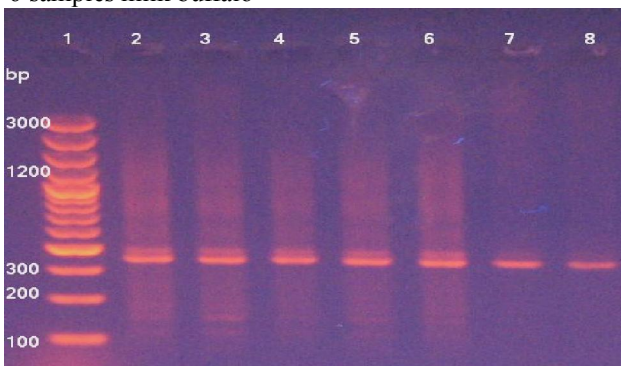
Fig(2):PCR amplification of purified baterial DNA by using Sau primer
Lane (1):100 bp DNA Ladder
Lane 2-8: samples from milk buffalo



Fig(3):PCR amplification of purified bacterial DNA by using E-coli primerLane (1):100 bp DNA LadderLane 2-6 samples milk buffalo



Fig(4)Electrophoretic pattern of 16SrRNA: gene of Mycoplasma from milk buffalo Lane (1):100DNA Ladder
Lane 2-8: samples from buffalo Milk.



Fig(5): Electrophoretic pattern of VspA gene of Mycoplasma from milk buffalo
Lane (1):100 bp DNA Ladder
Lane 2-8: samples from buffalo

2-Biochemical study of some milk serum constituents:

a-Clinical biochemical analysis:

Table(3)shows that LDH,ALP and GGT enzyme activities were significantly increased in SCM whey milk. However, the activity of AST was non-significantly changed in SCM buffaloes compared to healthy ones.

b-Milk serum immuno electrophoretic analysis:

Table (4) shows that an electrophoretic pattern of whey milk contains five fractions. Mastitis caused a significantly increased in non-casein total protein concentration of whey milk. Also, the content of milk serum albumin and immunoglobulin in whey milk were significant increased in mastitic buffaloes compared with that of healthy ones. While, the major whey proteins (beta- and alpha globulin) were significantly decreased in SCM-buffaloes compared with the healthy ones.

Table (1): Incidence of bacterial and mycoplasmal pathogens isolated from positive milk samples for CMT:

Species	Number	Incidence%
Staph.coagulase –ve	42	23.3
Staph.coagulase+ ve	14	7.8
Strept.agalactia	8	4.4
Strept.dysagalactia	10	5.6
Strept.uberis	6	3.3
Enterococcus species	16	8.9
E.coli	25	13.9
Klebsiella spp.	4	2.2
Acholeplasma spp.	4	2.2
Mycoplasma. bovis	24	13
Mycoplasma.bovirhinis	2	1.1
Mycoplasma.arginini	4	2.2
Negative samples for Bacterial Exam..	21	12
Total	180	100

Percentages were calculated according to total positive samples for CMT.

Table (2): Antibiogram of the isolated milk organisms

Antibiotic	*S..aureus*	*E. faecalis*	*E.Colli*	*K.pneumonae*	*Streptcoccus.species*	*Mycoplasma.sp*
Ampicillin	-	-	-	+	-	-
Tetracycline	-	-	+	+	+	+
Colistin	-	-	+++	+++	-	-
Streptomycine	+++	+++	+++	+++	+++	+
Gentamycin	+++	+++	+++	+++	+++	+
Ofloxacin	++	++	+++	+++	++	++
Ciprofloxacin	+	+	+++	+++	+	+++
Amikacin	+++	+++	+++	+++	+++	++

N.B:(0-30 %)= -, (35-45)=+, (65-75%)=++, (75-100 %)=+++

Table (3): The activities of some milk serum enzymes in mastitic and healthy buffaloes:

Milk enzyme Groups	AsparatateAminotransferase (AST)(IU/L)	Alkaline phospha-tase (ALP)(IU/L)	Gamma Glutamyl Tranferase (GGT)(IU/L)	LactateDehydrogenase (LDH-)(IU/L)
Control buf-faloes	21.40±2.80	282.8± 30.72	228.40± 42.	83.264± 8.98
Mastitic buffaloes	21.20^{NS}±3.22	551.6*± 90.71	754.60^{***}±60	188.502^{**}± 22.32

N.B.: *= Significant change between means (at $P \leq 0.05$), **= highly significant change between means (at $P \leq 0.01$), ***= very highly significant change between means (at $P \leq 0.001$); NS= non- significant change between means.

Table (4): The different protein fractions of why milk of mastitic and healthy buffaloes as determined by Immuno-electrophoresis:

Protein fractions of milk Animal Groups	Total protein	β-Lact. globulin	α-Lact. globulin	Serum albu-min	Immunoglobulin	Other protein
Control buffaloes	1.54 ±.034	0.51± 0.052	0.278± 0.026	0.289 ± 0.02	0.240± 0.025	0.22± 0.02
Mastitic buffaloes	1.77^{***} ±0.01	0.486^{***} ±0.03	0.158^{*} ±0.035	0.43 ^{NS}±0.03	0.37^{NS} ±0.02	0.33^{NS} - ±0.01

N.B.: *= Significant change between means (at $P \leq 0.05$), **= highly significant change between means (at $P \leq 0.01$), ***= very highly significant change between means (at $P \leq 0.001$),NS= non- significant change between means.

4. Discussion

Subclinical mastitis is one of the most serious diseases of cattle, as the infected animal shows no obvious symptoms and secretes apparently normal milk for a long time, during which causative organisms spread infection in the herd, This represents an important feature of the epidemiology of many forms of bovine mastitis **(Bakken and Gudding, 1982).** Early diagnosis of mastitis is a must for reduction of production losses and for enhancing the prospects of recovery. Also, the identification of subclinically infected gland is urgently required for successful control of mastitis in dairy animals **(Ahmed *et al.*, 2008).**The present study was designed to investigate subclinical mastitis in the main Egyptian dairy animals which are buffaloes th produce 65% of dairy product with special concept to find a practical marker for its early diagnosis From table (1) we can find that the incidence of subclinical mastitis in buffaloes depending on the bacterial cultivation was the highest in *S. aureus* (31.1%), *E. coli* infection (13.9%), followed by, *Enterococcus spp.(*8.9%), *S. dysgalactiae* (5.6%) and *S. agalactiae* (4.4%) and *Klebsiella spp*.(2.2%). This comes in agreement with **(El-Khodery and Osman, 2008)** who reported that the bacteriological examination of buffaloes milk samples with acute mastitis revealed that coliform bacteria was the most common pathogen followed by *S. aureus* then *S. uberis*, and *S. agalactiae*. This also the results came to some extent with (**Ahmed *et al.*, 2008)** who reported high incidence of bacteria isolated from milk samples of Egyptian buffalo-cows suffering from sub-clinical mastitis where the most prevalent isolates were *E. coli* (94.99%), *S. epidermidis* (78.33%), *C. bovis* (55%), *Klebsiella spp*. (51.67%), *S. uberis* (46/67%), *S. aureus* (33.33%) and *S. agalactiae* (31.67%).

An efficient vaccine against bacterial species causing bovine mastitis (BM)is not yet available, and prevention subclinical mastitis as a measure of control needs sensitive, rapid, and specific tests to identify the main bacteria that cause heavy losses in the dairy industry. Conventional procedures for the identification of BM pathogens are labor-intensive, and most of the commercial identification systems are not designed to identify important veterinary pathogens **(Watts, 1989).** We aimed to develop a detection and identification test for BM pathogens that produced results in 1 day, did not need a culture step, Sensitivity of the PCR assay in detecting DNA from milk new methods using PCR based on the 16S region sequences have been successfully applied for the identification of bacteria (**Bentley, and Leigh**. **1995 and Forsman *et al.*, 1997) .**The major advantages of PCR lay in the possibility of using only nanograms of nucleic acid samples, allowing the elimination of culture as well as rapidity, and easy analysis .The present work concerned with the incidence of Bacterial and Mycoplasmal mastitis in dairy buffaloes in of Egypt. PCR assay is a good alternate culture, where it is rapid, sensitive, specific and can detect non-viable bacteria. **(Biddle *et al.*, 2004).** In this work, the test directly performed from milk samples without a culture step, and is specific for *E. coli, S. aureus.*For *mycoplasma* two PCR assays used, a common 16S rRNA gene, (**Königsson et al., 2002**) used for detection of *mycoplasmas* from ruminant, while Vsp A gene specific for M. bovis.(**Alberto *et al.*, 2006).** The isolated *mycoplasma* needed to be investigated with molecular tests specific for any of the suspected species. However, the presence of *M. bovis* in dairy herd need to be established quickly to prevent an epidemic event that could lead to significant economic losses . The neuroendocrine system and immune system metabolism interact to co-ordinate physiological responses to infection and inflammation **(Ma *et al.*, 2006; Gera and Guha, 2011b).**

The CMT is a reliable, easy, rapid and cheap tool helping in diagnosis and still the gold-standard screening test for high somatic cell count(SCC) as it directs attention to individual mammary quarter that is secreting milk of high (SCC) **(Leslie *et al.*, 2002; Abdel-Rady and Sayed, 2009**) but CMT not suitable in early lactation so the measurment of enzyme activiting appear to be suitable diagnostic method for identifying SCM in early lactation or in dry period **(Babaei et.al.,2007).** Mastitis is associated with changes in physical, chemical, bacteriological and organoleptic properties of milk, besides causing health hazards to the public **(Riaz Hussain *et al.*, 2012).**It can be observed from Table (3) that the concentration of AST was non significantly changed comparable in infected and normal milk, which agrees with the previous findings of **Babaei *et al.* (2007) and Gera *et al.* (2011)**in why milk. Our study found significant elevation of ALP, GGTand LDH enzymes activities in mastitic milk compared with healthy one, this result is partially agree with (**Chemale., 1986)** which found an increased amount of ASTand GGT in53 milk samples of cows,which had been affected dy *E-coli* mastitis while our result is hand to hand with **(Babaei *et al.*, 2007**) who found increase in ALP and LDHenzyme activities whey milk in the early diagnosisof subclinical mastitis.

In SCM the whey milk might characterized by the presence of leucocytes, and interstitial cells damaged during inflammation, particularly from disintegrated Leukocytes by bacterial toxins (**Katsoulos *et al.*, 2010 and Mohammadian, 2011). Table (4)** showed that mastitis caused a significant increase ($P \leq 0.001$) in total protein concentration of whey milk. This mainly as a result of increased concentration of

albumin and immunoglobulins in whey milk derived from blood (**Ishikawa *et al.*, 1982).** Bacterial toxins alter the permeability of the secretory epithelium and capillary wall and the blood components are allowed to pass into milk by diffusion through the leaky blood milk barrier **(Schultz,1977).**The major whey proteins, (beta- and alpha-globulin) were significantly decreased while the content of serum albumin and immune globulin in milk increased significantly($P \leq$ 0.001and $P \leq$ 0.05)respectively in mastitic why milk compared with healthy one. These results are in agreement with the results previously recorded in subclinical mastitic whey milk by**(Rashed *et al.*, 2002).** The present effects can be attributed to both inflammatory damage of the mammary secretory tissue and destruction of blood-milk permeability barrier which restrict in transfer of protein from interstitial fluid into milk (**Katsoulos *et al.*, 2010**).

Conclusion

Based on the current study,it could be concluded that dispite the CMT still the gold standard for daignosis of SCM, it could not detect disease in early lactation and dry periods, so it may be recommended that the changes of whey milk enzymes activities(GGT, ALP and LDH)which are associated with the presence of isolated pathogenic bacteria and mycoplasma species(as confirmed by the current molecular study)could be used as indicators for the subclinical mastitis in buffaloes especially in early lactation and dry periods.

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