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A CONTRIBUTION TOWARDS MILK ENZYMES, SOMATIC CELL COUNT AND BACTERIAL PATHOGENS ASSOCIATED WITH SUBCLINICAL MASTITIS COWS MILK

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ABSTRACT

A total of 200 milk samples of 50 clinically healthy dairy cows were collected and examined using California mastitis test (CMT) to detect subclinical mastitis. Somatic Cell count (SCC) and milk enzymes activities; lactate dehydrogenase (LDH), alkaline phosphatase (ALP), catalase and glutathione peroxidase (GPx) were applied to all samples while bacteriological examination was applied to positive CMT only. Only 14 cows (28%) were subclinically mastitic containing 31 mastitic quarters (15.5%). The entire 31 SCM milk sample showed elevated aLDH, ALP, catalase and GPx activities; with averages of 236.9±40.9, 268.5±53.3, 631.6±87.9, and 344.2±43 U/ml, respectively. Meanwhile 27 (87.1%) only of them showed elevation in SCC with an average of 9.24±2.0 ($\times 10^5$cells/ml). Bacteriological examination of milk samples revealed that 21 samples (67.7%) had different bacterial infections, of which 16 quarters (51.6%) had single bacterial infections and 5 (16.1%) had mixed bacterial infections. The correlations between bacterial infections, SCC and enzymatic activities in SCM milk revealed that the mixed bacterial infections; especially with *S. aureus*+ *Str. Dysgalactiae*, showed higher elevation in SCC as well as enzymes activities than single bacterial infections. In conclusion, enzyme parameters in this study can be used as biomarkers for early detection of subclinical mastitis.

Key words: *subclinical mastitis, somatic cell count, lactate dehydrogenase, alkaline phosphatase, Catalase, glutathione peroxidase, intramammary infection.*

INTRODUCTION

Mastitis is the most costly disease of the dairy industry in Egypt (Seleim *et al.*, 2002) and throughout the world affects both quality and quantity of milk (Ashfaq and Muhammad, 2008). Annual losses in the dairy industry due to mastitis are over 1.7 billion dollars a year in losses in the US alone (Sahoo *et al.,* 2012). Economical losses are due to loss in milk production, discarding abnormal milk and milk withheld from cows treated with antibiotics, degrading of milk quality and price due to high bacterial or somatic cell count (SCC), costs of drugs, veterinary services and increased labor costs, increased risk of subsequent mastitis, herd replacement, and problems related to antibiotics residues in milk and its products (Abdel-Rady and Sayed, 2009).

Different types either environmental or infectious mastitis and also some types of mastitis pathogens are more virulent than others. The severity of the inflammation can be classified into subclinical, clinical and chronic forms; however detection of subclinical mastitis (SCM) is difficult to be detected due to the absence of any remarkable indications (Mohammadian, 2011). Karyak *et al*. (2011) summarized that the higher percentages of mastitis losses were due to subclinical mastitis where milk production decreases by 10-26% (Dhakal, 2007). Moreover, cows with subclinical mastitis should be considered as a risk for spread of mastitis pathogens within and between herds and are as such of national concern (Persson *et al*., 2011).

Various method of detection of SCM have been found including California Mastitis Test (CMT) which is considered the best cut off to correctly identify SCM mastitis (Zaki *et al*., 2008). Also, estimation of somatic cell counts (SCC); is an indication of inflammation, measurement of biomarkers or enzymes associated with the onset of the disease and identification of the causative microorganisms (Viguier *et al.*, 2009).

Identifying and eliminating intramammary infection (IMI) may have significant economic benefits as preventing clinical mastitis and decreasing the amount of discarded milk (Dingwell *et al*., 2003). Additionally, knowledge of the clustering of IMI, both within quarters of a cow or within a herd, may be of considerable interest and may lead to further understanding of the dynamics of the disease (Lam

et al., 1996). Otherwise, from public health view, the assessment of SCM etiological pathogens aids to classify the healthy sound milk samples from those of public health hazard as the limits recommended by European countries standards (IDF, 1996) and (Egyptian Standards, 2001).

Pathogenic organisms in milk can be derived from the cow itself, the human hand or the environment (Bradely, 2002). About 150 species of microorganisms were found as the etiological agent of mastitis (Ebrahimi and Nikookhah, 2005). The major mastitis causing organisms are *S. aureus*, *Streptococci, E. coli*, *Corynebacterium* species and *Klebsiella* species (Shrestha and Bindari, 2012).

Antimicrobials are the most important tool in mastitis control programs. Therefore, identification of mastitis pathogens and their antimicrobial susceptibilities is important when selecting appropriate antimicrobial therapy (Ebrahimi and Taheri, 2009) to ensure optimal results of antimicrobial's use and minimize the risk for selection and spread of antimicrobial resistance (Moroni *et al.,* 2006).

For many years there has been an interest in using different enzymes in milk as biomarkers for mastitis. Many studies have revealed that enzyme activities in the udder epithelium change markedly due to mastitic inflammation (Andrei *et al*., 2011). More practical attention has been given to detection of enzyme activity in milk, and many enzymes have been proposed and listed as reliable markers for early diagnosis of subclinical mastitis (Babaei *et al*., 2007; Guha *et al*., 2012). Among these enzymes, lactate dehydrogenase (LDH) has been suggested as a biomarker for udder health disturbances as well as a statistical model for mastitis detection (Chagunda *et al*., 2006). The connection between milk alkaline phosphatase (ALP) and mastitis has been recognized for decades (Bogin and Ziv, 1973; Babaei *et al*., 2007). However, in practice only a few studies on this enzyme have been published although it has recently been suggested as a biomarker for human mastitis (Rasmussen *et al*., 2008). Larsen *et al*., 2010, concluded that milk enzymes ALP and LDH were markedly increased in case of experimentally induced mastitis and they considered both are the early indicators of acute mastitis.

Antioxidant activity of milk is due to the presence of antioxidant enzymes such as catalase and glutathione peroxidase (GPx) and other enzymes which catalyze the reduction of different peroxides aided by glutathione or other reducing substrates. The activity of milk antioxidant enzymes increases when SCC increases (Andrei *et al*., 2011). Measurement of the activity of such enzymes in milk has been used to monitor udder health in dairy cows (Fox and Kelly, 2006; Andrei *et al*., 2011).

To our knowledge, the correlation between bacterial infection, SCC and milk enzymes activity has not yet been fully considered in cows. Therefore, the aim of the present study was to evaluate possible relationships between milk SCC, isolated pathogens and enzymes activity; LDH, ALP, catalase, and GPx in dairy cows with subclinical mastitis.

MATERIALS and METHODS

Animals: A total of 50 clinically healthy dairy cows of different breeds (Holstein Friesian and native breeds); free of clinical mastitis and any other udder lesions, were examined in a special dairy farm in Beni-Suef governorate, Egypt.

Milk sampling: Milk samples were collected from 200 quarters before morning milking. Cows had no evidence of clinical mastitis at the time of sampling. Teat orifices were cleaned firstly then swabbed with cotton soaked in 70% ethyl alcohol, discarding the first streams of foremilk and then 20 ml of milk was collected aseptically from each teat into sterile tubes. Milk samples were kept cold during transportation, at 4°C and reached to the laboratory to be examined within 2 hours after collection. Milk samples were not collected from cows, which were treated with antibiotics by any route, till 96 hours after last treatment.

California mastitis test (CMT): CMT was applied directly in the farm to milk samples from each quarter using the method of Schalm *et al*. (1971). It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and lead to the formation of a 'gel-like' matrix consistency. According to the visible reactions the results were classified in four scores; 0 "negative or trace", 1 "weak positive", 2 "distinct positive" and 3 "strong positive". A cow was considered mastitic if one or more quarters were CMT positive with or without isolation of microorganisms.

Somatic cell counts: The SCC was applied to all milk samples. Milk samples showed positive CMT were used to detect any possible SCC variation as well as bacteriological examination to correlate and investigate the possible associated causal agent while the samples showed negative CMT were used as control. The samples were warmed in waterbath 40ºC for 5min, then mixed autonomically before autonomic reading of SCC by Bentley Soma count 15º for dispersion of fat globules (Zecconi *et al.,* 2002). The SCC measures the number of WBCs including neutrophils, macrophage, lymphocytes, eosinophils and various epithelial cell types of mammary gland in milk that were present in large number in case of subclinical mastitis.

Bacteriological examination of milk samples: Milk samples (10 µl) were cultured from each positive CMT milk sample onto 10% sheep blood agar, nutrient agar and MacConkey agar plates according to Abdel-Rady and Sayed (2009). Plates were incubated aerobically at 37°C for 24-48 h. The plates were examined for gross colony morphology, pigmentation and haemolytic characteristics at 24-48h. Presumptive identification of bacterial isolates was according to their colonial characteristics, Gram's reaction and morphology. Identification was confirmed by additional laboratory tests according to Quinn *et al*. (1994); Abera *et al*. (2010) and Persson *et al*. (2011).

Enzymatic assays: It was applied to all milk samples. Firstly, milk samples were defatted by centrifugation at 3000×g for 10min at 4°C. Defatted milk samples were used for enzymatic estimation. LDH, ALP, catalase and GPx enzymes activities were estimated using commercial colorimetric assay kits (Biodiagnostic Company, Egypt). The standardized protocol provided with the kit was followed for estimation. The final results were reported in units per ml of milk (U/ml milk).

Antimicrobial susceptibility testing: Antimicrobial susceptibility test was performed on the isolates using Mueller Hinton agar by disc diffusion method according to NCCLS Standards (2002). The isolates were tested for 10 antibiotic discs. The following antimicrobial discs (Oxoid, Basing Stoke, UK) with their corresponding concentration were used: Ampicillin (AMP, 10µg), Spiramycin (SP, 100µg), amoxacillin (AML, 10µg), Clindamycin (DA, 2µg), Doxycycline (DO, 30µg), Florophenicol (F, 30µg), Spectinomycin (SH, 100µg), Enrofloxacin (ENR, 5µg), Ciprofloxacin (CIP, 5µg) and Flumequine (UB, 30µg). The inhibition zone was reported as the diameter of the zone surrounding the individual disk in which bacterial growth was absent. Based on this, the isolates were defined as resistant, intermediate and susceptible according to the guide lines of the NCCLS Standards (2002).

Statistical analysis: Statistical analysis was performed using Sigmastat version 3.5 (Systat Software Inc., London, UK). For all treatments, data are the means ± the standard deviation of the results. Data were analysed using analysis of variance (ANOVA). A significant difference was defined as a P value of ≤ 0.05. In case of a statistical difference, treatment means were compared with the Holm–Sidak or Tukey test for multiple pairwise comparisons.

RESULTS

Results of CMT: Out of the 50 clinically healthy dairy cows examined by the CMT, 14 (28%) were subclinically mastitic. Results of positive CMT realized on quarters showed that out of 200 quarters, 31 (15.5%) were positive with variable scores; 3 (21.5%) showed degree 3, 16 (8%) showed degree 2, 12 (6%) showed degree 1 and the rest; 169 (84.5%) showed degree 0 (Table 1).

Table 1: Relationship between the positive CMT and SCC, enzymes activities and the infection status of the quarters.

The CMT score	No. of examined quarters	Positive quarters		High SCC		Enzymes activities		Bacterial isolation	
		No	%	No	%	No	%	No	%
1	200	12	6	9	75	12	100	4	33.3
2		16	8	15	93.75	16	100	14	87.5
3		3	1.5	3	100	3	100	3	100
Total		31	15.5	27	87.1	31	100	21	67.7

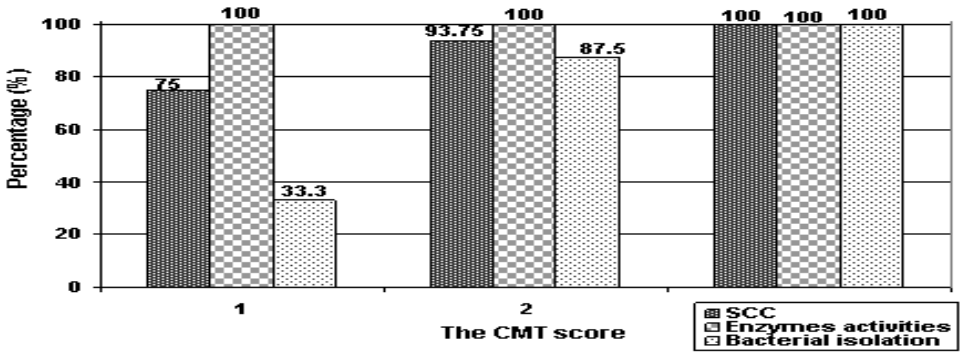

Fig. 1: Relation between CMT score and SCC, enzymes activities and the infection status.

Results of somatic cell counts: Out of the 31 positive CMT quarters, 27 (87.1%) were high in SCC with an average of 9.24±2.0×10^5cells/ml. The other 173 quarters (4 positive CMT and 169 negative CMT) were normal SCC with an average of 1.2± 0.21×10^5cells/ml (Tables 1 and 2).

Table 2: Changes in SCC in both normal and sub clinical mastitis cow's milk according to CMT.

Animal groups	No. of samples	SCC (×10^5 cells/ml)
Group I (Normal)	173	1.2 ± 0.21
Group II (Subclinical mastitis)	27	9.24±2.0

Somatic cell counts and bacteriological analysis: Of the 27 quarters with high SCC, 17 (63%) gave positive bacterial isolation while 10 (37%) were negative. On the other hand, out of the 173 quarters with normal SCC, 4 (2.3%) showed positive bacterial isolation while 169 (94.4%) were negative (Table 3).

Table 3: Classification of quarters according to SCC and bacteriological findings in SCM cases.

SCC	No.	Positive isolation		Negative isolation	
		No.	%	No.	%
>5×10^5cells/ml	27	17	63	10	37
<5×10^5cells/ml	173	4	2.3	169	97.7

Results of bacteriological examination of milk samples. Out of the 31 SCM cases, 21 (67.7%) showed positive bacterial isolation of which 16 (51.6%) had single bacterial infections and 5 (16.1%) had mixed bacterial infections (Tables 1&4). Of the 16 single bacterial infections, 5 (16.1%) were *Str. dysgalactiae*, 4 (12.9%) *E. coli*, 2 (6.5%) *Str. agalactiae*, 2 (6.5%) were *S. aureus*, 2 (6.5%) were *Bacillus subtilis* and 1 (3.2%) were *Corynebacterium bovis*. The 5 mixed infections were 2 (6.5%) *Str. agalactiae*+ *E. coli* and one (3.2%) for each of *S. aureus*+ *E. coli*, *S. aureus*+ *Str. dysgalactiae* and *S. aureus*+ *B. subtilis* (Table 4).

Table 4: Incidence of bacterial pathogens in SCM quarters milk samples in subclinical mastitis.

	Microbial isolates	Positive isolation	
		No	%
Single isolates	*Str. dysgalactiae*	5	16.1
	Escherichia coli	4	12.9
	Str. Agalactiae	2	6.5
	S. aureus	2	6.5
	Bacillus subtilis	2	6.5
	Corynebacterium bovis	1	3.2
	Total	16	51.6
Mixed isolates	*Str. agalactiae* + *E. Coli*	2	6.5
	S. aureus + *E. Coli*	1	3.2
	S. aureus + *Str. dysgalactiae*	1	3.2
	S. aureus + *B. Subtilis*	1	3.2
	Total	5	16.1
	Overall Total	21	67.7

Results of enzymatic assays: All the 31 mastitis milk showed samply high levels of milk enzymes compared with the 169 normal milk where the average levels of LDH, ALP, catalase and GPx in SCM cases were 236.9±40.9, 268.5±53.3, 631.6±87.9, and 344.2±43 U/ml, respectively, compared with 66.6±13.7, 32.8±8, 125.2±10.2, and 125.2±20.7 U/ml in normal cases, respectively (Tables 1 and 5).

Table 5: Changes in milk enzymes in both normal and subclinical mastitis cow's milk samples.

Animal groups	No. of samples	LDH (U/ml)	ALP (U/ml)	Catalase (U/ml)	GPx (U/ml)
Group I (Normal)	169	66.6 ±13.7	32.8 ±8	125.2±10.2	125.2±20.7
Group II Subclinical mastitis)	31	236.9±40.9	268.5±53.3	631.6±87.9	344.2±43

Correlation between bacterial infections, SCC and enzymatic activities in subclinical mastitis milk: Among the bacterial infections, the mixed *S. aureus*+ *Str. dysgalactiae* infection was the highest in SCC with an average of 12.44×10^5cells/ml. Moreover, there were 4 bacterial infections associated with normal SCC including 2 *E. coli* isolates ($1.5\pm0.04\times10^5$cells/ml) and 2 *B. subtilis* isolates ($1.39\pm0.04\times10^5$ cells/ml), but having high levels of milk enzymes. On the other hand, there were 10 cases with negative bacterial isolation but with high SCC with an average of $7.18\pm0.62\times10^5$cells/ml. Concerning milk enzymes, the mixed *S. aureus*+ *Str. dysgalactiae* infection also gave the highest levels of LDH, ALP and GPx with levels of 307.6, 408 and 454 U/ml, respectively, while catalase enzyme showed the highest level with the mixed *S. aureus*+ *E. coli* infection; 781 U/ml (Table 6).

Table 6: Correlation between bacterial infection, SCC and enzymatic activities in subclinical mastitis milk.

Bacterial isolates	No.	Mean SCC (10^5 cells/ml)	Enzymes activities (U/ml)			
			LDH	ALP	Catalase	GPx
Str. Dysgalactiae	5	9.1±0.12	238.3±17.7	249.2±34.8	588.8±77.8	337.1±18.5
E. coli	2	8.69±0.41	220.7±1.5	267.8±27.7	665±35.4	354.2±15.3
E. coli[*]	2	1.50±0.04	203.6±5.4	264±9.9	590.5±44.5	348.9±5.6
Str. agalactiae	2	11.30±0.45	282±7.1	287.5±14.8	621.5±40.3	341±5.8
S. aureus	2	11.84± 0.09	299.7±1.8	309.5±23.3	759±38.2	370.9±26.7
Bacillus subtilis[*]	2	1.39±0.04	193.7±7.6	207.1±14.1	605±9.9	302.6±13.2
Corynebacterium bovis	1	9.1	225.3	256	660	347.6
Str. agalactiae + *E. coli*	2	11.48±0.85	290.5±19.1	328.8±24.3	700±11.3	399.9±45.4
S. aureus + *E. coli*	1	12.0	303	332.6	781	425.4
S. aureus+ *Str. dysgalactiae*	1	12.44	307.6	408	774	454
S. aureus + *B. subtilis*	1	11.92	288.3	316	736	420.2
Negative isolation[**]	10	7.18±0.62	204.8±14.4	243.7±52.6	580.2±82	310.4±19

No.= Number of bacterial isolates, [*] = Latent infections (Bacterial isolates associated with normal SCC).
[**] = Non specific mastitis (High SCC with no bacterial isolation).

Results of antimicrobial susceptibility testing: Using 10 antimicrobials; all Streptococcus species (*Str. agalactiae* and *Str. dysgalactiae*) as well as *E. coli* were sensitive to spectinomycin, enrofloxacin and ciprofloxacin while Streptococci were more resistant to flumequine; 60%, and clindamycin; 50, meanwhile 71.4% of *E. coli* isolates were resistant to ampicillin. All *S. aureus* isolates were sensitive to clindamycin, florophenicol and enrofloxacin but 80% of them were resistant to ampicillin. Moreover, all *B. subtilis* isolates were sensitive to spiramycin, doxycycline and florophenicol while they were more resistant to ampicillin and amoxacillin; 66.7% for each. *C. bovis* was resistant to clindamycin only (Table 7).

Table 7: Sensitivity of bacterial isolates to different antimicrobial drugs.

Antibiotics	Streptococci (10)			*E. coli* (7)			*S. aureus* (5)			*B. subtilis* (3)			*C. bovis* (1)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Ampicillin	0	20	80	71.4	0	28.6	80	20	0	66.7	33.3	0	0	100	0
Spiramycin	20	40	40	28.6	42.9	28.6	20	20	60	0	0	100	0	0	100
amoxacillin	10	50	40	14.3	57.1	28.6	0	20	80	66.7	33.3	0	0	0	100
Clindamycin	50	10	40	42.9	14.2	42.9	0	0	100	0	33.3	66.7	100	0	0
Doxycycline	40	20	40	0	28.6	71.4	0	20	80	0	0	100	0	100	0
Florophenicol	0	20	80	0	28.6	71.4	0	0	100	0	0	100	0	0	100
Spectinomycin	0	20	100	0	0	100	0	20	80	33.3	66.7	0	0	100	0
Enrofloxacin	0	0	100	0	0	100	0	0	100	0	33.3	66.7	0	0	100
Ciprofloxacin	0	0	100	0	0	100	20	0	80	0	33.3	66.7	0	0	100
Flumequine	60	20	20	0	28.6	71.4	0	20	80	0	0	100	0	0	100

R= Resistant, I= Intermediate, S= Sensetive

DISCUSSION

Mastitis is one of the most critical diseases in dairy production as it is an economically devastating disease causing immense economic losses in the dairy industry. Subclinical mastitis is the most serious type as the infected animal shows no obvious symptoms and secrets apparently normal milk for a long time, during which causative organisms spread infection in herd, so it is an important feature of the epidemiology of many forms of bovine mastitis (Abdel-Rady and Sayed, 2009). Early detection of mastitis is of utmost importance in order to take steps toward isolation and/or treatment of the cow (Larsen *et al*., 2010). Clinical mastitis is easy to detect but also cows with SCM should be identified. The diagnosis of mastitis according to the IDF recommendations is based on SCC and microbiological status of the quarter (Guha *et al*., 2010).

CMT is still the superior screening diagnostic aid for subclinical mastitis, while bacteriological examination is still the most suitable, accurate and reliable method to confirm the causative organisms (El-Balkemy *et al*., 1997). Tests for indicators of inflammation are therefore necessary as screening tests to identify quarters with IMI and to select cows for subsequent bacteriological sampling. Over the past 50 years, many studies have been conducted in attempts to validate the CMT as a predictor of IMI. So, the CMT is a screening test for subclinical mastitis that can be used easily at cow-side (Leslie *et al*., 2002). It is widespread used in dairy fields and recommended as rapid and characteristic indicator for the infection of mammary gland (Al-Anbari *et al*., 2006). CMT can estimate SCC indirectly based on estimation of the DNA content of the milk; the reagent is simply a detergent (Sandholm *et al.*, 1995) and the results were scored from 0 to 3 where 0 represents SCC less than 500,000cells/ml.

From the aforementioned results presented in Table 1, the recorded overall quarter incidence of SCM by the CMT was 15.5% (31 quarters out of totally 200). Nearly the same incidence was obtained by Abdel-Rady and Sayed (2009) in Egypt. Higher incidences were obtained by Mahmoud (1988) in Egypt, Ismail and Hatem (1998) in Saudi Arabia, Abera *et al*. (2010) in Ethiopia and Heleili *et al*. (2012) in Algeria.

Furthermore, among positive CMT milk samples, elevated enzymatic activity was shown in 100% of them while 87.1% only of them showed elevation in SCC. Meanwhile, 67.7% only were accompanied with bacterial isolation (Table 1 and Fig. 1). These results indicated that enzyme activity were more proportional with CMT, followed by SCC and finally bacterial isolation.

Milk SCC has been used extensively as indicator of IMI. SCC has been included as a component of the definition of mastitis, and the original limit for SCC of a healthy quarter is 500,000cells/ml (IDF, 1971). The definition was a guide for diagnosis, even though 50% of truly infected quarters could at any time have a cell count less than the break-point of 500,000cells/ml (IDF, 1971). The somatic cells present in the milk of a healthy cow belong mainly to the macrophages (66-88%); in addition there are neutrophils (1-11%); which increases up to 90% and more in case of IMI, as well as epithelial and mononuclear cells (Sandholm *et al.*, 1995).

The obtained results from tables 1 and 2 revealed that 87.1% of the positive CMT samples were high in SCC with an average of $9.24\pm2.0\times10^5$cells/ml which were significantly different ($P<0.001$) comparing to

the normal ones. These results were higher than that of Heleili *et al*. (2012) who found that 34% only out of positive CMT milk samples were high in SCC. The rest of samples; 12.5%, were normal SCC although they were positive CMT and bacterial isolation and that occurs due to presence of latent infections (IDF, 1971). Consequently, SCC is always compared with bacteriology, and these tests can never completely agree (Pyorala, 2003). It is evident that a healthy quarter with no bacterial growth contains less than the original limit, and if the cell count exceeds it, the quarter is very likely to be infected. It is not relevant if the bacteria are minor or major pathogens, even though the so-called minor pathogens cause less elevation in the SCC (Hillerton, 1999).

The terms latent infection; positive bacterial isolation with normal SCC, and non specific (non infectious) mastitis; negative bacterial isolation with high SCC, have been in use (IDF, 1971). The comparison of SCC and bacteriological analysis results, illustrated in Table 3, showed that 63% of quarters with high SCC suffered from infectious mastitis while 37% suffered from non-infectious mastitis. On the other hand, latent infections were observed in 2.3% of the quarters with normal SCC. These results were nearly similar to those of Gianneechini *et al.* (2002) but differed from that of Heleili *et al*. (2012) who found that infectious mastitis cases were 94.4% while non infectious mastitis represented 5.6% only and latent infections were 83.8%.

Additionally, Dingwell *et al*. (2003) had assured that bacteriological culture is the gold standard method for identifying IMI but till nowadays the bacteriological sampling is not feasible as a routine test to identify subclinical mastitis. The indirect tests of mastitis seem to be more suitable for selecting cows with IMI (Pyorala, 2003). Also, Sargeant *et al*. (2001) added that logistic and financial considerations involved with sampling all fresh cows for bacteriological culture have precluded the widespread adoption of this strategy in the dairy industry.

Concerning the results of bacteriological examination shown in table 4, 67.7% of SCM milk samples showed positive bacterial isolation. These results were higher than that of Kapronezai *et al*. (2005); 26.8 %, Argaw and Tolosa (2008); 56.7%, Abdel-Rady and Sayed (2009); 40% and Rahman *et al*. (2010); 51.3%. On the contrary, our results were lower than that of Heleili *et al*. (2012); 87.3%. There were 10 positive CMT without isolation of the causative agent may attributed to that single milk sample has been criticized for not being sufficiently sensitive and for requiring more than one bacteriological sampling to determine whether the quarter is infected (Pyorala and Pyorala, 1997). Also, Heleili *et al*. (2012) reported that some of the microorganisms like listeria, mycoplasma and fungi require specific culture media. They also added that freezing of milk samples destroys some germs or reduces their number and makes the isolation of bacteria difficult. Persson *et al*. (2011) elucidated the reason for the number of colony forming units in milk is below the detection limit of the assay as well as the spontaneous bacteriological recovery. Additionally, the presence of antibiotic residues may explain falsely negative bacteriological results (Longo *et al.,* 1994) because the withdrawal time is not respected in our herds.

Of the positive bacterial isolation, 51.6% had single bacterial infections and 16.1% had mixed bacterial infections. These results were higher than that of Rahman *et al*. (2010) who found that 17.1% of samples had mono-bacterial infection and 10.8% had mixed bacterial infections. Among the bacterial infections, *Str. dysgalactiae* was the most prevalent; 16.1%, followed by *E. coli* (12.9%), then *Str. agalactiae*, *S. aureus*, *Bacillus subtilis* and mixed *Str. agalactiae*+ *E. coli* infections (6.5% for each), and *Corynebacterium bovis* as well as mixed infections of *S. aureus*+ *E. coli*, *S. aureus*+ *Str. dysgalactiae* and *S. aureus*+ *B. subtilis* (3.2% for each). These bacterial infections were recorded also by Kapronezai *et al*. (2005); Elango *et al.* (2010); Rahman *et al*. (2010) and Shrestha and Bindari (2012) but they all differed in that *S. aureus* was the most prevalent. Our results also are in agreement with Andrei *et al*. (2011) who reported that most mammary gland infections are caused by only a few types of bacteria, including streptococci, staphylococci, coliforms and *Corynebacterium bovis*

Earlier investigations have revealed that enzyme activities in the udder epithelium change markedly due to mastitic inflammation (Bogin and Ziv, 1973; Kitchen, 1981). Many of the indigenous enzymes increase in milk during inflammation. The enzymes dealing with the synthesis of milk decrease and the enzymes related to inflammation increase. This increase is due to that the enzymes originating from phagocytes increase exponentially and they include NAGase, catalase and beta-glucuronidase (Pyorala, 2003). Also, the activity of the enzymes originating from the blood increase and leak from the blood into the milk due to an increased permeability (Pyorala, 2003). Moreover, Kitchen (1981); in his review article, listed many enzymes as reliable markers of bovine mastitis such as ALP, LDH, different lipases, esterases, etc. Recently, it had shown that LDH possesses comparable qualities for mastitis detection (Chagunda *et al*., 2006).

Regarding the results of enzymatic analysis in milk samples; reported in Tables 1and 5, it was observed that enzyme activity of LDH, ALP, catalase and GPx were significantly increased ($P< 0.001$) in mastitis (positive CMT) milk compared to normal milk. Our results of higher activity of LDH and ALP in SCM

milk were supported with that of Babaei *et al*. (2007) and Batavani *et al*. (2007). Also, LDH and ALP were found to be elevated in mastitis buffalo milk infected with both gram positive and negative bacteria (Guha *et al*., 2012).

The early investigations indicated that elevated LDH activity in mastitic milk would not originate from blood plasma alone, but were more likely from parenchyma cells as well as disintegrated leukocytes (Bogin and Ziv, 1973; Kitchen, 1981). Kato *et al*. (1989) confirmed the contribution of LDH activity in mastitic milk from leukocytes and further specified the major increase in activity to originate from granulocytes and lymphocytes. Furthermore, Heyneman and Burvenich (1992) concluded that the leukocyte ALP enzyme from healthy and mastitic cows displayed very similar characteristics, suggesting that the increase in activity during mastitis is most probably related to the enhanced expression of the normal leukocyte ALP enzyme under direct or indirect influence of inflammatory mediators. Recently, Batavani *et al.* (2003) attributed that to the blood enzymes leaking from the blood into the milk due to an increased permeability. Katsoulos *et al.* (2010) and Mohammadian (2011) reported that the origin of elevated LDH and ALP activity was from leukocyte and mammary epithelial and interstitial cells damaged during inflammation, particularly from disintegrated leukocytes.

Catalase activity present in milk varies according to diet, lactation stage and especially for mastitis. It was demonstrated that catalase activity can be used as a marker of mastitis, in these cases the activity of catalase is much higher compared with normal milk (Fox and Kelly, 2006). Our results confirmed those of Hamed *et al*. (2008) who found a positive correlation between the catalase activity and the milk SCC. They signalled that catalase plays a central role in milk redox control. Especially during mastitis, catalase activity increases markedly, making it a useful indicator of mastitis. Also, they demonstrated that the catalase activity increased more with neutrophils than with macrophages and lymphocytes, indicating its role in free-radical decomposition in the case of bacterial infection resulting in an important increase of milk somatic cells. Elevated catalase activity also originated from the mammary gland and or bacterial cells (Fox and Kelly, 2006).

Glutathione-peroxidase is widespread in the cytoplasm of animal cells. The function of this enzyme is to protect cells against the damaging effects of peroxides, as part of an antioxidant enzymatic system (Andrei *et al*., 2011). Milk GPx varies according to species and diet (Fox and Kelly, 2006). Two different classes of GPx; selenium-dependent and selenium-independent, are known and both utilize glutathione for reducing hydroperoxides, but selenium-dependent enzymes are also capable of reducing hydrogen peroxide (Andrei *et al*., 2011). Our results of increased GPx activity in SCM milk compared to normal milk were supported with that of Andrei *et al*. (2011) who issued three hypotheses to explain this variation: (1) increased activity in milk is due to increasing enzyme concentration/activity in blood; (2) in milk, the enzyme is associated with caseins, an intensive hydrolysis process of casein can release the enzyme, and thus increase its activity; (3) peroxidases can be produced in milk by pathogens.

The correlations between bacterial infections, SCC and enzymatic activities in SCM milk were illustrated in Table 6. Concerning SCC, the mixed bacterial infection showed higher level than single infections and the mixed *S. aureus*+ *Str. dysgalactiae* infection were the highest in SCC with an average of 12.44×10^5cells/ml while the 4 latent infections; 2 *E. coli* and 2 *B. subtilis*, showed normal SCC. On the other hand, the 10 cases of non specific mastitis showed high SCC with an average of $7.18\pm0.62\times10^5$cells/ml. Concerning the milk enzymes, all bacterial infections; even latent infections, as well as non specific mastitis showed high milk enzymatic activity. Generally, the mixed infections were associated with the highest activity and the mixed *S. aureus*+ *Str. dysgalactiae* infection gave the highest levels of LDH, ALP and GPx with a level of 307.6, 408 and 454 U/ml, respectively, while catalase enzyme showed the highest level with the mixed *S. aureus*+ *E. coli* infection (781 U/ml). The high levels of mixed infections may be due to the more powerful effect of two or more pathogens than single pathogen especially if they were highly virulent, therefore this point needs more investigations.

Results of antimicrobial susceptibility testing illustrated in Table 7 showed that the susceptibility of Streptococci and *E. coli* to the tested antibiotics has revealed a complete (100%) susceptibility to spectinomycin, enrofloxacin and ciprofloxacin. Streptococci were more resistant to flumequine; 60%, and clindamycin; 50, while 71.4% of *E. coli* isolates were resistant to ampicillin. Concerning *S. aureus* strains, we noted a complete susceptibility against clindamycin, florophenicol and enrofloxacin but 80% of them were resistant to ampicillin. *B. subtilis* isolates were completely susceptible to spiramycin, doxycycline and florophenicol while they were more resistant to ampicillin and amoxacillin; 66.7% for each. *Corynebacterium bovis* was sensitive to most antimicrobials while it was resistant to clindamycin only.

CONCLUSION

SCM influences milk enzyme activity. From the current study it is evident that there is not a single

indicator to detect SCM. Although, LDH, ALP, Catalase and GPx were found to be indicators of SCM, all are being better and more reliable in the present study as they showed the highest agreement amongst all with IDF criteria of SCM diagnosis. It is recommended that all of mentioned enzymes are considered as markers for screening large herds for SCM. Standardizing easy biochemical methods or qualitative tests for estimating these indicators is recommended to develop kit for diagnosing SCM in the field. An attempt to develop a pathophysiological explanation of the ascertained association is also recommended for further study.

REFERENCES

Abdel-Rady, A. and Sayed, M. (2009): Epidemiological Studies on Subclinical Mastitis in Dairy cows in Assiut Governorate. Vet. World, 2(10): 373-380.

Abera, M.; Demie, B.; Aragaw, K.; Regassa, F. and Regassa, A. (2010): Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. J. Vet. Med. Anim. Hlth., 2(3): 29-34.

Al-Anbari, N.N.; Alkass, J.E. and Al-Rawi, A.A. (2006): California mastitis test as dependant and independent variable in Holstein cows. The 2nd Inter. Sci. Cong. Environ. South Valley Univ., Egypt.

Andrei, S.; Matei, S.; Fit, N.; Cernea, C.; Ciupe, S.; Bogdan, S. and Groza, I.S. (2011): Glutathione peroxidase activity and its relationship with somatic cell count, number of colony forming units and protein content in subclinical mastitis cows milk. Romanian Biotechnological Letters. 16(3): 6209-6217.

Argaw, K. and Tolosa, T. (2008): Prevalence of sub clinical mastitis in small holder dairy farms in Selale, North Shewa Zone, Central Ethiopia. Inter. J. Vet. Med., 5(1): 72-75.

Ashfaq, K. and Muhammad, G. (2008): Pathogens Associated with Bovine and Bubaline Mastitis in Peri-Urban Areas of Faisalabad, Pakistan. Pak. j. life soc. sci., 6(2): 86-88.

Babaei, H.; Mansouri-Najand, L.; Molaei, M.M., Kheradmand, A. and Sharifan, M. (2007): Assessment of lactate dehydrogenase, alkaline phosphatase and aspartate aminotransferase activities in cow's milk as an indicator of subclinical mastitis. Vet. Res. Comm., 31: 419-425.

Batavani, R.A.; Asri, S. and Naebzadeh, H. (2007): The effect of subclinical mastitis on milk composition in dairy cows. Ir J. Vet Res, Shiraz Univ., 8(3): Ser. No. 20, p. 205-220.

Batavani, R.A.; Mortaz, E.; Falahian, K. and Dawoodi, M.A. (2003): Study on frequency, etiology and some enzymatic activities of subclinical ovine mastitis in Urmia, Iran. Small Ruminant Research, 50: 45-50.

Bogin, E. and Ziv, G. (1973): Enzymes and minerals in normal and mastitic milk. Cornell Veterinary 63: 666-676.

Bradely, A.J. (2002): Bovine mastitis an evolving disease. Vet. J. 164:116-128.

Chagunda, M.G.G.; Larsen, T.; Bjerring, M. and Ingvartsen, K.L. (2006): L-actate dehydrogenase and N-acetyl-b-D-glucosaminidase activities in bovine milk as indicators of clinical mastitis. J. Dairy Res., 73: 431-440.

Dhakal, I.P. (2007): Normal somatic cell count and subclinical mastitis in murrah buffaloes. Buffalo J., 20: 261-270.

Dingwell, R.T.; Leslie, K.E.; Schukken, Y.H.; Sargeant, J.M. and Timms, L.L. (2003): Evaluation of the California Mastitis Test to detect an intramammary infection with a major pathogen in early lactation dairy cows. Can. Vet. J. 44: 413.

Ebrahimi, A. and Nikookhah, F. (2005): Identification of fungal agents in the milk samples of mastitic cows, Ind. Vet. J., 82: 52-54.

Ebrahimi, A. and Taheri, A.M. (2009): Characteristics of staphylococci isolated from clinical and subclinical mastitis cows in Shahrekord, Iran. Iranian J. Vet. Res., Shiraz Univ.,. 10(3): 273-277.

Egyptian Standards (2001): Raw milk. Egyptian organization for standardization and quality control, No.154.

Elango, A.; Doraisamy, K.A.; Rajarajan, G. and Kumaresan, G. (2010): Bacteriology of sub clinical mastitis and antibiogram of isolates recovered from cross bred cows. Ind. J. Anim. Res., 44(4): 280-284.

El-Balkemy, F.A.; Esmat, M.; Menazie, A. and Farag, A.N. (1997): Evaluation of screening tests used for detection of subclinical mastitis. 4th Sci. Cong. Egyptian Society for Cattle Diseases, Assiut, Egypt. pp 181.

Fox, P.F. and Kelly, A.L. (2006): Indigenous enzymes in milk: Overview and historical aspects-Part 2, Inter. Dairy J., 16: 517-532.

Gianneechini, R.; Concha, C.; Rivero, R.; Delucci, I. and Moreno Lopez, J. (2002): Occurrence of clinical and sub-clinical mastitis in dairy herds in the West Littoral Region in Uruguay. Acta. Vet. Scand., 43(4): 221-230.

Guha, A.; Gera, S. and Sharma, A. (2010): Assessment of chemical and electrolyte profile as an indicator of subclinical mastitis in riverine buffalo (Bubalus bubalis). Har. Vet., 49: 19-21.

Guha, A., Gera, S. and Sharma, A. (2012): Evaluation of milk trace elements, lactate dehydrogenase,

alkaline phosphatase and aspartate aminotransferase activity of subclinical mastitis as an indicator of subclinical mastitis in riverine buffalo (Bubalus bubalis). Asian-Aust. J. Anim. Sci., 25(3):353-360.

Hamed, H.; El-Feki, A. and Gargouri, A. (2008): Total and differential bulk cow milk somatic cell counts and their relation with antioxidant factors, C.R.Biologies, 331: 144-151.

Heleili, N.; Ayachi, A.; Melizi, M.; Kassah, A.L. and Mamache, B. (2012): Prevalence of Subclinical Bovine Mastitis and the *In Vitro* Sensitivity of Bacterial Isolates in Batna Governorate, East of Algeria J. Anim. Sci. Adv., 2(6): 576-582.

Heyneman, R. and Burvenich, C. (1992): Kinetics and characteristics of bovine neutrophil alkaline phosphatase during acute *Eschericia coli* mastitis. J. Dairy Sci., 75: 1826-1834.

Hillerton, J.E. (1999): Redefining mastitis based on somatic cell count, IDF Bulletin, 345:4-6.

IDF; International Dairy Federation (1971): A monograph of bovine mastitis.

IDF; International Dairy Federation (1996): Bacteriological quality of raw milk. 41 Square Vevgote, 13-1030, Brussels, Belgium.

Ismail, T.M. and Hatem, M.E. (1998): Prevalence of subclinical mastitis in a dairy cattle herd in the eastern region of Saudi Arabia. 8th Sci. Con. Fac. Vet. Med. Assiut Univ. Egypt.

Kapronezai, P.; Melville, N.R. and Benites, J. (2005): Microbiological analysis, Tamis test and the California Mastitis Test in milk samples of female buffaloes from herds in the state of Sao Paulo. Arch. Biol. Inst., 72(2): 183-187.

Karyak, G.O.; Safi, S.; Rahimi Froushani, A. and Bolourchi, M. (2011): Study of the relationship between oxidative stress and subclinical mastitis in dairy cattle. Iranian J. of Vet. Res., 12(4): 350-353.

Kato, K.K.; Mori, K. and Katoh, N. (1989): Contribution of leucocytes to the origin of lactate dehydrogenase isoenzymes in milk of bovine mastitis. Japanese J. Vet. Sci., 51: 530-539.

Katsoulos, P.D.; Christodoulopoulos, G.; Minas, A.; Karatzia, M.A.; Pourliotis K. and Kritas, S.K. (2010): The role of lactate dehydrogenase, alkaline phosphatase and aspartate aminotransferase in the diagnosis of subclinical intramammary infections in dairy sheep and goats. J. Dairy Res. 77: 107-111.

Kitchen, B. (1981): Review of the progress of dairy science: Bovine mastitis: Milk compositional changes and related diagnostic tests. J. Dairy Res., 48: 167-188.

Lam, T.J.G.M.; De Jong, M.C.M.; Schukken, Y.H. and Brand, A. (1996): Mathematical modeling to estimate efficacy of postmilking teat disinfection in split udder trials of dairy cows. J. Dairy Sci., 79: 62.

Larsen, T.; Rontved, C.M.; Ingvartsen, K.L.; Vels, L. and Bjerring, M. (2010): Enzyme activity and acute phase proteins in milk utilized as indicators of acute clinical *E. coli* LPS-induced mastitis. Animal, 4(10): 1672-1679.

Leslie, K.E.; Jansen, J.T. and Lim, G.H. (2002): Opportunities and implications for improved on-farm cowside diagnostics. Proc. De Laval Hygiene Symp. pp. 147.

Longo, F.; Beguin, J.C.; Consalvi, P.J. and Deltor, J.C. (1994): Quelques données épidémiologiques sur les mammites subcliniques de la vache laitière. Rev. Med. Vet., 145(1): 43-47.

Mahmoud, A.A. (1988): Some studies on subclinical mastitis in dairy cattle. Assiut Vet. Med. J. 20: 150.

Mohammadian, B. (2011): The effect of subclinical mastitis on Lactate Dehydrogenase in dairy cows. Inter. J. Anim. Vet. Adv., 3(3): 161-163.

Moroni, P.; Pisoni, G.; Antonini, M.; Villa, R.; Boettcher, P. and Carli, S. (2006): Antimicrobial Drug Susceptibility of *Staphylococcus aureus* from Subclinical Bovine Mastitis in Italy. J Dairy Sci., 89: 2973-2976.

National Committee for Clinical Laboratory (NCCLS) Standards (2002): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. 2nd ed. NCCLS document M31-A2. Natl. Committee for Clinical Laboratory Standards, Wayne, PA.

Persson, Y.; Nyman, A.K.J. and Andersson, U.G. (2011): Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. Acta Veterinaria Scandinavica, 53: 36-43.

Pyorala, S. (2003): Review article: Indicators of inflammation in the diagnosis of mastitis. Vet. Res., 34: 565-578.

Pyorala, S. and Pyorala, E. (1997): Accuracy of methods using somatic cell count and N-Acetyl-β-d-Glucosaminidase activity in milk to assess the bacteriological cure of bovine clinical mastitis. J. Dairy Sci., 80: 2820-2825.

Quinn, P.J.; Carter, M.E.; Markey, B. and Carter, G.R. (1994): Clinical Veterinary Microbiology, Wilfe Publishing, London pp. 95-101.

Rahman, M.M.; Islam, M.R.; Uddin, M.B. and Aktaruzzaman, M. (2010): Prevalence of subclinical mastitis in dairy cows reared in Sylhet district of Bangladesh. Int. J. BioRes., 1(2): 23-28.

Rasmussen, L.B.W.; Hansen, D.H.; Kæstel, P.; Michaelsen, K.F.; Friis, H. and Larsen, T. (2008): Milk enzyme activities and subclinical

mastitis among women in Guinea-Bissau. Breastfeeding Medicine 3: 215-219.

Sahoo, N.R.; Kumar, P.; Bhusan, B.; Bhattacharya, T.K.; Dayal, S. and Sahoo, M. (2012): Lysozyme in Livestock: A Guide to Selection for Disease Resistance: a Review. J Anim Sci Adv, 2 (4): 347-360.

Sandholm, M.; Honkanen-Buzalski, T.; Kaartinen, L. and Pyorala, S. (1995): The bovine udder and mastitis (Eds.), Gummerus, Jyväskylä, Finland, pp.89-104.

Sargeant, J.M.; Leslie, K.E.; Shirley, J.E.; Pulkrabek, J.L. and Liim G.H. (2001): Sensitivity and specificity of somatic cell count and California Mastitis Test for identifying intramammary infection in early lactation. J. Dairy Sci., 84: 2018-2024.

Schalm, O.W.; Carroll, E.J. and Jaire, N.C. (1971): Bovine mastitis. Lea and Febiger, Philadelphia, PA, pp: 80-89.

Seleim, R.S.; Rashed, A.Y.M. and Fahmy, B.G.A. (2002): Mastitis pathogens: attachment-related virulence features, whey protein markers and antibiotic efficacy in cows. Vet. Med. J. Giza, 50:405.

Shrestha, S. and Bindari, Y.R. (2012): Prevalence of Sub-Clinical Mastitis among Dairy Cattle in Bhaktapur District, Nepal. Inter. J. Agri. Biosci., 1(1): 16-19.

Viguier, C.; Arora, S.; Gilmartin, N.; Welbeck, K. and O'kennedy, R. (2009): Mastitis detection. current trends and future perspectives. Trend. Biotechnol., 27(8): 486-493.

Zaki, M.S.; Sharaf, N.E.; Mostafa, S.O.; Fawzi, O.M. and Batrawy, N.E.L. (2008): Effect of subclinical mastitis on some biochemical and clinicopathological parameters in Buffalo. Am. Eur. J. Agric. Environ. Sci., 3(2): 200-204.

Zecconi, A.; Casirani, G.; Binda, E. and Piccinini, R. (2002): The importance of assess the effects of voluntary milking system on teat tissues, inflammatory, infectous and somatic cell counts. Dept. Anim., path- infect. Dis. Lab., Univ. of Milan. Delval. Hygeine, Technology center imaugal symposium, 15- 16.

اطلالة علي انزيمات الألبان والخلايا الجسدية والممرضات البكتيرية المرتبطة بالتهاب الضرع تحت الإكلينيكي فى لبن الابقار

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أجريت هذه الدراسة على عدد ٢٠٠ عينة لبن تم تجميعها من ٥٠ بقره حلاب بمحافظة بنى سويف وتم فحصها بواسطة اختبار الكاليفورنيا. هذا وقد تم تعيين عدد الخلايا الجسدية وبعض الانزيمات منها LDH، ALP، Catalase و GPx فى جميع العينات ولكن تم الفحص البكتريولوجي في العينات الايجابية لاختبار الكاليفورنيا فقط. وقد أظهرت النتائج تواجد التهاب الضرع تحت الإكلينيكي في ١٤ بقرة بنسبة ٢٨% في حين كان العدد على مستوى الضرع ٣١ عينة بنسبة ١٥.٥ **%**. أظهرت جميع العينات الايجابية لاختبار الكاليفورنيا ارتفاعا ملحوظا في جميع الإنزيمات قيد الدراسة منها LDH، ALP، Catalase **و** GPx بمتوسط 40.9±236.9، 53.3±268.5، 87.9±631.6 و 43±344.2 وحده/ مل على التوالي. بينما بلغ عدد العينات التي شهدت ارتفاعا فى عدد الخلايا الجسدية ٢٧ عينة بنسبة ٨٧.١% بمتوسط ٩.٢٤± ٢.٠×١٠^٥ خلية/ مل. وقد أوضح الفحص البكتريولوجى للعينات بأن عدد ٢١ عينة (٦٧.٧%) كانت ايجابية للعزل منها ١٦ عينة (٥١.٦%) احتوت على عدوى فردية و ٥ عينات (١٦.١%) احتوت على عدوى مختلطة. هذا وقد درست العلاقة بين الفحص البكتريولوجى والخلايا الجسدية ونشاط الإنزيمات وتبين منها أن العدوي المختلطة شهدت معدلات أعلي من العدوى الفردية. ويستخلص من هذه الدراسة أن إنزيمات الألبان التي تم دراستها تصلح لأن تكون كاشف للتعيين المبكر لحالات التهاب الضرع تحت الإكلينيكي.