



Research Article

Incidence of Subclinical Mastitis with Special Reference to Lactate Dehydrogenase (LDH) Enzyme as a Biomarker

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Abstract

Background and Objective: Subclinical mastitis is considered as one of the most costly diseases in dairy production throughout the world and the second problem in dairy Egyptian farms after laminitis, therefore this study focused on determination of the prevalence of subclinical mastitis (SCM) in two dairy herds located in Ismailia and El-Sharkia governorate, Egypt with special reference to Lactate Dehydrogenase (LDH) enzyme as a biomarker with isolation of some of the etiological agents. **Materials and Methods:** About 230 quarter milk samples (QMS) were collected from apparently healthy 60 cows and subjected to California Mastitis Test (CMT). The positive CMT samples were subjected to some of the screening tests (Milk Somatic Cell Count [MSCC], Koestler value and Lactate Dehydrogenase (LDH) enzyme level in milk and blood serum samples and isolation and identification of some of the etiological agents. **Result:** The CMT revealed that 43.5% of the examined samples had subclinical mastitis, the mean values of Milk Somatic Cell Count [MSCC], Koestler value and Lactate Dehydrogenase (LDH) enzyme level in milk and blood serum samples were $1.1 \times 10^6 \pm 61554.12$ cells mL^{-1} , 4.62 ± 0.08 , 621.15 ± 55.45 and 633.17 ± 30.20 UL^{-1} , respectively. Bacteriological examination showed the isolation of Streptococci and Coliforms from 88 and 100% of the examined samples, respectively, Polymerase Chain Reaction (PCR) revealed the isolation of *Streptococcus agalactiae* and *E. coli* strains with percentages of 21.74 and 1.36%, respectively. **Conclusion:** The poor management and udder health practices and inadequate screening tests applying by most farmers in the study area resulted in high prevalence rate of SCM with isolation of pathogenic *E. coli*.

Key words: Lactate dehydrogenase enzyme, CMT, MSCC, *E. coli*, PCR

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mastitis is the most common inflammatory diseases of mammary gland in ruminants, which leads to physical, chemical and microbiological changes in milk of dairy animals¹. The disease is usually classified into two forms, clinical in which the disease is diagnosed clinically and subclinical which is mainly diagnosed via assessment of somatic cell count and bacterial examination².

Over 100 different micro-organisms have been recorded as a cause of intra mammary infection in dairy cow, the major causative micro-organisms were classified into contagious pathogens including (*Staphylococcus aureus* and *Streptococcus agalactiae*) and environmental pathogens most frequently encountered are species of streptococci other than *Streptococcus agalactiae* and Gram-negative bacteria such as *E. coli* and *Klebsiella*^{3,4}.

Subclinical mastitis has been considered as a major problem for the dairy industry, the infected quarters look apparently normal and could act as an invisible source of infection among the dairy cattle⁵. This disease inflicts heavy economic losses on account of reduced milk production, treatment costs, increased labor, milk withheld for human consumption following treatment and premature culling, therefore early detection of mastitis at the subclinical stage is important for most dairy farmers to take appropriate measures toward treatment and prevention of transmission of infection to other healthy one in the herd and hence reduce the production losses.

Diagnosis of mastitis according to the IDF recommendations is based on the SCC and microbiological status of the quarter. Bacteriological culture of milk samples is the standard method for identifying mastitis⁶ but it consumes a long time. Subclinical mastitis milk appears normal but usually has an elevated somatic cell count⁷, animals are considered to have a subclinical mastitis if milk contains a threshold more than^{8,9} 2×10^5 cells mL⁻¹. The use of advanced tests like real time PCR and acute phase protein estimation as routine tests are costly. Therefore, other inflammatory markers with high indicative value and faster times are necessary for early diagnosis of SCM, from these markers lactate dehydrogenase (LDH) enzyme which increases during inflammation of mammary glands so it has the potential to be used as a screening test and biomarkers for detection of subclinical mastitis¹⁰.

Due to the undesirable effect of subclinical mastitis economically and on the public health, this study was planned to determine the prevalence of subclinical mastitis (SCM) in two dairy herds located in Ismailia and El-Sharakia

governorates, Egypt with special reference to Lactate Dehydrogenase (LDH) enzyme as a biomarker and isolation and identification of some of the etiological agents for control these big problem.

MATERIALS AND METHODS

Collection of samples¹¹: This study was carried out on two dairy herds located in Ismailia and El-Sharakia Governorates during the period from October, 2016 to August, 2017. About 230 quarters milk samples (QMS) were collected from 60 apparently healthy (Holstein-Friesian) cows at all stages of lactation. All cows were milked twice daily mechanically. Teats were washed, dried and sterilized with cotton soaked in 70% ethyl alcohol, the first 3-4 streams of milk were discarded and milk samples were collected from each quarter into sterile vials. Collected samples were transferred to the laboratory in an insulating ice box with a minimum of delay to be immediately examined.

Screening tests:

- California Mastitis Test (CMT) was adopted according to APHA¹¹

All samples which showed positive CMT were subjected to the following tests:

- Milk Somatic Cell Count (MSCC) was determined according to Zecconi *et al.*¹²
- Koestler value was cited after Kifaro *et al.*¹³
- Evaluation of enzymatic activity (lactate dehydrogenase, LDH) according to Bergmeyer¹⁴

Bacteriological examination:

- Isolation of Streptococci was adopted according to APHA¹¹
- Biochemical identification of the isolated organisms was determined according to Quinn *et al.*¹⁵ and BAM¹⁶
- Molecular identification of *Streptococcus agalactiae* by polymerase chain reaction (PCR) was adopted according to Ke *et al.*¹⁷
- *S. agalactiae* *fb* TTTCACCAGCTGTATTAGAAGTA 53 bp
- GTTCCCTGAACATTATCTTTGAT
- Isolation of coliform organisms was carried out according to BAM¹⁶
- Biochemical identification of the isolated organisms was adopted according to Quinn *et al.*¹⁵ and BAM¹⁶
- Serological identification of *E. coli* was adopted according to Kok *et al.*¹⁸

- Molecular identification of *E. coli* by polymerase chain reaction (PCR) was adopted according to Louie *et al.*¹⁹
- Strain O₁₅₇..... rfb Gene....CGGACATCCATGTGATATGG (259 Bb)
- TTGCCTATGTACAGCTAATCC
- Strain O₂₅.... wzy Gene... AGAGATCCGTCTTTATTTCTTCGG (230 Bb)
- GTTCTCGATACCTAACGCAATACCC

Statistical analysis: Results were expressed as Mean±Standard Deviation with correlation matrix between the chemical parameters were investigated by ANOVA. Statistical analysis were performed using the program Statistical Package for Social Science (SPSS), version 17.

RESULTS

California mastitis test (CMT): Data illustrated in Fig. 1 Showed that 230 quarter milk samples were examined by CMT and found that 43.5% of the examined samples reacted positively to CMT.

Milk somatic cell count (MSCC): Study showed that Milk Somatic Cell Count ranged from 263×10^3 - 263×10^3 cells mL⁻¹ with a mean count of $1.1 \times 10^6 \pm 61554.12$ cells mL⁻¹ in the positive CMT quarter milk samples (Table 1). Egyptian standards considered udder is affected with subclinical mastitis when MSCC exceed 500.000 cells mL⁻¹ (Egyptian Standards for raw milk)²⁰ according to these Standards, the acceptable samples represent 9.00% of the examined samples, while the unacceptable samples that had subclinical mastitis were 91.00% (Fig. 2).

Koestler value: Data illustrated in Table 1 revealed that the minimum Koestler value was 3.12, the maximum was 7.39 with a mean value of 4.62 ± 0.08 .

Lactate dehydrogenase enzyme (LDH): Results represented in Table 1 revealed that the LDH level in the examined milk samples ranged from 236.00-1033.00 (U L⁻¹) with mean value of 621.15 ± 55.45 (U L⁻¹), while its minimum, maximum and average value in blood serum samples were 496.00, 867.00 and 633.17 ± 30.20 U L⁻¹, respectively.

Correlation matrix between the examined chemical parameters: Data of the correlation matrix of the examined parameters showed that Koestler's value and LDH activity

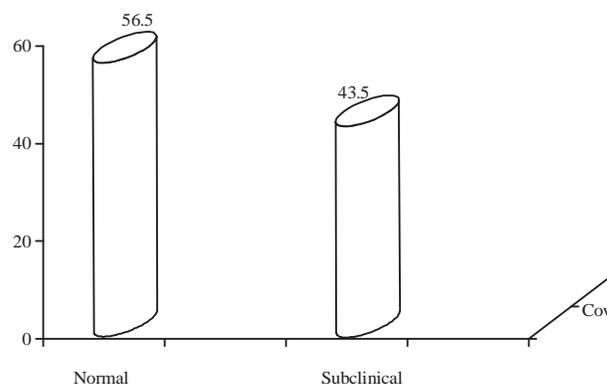


Fig. 1: Incidence of subclinical mastitis in the examined Quarter Milk Samples (QMS) using California Mastitis Test (CMT)

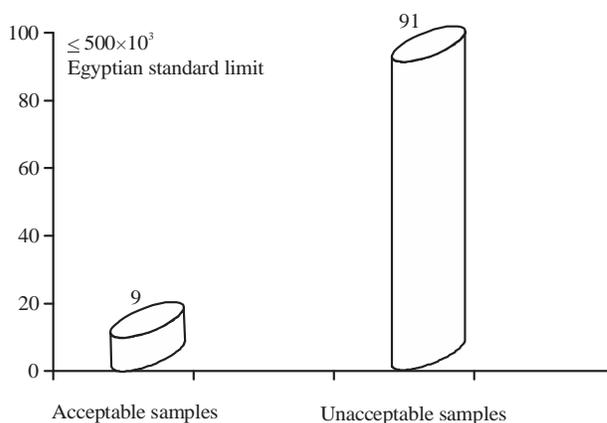


Fig. 2: Acceptability of the examined quarter milk samples in relation to SCC

Source: Egyptian standards of Raw milk²⁰

were positively correlated with SCC. The LDH of blood serum had a strong significant positive correlation with SCC ($p < 0.01$).

Incidence of the isolated micro-organisms from the examined samples: This study conducted a bacteriological examination to 100 quarter milk samples collected from sub clinically mastitis cows using CMT. The obtained results showed that Streptococci and Coliforms were present in 88 and 100% of the examined positive CMT quartet milk samples, respectively (Table 2).

Prevalence of *Streptococcus agalactiae* in the examined QMS: The results obtained in Table 3 showed that the incidence of contamination of the examined positive CMT quarter milk samples with *S. agalactiae* biochemically was 17.7% (23 isolates) among 130 isolated strains of streptococci, 21.74% of the isolated *S. agalactiae*

Fig. 3: PCR results for *S. agalactiae* *fb* gene showing positive amplification of 153 bp in the 5 tested samples
Lanes 1: 5 represent 5 tested samples, Pos: Positive control, Neg: Positive control, L [Gelpilot100 bp ladder (Qiagen, 100-600 bp)]



Fig. 4: PCR results for *E. coli* genes (*rfb* Gene and *wzy* Gene)

Lane 1: 1 kb DNA marker Fermentas Lane 4: Sample 2 (*E. coli* O₂₅), Lane 2: Sample 1 (*E. coli* O₁₅₇) Lane 5: Control negative, Lane 3: Control negative

Table 1: Statistical analytical results of screening tests in the examined positive CMT quarter milk samples

Screening tests	Number of QMS	Minimum	Maximum	Mean ± SEM
Milk somatic cell count (MSCC)	100	263 × 10 ³	2 × 10 ⁶	1.099 × 10 ⁶ ± 0.062 × 10 ⁶
Koestler value	100	3.12	7.39	4.62 ± 0.084
LDH level (U L ⁻¹) in QMS	100	236.00	1033.00	621.15 ± 55.45
LDH level (U L ⁻¹) in blood serum	100	496.00	867.00	633.17 ± 30.20

Table 2: Prevalence of the isolated micro-organisms in the examined positive CMT quarter milk samples (QMS)

	Streptococci				Coliforms			
	-ve		+ve		-ve		+ve	
Total number of positive CMT QMS	No.	%	No.	%	No.	%	No.	%
100	12	12.0	88	88.0	0	0.0	100	100.0

identified biochemically were examined by PCR and the obtained results confirmed positive amplification of 153 bp in all of the examined isolates (21.74%) (Fig. 3).

Coliforms: Regarding the results represented in Table 4 Coliforms could be detected in all of the examined positive CMT quarter milk samples with isolation of 147 strains, the biochemical identification of coliform organisms revealed that *Citrobacter diversus* 82 (55.78%) was the most common one followed by *Serratia fonticola* 18 (12.25%) and *Citrobacter freundii* 13 (8.84%) then *Klebsiella oxytoca* 9 (6.13%) and *Enterobacter aerogenes* 8 (5.44%), *E. coli* 7 (4.76%) and *Enterobacter intermedius* 7 (4.76%), finally *Klebsiella pneumoniae* subsp. *Azoenae* 3 (2.04%).

Table 3: Incidence of isolated *Streptococcus agalactiae* in the positive CMT quarter milk samples (total streptococcus isolates = 130)

Results depending on biochemical tests	No. of isolated <i>S. agalactiae</i>	
	No.	%
(CAMP and sodium hippurate test)	23	17.70
PCR	5	21.74

Incidence of *Escherichia coli* in the examined QMS:

Results illustrated the serological identification of the suspected isolates of *E. coli* by slide agglutination test. The results showed that two (1.36%) isolates out of 7 (4.76%) were positive and they were *E. coli* O₂₅ and *E. coli* O₁₅₇, when these isolates 2 (1.36%) were investigated by PCR, the results confirmed the obtained results serologically (Fig. 4, 5).

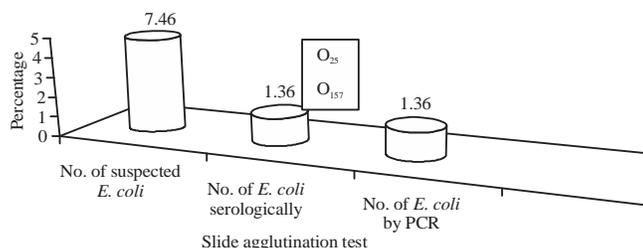


Fig. 5: Prevalence of *E. coli* using different methods of identification

Table 4: Incidence of coliform isolates obtained from the positive CMT quarter milk samples

Coliform isolates	No. of isolates	%
<i>Citrobacter diversus</i>	82	55.78
<i>Serratia fonticola</i>	18	12.25
<i>Citrobacter freundii</i>	13	8.84
<i>Klebsiella oxytoca</i>	9	6.13
<i>Enterobacter aerogenes</i>	8	5.44
<i>E. coli</i>	7	4.76
<i>Enterobacter intermedius</i>	7	4.76
<i>Klebsiella pneumoniae</i> subsp. <i>Azoenae</i>	3	2.04
Total	147	100.00

DISCUSSION

Subclinical mastitis milk appears grossly normal and there are no visible signs of inflammation in the udder. However there are many changes encountered in milk from udder with subclinical infection, cellular, biochemical and enzymatic changes are present in such milk. These changes are detected in most of screening and routine tests.

The CMT is the most common test used for diagnosis of subclinical cases depending on detection of increased leukocyte count in milk. Data illustrated in Fig. 1 showed that 43.5% of the examined samples had subclinical mastitis and these results were in accordance with that reported by Ayano *et al.*²¹, who evaluated 546 milking cows by CMT and found that 41.02% of the examined samples were positive for subclinical mastitis, while lower than that obtained by Mureithi and Njuguna²², who found that 64% of samples had SCM, where as higher than that recorded by Sanotharan *et al.*²³, who illustrated that 19.1% of the examined quarters milk samples were positive to CMT. This difference in results may be attributed to kind of milking (manual, automatic), numbers of milking time, farm hygiene, milking cows like (age, climate, state of udder and numbers of lactation) and labor hygiene.

Somatic Cell Count (SCC) has been recognized as an available tool for identifying cows with intra mammary infection (IMI) with major pathogens or for

monitoring the udder health at the herd level²⁴, also there are some other factors have a direct and dramatic effect on SCC as: Stresses, seasonal variations, age of cow, stage of lactation and time of milking²⁵. In healthy udder, Milk Somatic Cell Count (MSCC) usually ranged between 50,000 and 100,000 cells mL⁻¹ and in some cases up to 200,000 cells mL⁻¹. If the SCC exceeded 200,000 cells mL⁻¹, animal is considered affected with subclinical mastitis²⁶, while Egyptian standards considered udder is affected with subclinical mastitis when MSCC exceed²⁰ 500.000 cells mL⁻¹.

Results of MSCC that recorded in Table 1 were nearly similar to that mentioned by Mohamed *et al.*²⁷, who found that the average count of somatic cell were 9.24±2.0 (×10⁵ cells mL⁻¹), while higher than that reported by Andrea *et al.*²⁸, who showed that the average SCC count was 721,000 cells mL⁻¹ in the examined milk samples indicating presence of subclinical mastitis. The obtained results proved the presence of good correlation between CMT and SCC as a tool for monitoring subclinical mastitis in dairy farms and these results were agreed with that reported by Abd El-Fatah²⁹ and Khalil³⁰.

Subclinical Mastitis declined milk lactose in the affected quarters due to the damage in the alveolar epithelial cells, also had markedly changed the ionic environment, sodium and chloride are increased, while potassium levels was decreased, by necessity to maintain udder osmotic equilibrium^{31,32} and this resulting in higher Koestler values which computed by using percentages of chloride and lactose, showing an increase in values with severity of mastitis¹³ and this was in accordance with that reported by Morsi *et al.*³³, who proved that the decrease in milk lactose content and/or the increase in milk chloride content can be used as an index for suspecting the presence of sub-clinical mastitis.

Results illustrated in Table 1 which revealed that the mean Koestler value were 4.62±0.08 were lower than that mentioned by Kifaro *et al.*¹³, who stated that the mean Koestler value in its study was very high (6.4).

Although CMT and SCC are field, rapid and cheap tools helping as a screening tests for detection of subclinical mastitis³⁴, CMT is unsuitable in early lactation or in dry period and SCC lacks the needed specificity because its affected by many other factors such as the number of lactations, stage of lactation, level of milk production, season, age and breed of cattle^{35,36}.

The detection of milk enzymatic activities of lactate dehydrogenase enzyme (LDH) might represent a reliable diagnostic method for identifying subclinical mastitis in early lactation and dry period³⁷.

Some enzyme levels increase in milk during intra mammary infections^{38,39}, one of these enzymes is LDH which is a cytoplasmic enzyme that has been proposed as a biomarker for udder health check, it gets released into milk from ruptured mammary epithelial cells, phagocytes and from serum, the activity of this enzyme significantly increase in milk obtained from quarters with subclinical mastitis and its activity has a high and positive correlation with SCC especially in infected quarters⁴⁰⁻⁴².

Results obtained in Table 1 were in accordance with Batavani *et al.*⁴³, who compared the level of LDH in milk samples from healthy quarters and milk from quarters with subclinical mastitis and found that the activity of LDH elevated in quarters affected with subclinical mastitis (1524.04 vs. 485.94 U L⁻¹) and Mohammadian⁴⁰, Kalantari *et al.*⁴² and Sorensen *et al.*⁴⁴, who reported marked elevation in LDH activity in milk serum from sub clinically infected bovine mammary gland but Babaei *et al.*³⁷ reported that LDH was not a sensitive marker and the blood LDH may be transferred to milk due to damage of blood-milk barrier.

Measuring enzymatic activities in milk which is both easy and of low cost compared to other methods could be used as a diagnostic test with acceptable sensitivity and specificity for detecting quarters with subclinical mastitis and these was assured by Kalantari *et al.*⁴², who reported that Milk LDH had the most clinical accuracy with 94.8 sensitivity and 94.1% specificity.

The positive correlation between SCC and Koestler value was agreed with that cited by Kifaro *et al.*¹³, who reported that lactose content tended to decrease with a tendency for chloride content to increase with increase in CMT score. The strong positive correlation of blood serum LDH with SCC ($p < 0.01$) was approved by Chagunda *et al.*⁴⁵, Hiss *et al.*⁴⁶ and Guha *et al.*⁴⁷, who reported that LDH is positively correlated with SCC. Kalantari *et al.*⁴² showed that there is a significant positive correlation between LDH in milk and somatic cells and oppositely no significant increase was seen in the activity of

these enzymes in the blood serum of dairy cows with subclinical mastitis compared to the healthy cows.

From the results, study showed a high prevalence of SCM which could be explained by the poor management and udder health practices, inadequate milking procedures, non-use of teat dips and other mastitis control techniques and inadequate screening tests for sub clinical mastitis detection at earlier stages.

In order to establish a specific and efficient management of dairy herd and to avoid the development of clinical mastitis, identification of micro-organisms responsible for subclinical mastitis is significant⁴⁸.

Bacteria that commonly cause mastitis are generally classified into contagious and environmental pathogens depending on the source of the pathogen and mode of transmission. *Streptococcus agalactiae* and *Staphylococcus aureus* are considered as typical contagious pathogens, these pathogens have adapted to survive within the mammary gland and are spread from cow to cow at or around the time of milking. Typical environmental organisms are called environmental streptococci (Streptococci other than *Streptococcus agalactiae*) and the enterobacteriaceae⁴⁹.

The obtained results of Streptococci showed in Table 2 were nearly similar to that obtained by Merl *et al.*⁵⁰, while lower results were determined by Hanan *et al.*⁵¹ and Mpatswenumugabo *et al.*⁵², where as lower findings of Coliforms were reported by Ferguson *et al.*⁵³ and Mbuk *et al.*⁵⁴.

El-Attar *et al.*⁵⁵ considered these micro-organisms as major causative agents of sub clinical mastitis worldwide due to teat-to-teat and cow-to-cow spread, feasibly via milking machines and the milker's hands under the lack of hygiene and this opinion was proven by Radostits *et al.*⁵⁶, who cited that *Streptococcus* spp. was the most prevalent bacteria along with *Staphylococcus* spp.

It is important to identify the presence of *S. agalactiae* in a herd with the appearance of the first infected animal as this pathogen is highly infectious bovine mastitis pathogen that can rapidly spread throughout a herd from a single infected animal⁵⁷. Moreover mastitis caused by *S. agalactiae* is extremely costly, once these bacteria appear usually infect many quarters of the mammary gland causing extensive damage to the alveolar cells and resulting in decrease of milk production^{58,59}.

The results obtained in Table 3 showed the incidence of contamination of the examined positive CMT quarter milk samples with *S. agalactiae* biochemically were in

accordance with that obtained by Bhagat *et al.*⁶⁰, who found that 22% of the isolated strains were *Streptococcus agalactiae*, whereas Lower results were determined by Mpatswenumugabo *et al.*⁵², who found that *Streptococcus agalactiae* were isolated from 5.8% of the examined isolates. The isolated *S. agalactiae* were confirmed by PCR as an accurate and rapid molecular technique in comparison to conventional biochemical test^{61,62}.

The primary source of environmental pathogens is the bedding used to house cattle but contaminated teat dips, intramammary infusions, water used for udder preparation before milking, water ponds or mud holes, skin lesions, teat trauma and flies have all been incriminated as sources of infection^{63,64}. Coliforms are able to produce subclinical infections persist for longer periods of time⁶⁵.

Recently, all species and all serotypes of Klebsiella, Citrobacter, Enterobacter and Serratia are listed by the United States Public Health Service, Department of Health and Human Services bioterrorism list of dangerous biological agents that have the potential to pose a severe threat to public health and safety to animal health, plant health or to animal and plant products⁶⁶.

The *E. coli* is considered an environmental pathogen and one of the most important causes of bovine mastitis, which is mostly observed in the early lactation period and in high-producing cows with low somatic cell counts⁶⁷ and one of the major sources of economic loss in the dairy industry due to reduced milk production, treatment costs, discarded milk and occasional fatal disease⁶⁸.

Results presented in Fig. 5 illustrated the serological identification of the suspected isolates of *E. coli* by slide agglutination test and confirmed by PCR were nearly similar to that reported by Vasquez-Garcia *et al.*²⁸, who isolated *Escherichia coli* with the percentage of 4.5% from subclinical mastitis milk, higher obtaining were recorded by El-Bagory and Zayda⁶⁹, who cited that *E. coli* could be isolated from cows suffered from subclinical mastitis with the percentages of 15 and 2% and Singh *et al.*⁷⁰, who stated that out of 24 isolates, 4 isolates (16.66%) proved to be *E. coli*, while lower results were reported by Mpatswenumugabo *et al.*⁵² who could isolate *Escherichia coli* with the percentage of 1.5%.

Milk collected for human consumption can become contaminated with *E.coli* directly via animal faeces and infected udder or indirectly via contaminated farm and dairy

parlor environments, equipment and workers. Most strains of *E. coli* are harmless commensals, some area because human gastro in test in disease with mild to severe symptoms that may progress to long-term sequelae or fatal outcomes in high-risk individuals. Also *E. coli* was found to be responsible for cases of cystitis, pyelitis, pyelonephritis as well as appendicitis and peritonitis^{71,72}.

Finally subclinically infected udder quarters can develop clinical mastitis with high rate of infection⁷³, cows with subclinical mastitis maintain a reservoir of infection within the dairy herd and increase the potential exposure of uninfected cows to contagious pathogens⁷⁴. Milk and dairy products made from these sub clinical mastitis milk may cause food borne diseases^{75,76}, so early detection of this disease at the sub clinical stage is very important for control and prevention.

CONCLUSION

To reduce the prevalence of SCM which is a hidden problem facing dairy herds proper sanitation and hygiene during milking procedures and management practices of dairy cows, application of screening tests to detect SCM at earlier stages, High correlation between LDH and SCC in the present study advised that LDH activity which is both easy and cost effective with high sensitivity and specificity can substitute SCC for detection of SCM, establishment of correct in vitro anti biogram that were the main prerequisites for implementation of effective treatment of SCM with timely treatment might prevent the occurrence of clinical mastitis and transmission to herd mates with improvement in milk production and heat treatment of raw milk before human consumption.

SIGNIFICANCE STATEMENT

The high incidence of Subclinical Mastitis which is the major hidden problem in the dairy farms can be reduced by many ways, the most important one is the early detection of SCM by using more recent screening methods as the determination of the enzymatic activity of LDH (Lactate Dehydrogenase enzyme) as well as the isolation of the etiological agents of SCM using culture methods.

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