Detection Of Subclinical Bovine Mastitis Using Conventional Indirect Laboratory Methods

By

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Summary

Milk samples were collected from seventeen clinically normal cross breed dairy cows raised in private farms in Diyala districts for the detection of subclinical mastitis. The sixty eight milk samples were subjected to four cow-side indirect mastitis tests; the Arial mastitis test (AMT), White side (WST), chloride test and the pH card test for the detection of subclinical mastitis. Positive reactions were detected in 88%, 85%, 100% and 79% of milk samples using AMT, WST, chloride test and the PH card test, respectively. About 65% of positive scores were similar in the AMT and the WST. The chloride test showed increase in chloride % of all milk samples tested; this test needs a rearrangement to be used as mastitis detector and till then we don't advice its use in this field. 83% of samples giving negative reaction to the AMT and WST were of normal pH, we realized that measuring milk pH is a good subclinical mastitis detector. Through conducting this concise study, we detected high prevalence of subclinical mastitis among dairy cattle raised in small private farms at Diyala province districts; and we realized that the use of the locally prepared AMT, WST and the imported Bovi card test are suitable for early detection of subclinical mastitis in cattle in spite of slight differences in the scores recorded.

Introduction

Mastitis is inflammation of the mammary gland that causes physical and chemical changes in milk and leads to pathological condition of the glandular tissue (1).

It is the most common infectious diseases in dairy industry causing anomic losses for the farmers and dairy industry (2, 3, and 4). Moreover milk from an affected animal may harbor organisms potentially pathogenic for human (5).

Almost any bacteria or fungus that can opportunistically invade tissue can cause mastitis. However, most infections are caused by various species of streptococci, staphylococci, and gram-negative rods, especially lactose-fermenting organisms of enteric origin, commonly termed Coliforms (6).

The ideal means of dealing with mastitis is to prevent it from happening. However, even under the best prevention and control programs, mastitis will occur. Mastitis can generally be classified as clinical and subclinical (7). Diagnosis of clinical mastitis is based on abnormally appearing milk and clinical signs observed on the cow, particularly the udder. However, diagnosis of subclinical mastitis is more problematic, since it is not associated with apparent changes in the udder or milk composition, although microorganism can be isolated by appropriate culture techniques .Compositional change and increased somatic cell count (SCC) in milk usually accompanies subclinical mastitis and can be detected by appropriate tests for early detection of mastitic cows. This is essential to reduce production losses and to enhance prospects of recovery (8, 9).

As a common perception, the inflammation of udder strikingly augments the somatic cell count (SCC) in milk. For this reason, the most reliable index next to bacterial culture examination for the diagnosis of subclinical mastitis (SCM) is by somatic cell count (SCC) (10). Direct somatic cell count using microscopy or automated electronic counters is time and money consuming. However, many cow-sides indirect leukocyte counting methods has been established; they require relatively little training and can be conducted by the farmer and veterinarian. One of the oldest and best known indirect tests is the California mastitis test (CMT, it is a simple cow-side indicator of the somatic cell count of milk. It operates by disrupting the cell membrane of any cells present in the milk sample, allowing the DNA in those cells to react with the test reagent, forming a gel (11). It was developed in 1957 as a modification of the white side test (WST) (12, 13). When the classical CMT reagent is not available; any detergent may be added with bromocresol purple to conduct the test as the Surf field mastitis test (SFMT) (1).

Another indirect cow- side test is the Modified Whiteside Test (MWST), a precursor of the "California mastitis test" and replaced by it; based on the development of viscosity in the milk when sodium hydroxide is added. The original test was described by Whiteside (1939) (14), then it was modified by Murphy and Hanson in 1941 (15)

and by Schalm et al., in 1971 (16).This test depends upon the increased Leukocytes content of milk, it is quick, simple and inexpensive (17).

Increase in the pH of normal cow's milk has long been used as a cow- side test for the detection of mastitis; normal pH lies in the range from 6.4 to 6.8 but may exceed 7.0 in milks with increase in cell counts associated with clinical and subclinical mastitis (18), with the degree of alkalinity depending on the severity of inflammation. Several commercial tests have been devised for detecting the pH of milk as the card test; by impregnating absorbent heavy filter paper with an indicator (17).

Detecting the concentration of chloride ions in a milk sample has long been used as an indirect mastitis detecting method; the chloride test is dependent upon the determination of an abnormal quantity of chloride in the milk. Normal milk contains 0.08-0.14 % chloride (17). Reports on the effect of bovine mastitis on milk composition show consistently lower lactose and higher chloride contents in quarters with mastitis than in those without mastitis (19).

Electrical conductivity test and measurement of N-acetyl-b- D-glucosaminidase (NAG-ase) and lactate dehydrogenase (LDH) are other promising tests for more accurate detection of subclinical mastitis. Other type of diagnosis is more specific and based mainly on isolation and identification of the causative pathogen of mastitis or the immune response (antibodies) such as bacteriological culture (BC) of milk, biochemical tests, Milk Elisa, and polymerase chain reaction (PCR) (20). Recently, a commercially available multiplex real-time PCR technique has been introduced as a faster and highly accurate alternative to BC [21].
Many of these highly advanced techniques are beyond the use in underdeveloped countries, including Iraq.
This study aimed at diagnosing subclinical mastitis in cows raised at different districts of Diyala Province. To accomplish that; simple, mostly locally prepared indirect tests including; Arial mastitis test (AMT), White side test (WST), chloride test and card test for milk pH were applied.

Materials and Methods:

Animals: Seventeen cross breed cows were randomly selected from five private farms in Diala province districts. Cows were subjected to general clinical examination with special focusing on the appearance of the udder; the farmers were asked for any observed clinical problems in the milk or udder of these cows. Only those with apparently normal udders and milk were subjected to this study. Recently parturated cows and those in late lactation period were excluded from the study.

Samples: About five ml of fore milk was aseptically collected from each quarter (Fig.1) according to instructions supplied by Coles, 1986 (17). Samples were kept in the refrigerator (4°C) until delivered to the clinical pathology lab. in cooled boxes (within 8-10 hours post collection).

Fig.1- Collection of samples.

Laboratory tests: All milk samples were subjected to the following tests to detect subclinical mastitis: Arial mastitis test (AMT), Whiteside test (WST), Chloride test (CT) and the Bovi-mastitis test (Card test) for detection of milk pH.

AMT: It was prepared similar to the Surf field mastitis test (SFMT) according to (1) with slight modification. 3% solution was prepared by addition of three grams of commonly used detergent powder (Arial) in 100 ml of warm distilled water; Bromocresol purple at the ratio of 1:10,000 was added to the solution. A four-well plastic paddle is used, one well being for each quarter of the cow to be tested (It was locally constructed from Lab. wastes) (Fig.2).

Fig.2- Paddle used for AMT.

Equal amounts of Arial solution and properly homogenized milk sample were put in the paddle, mixed by rotation swirling and using wooden sticks, looking for changes in the colour and consistency of the mixture within 30-40 seconds. Scores were registered as the California mastitis test (CMT) (22), following table -1 (23).

CMT score	Interpretation	Visible reaction	Total cell count (/ml)
0	Negative	Milk fluid and normal	0-200,000 0-25% neutrophils
T	Trace	Slight precipitating particles, reaction disappears in 10 seconds	150,000-500,000 30-40% neutrophils
1	Weak positive	Distinct precipitation but no gel formation	400,000-1,500,000 40-60% neutrophils
2	Distinct positive	Mixture thickens with a gel formation	800,000-5,000,000 60-70% neutrophils
3	Strong positive	Thick gel, viscosity greatly increased. Strong gel that is cohesive with a convex surface.	>5,000,000 70-80% neutrophils

Table-1. Correlation between CMT result and milk somatic cell count.

Whiteside Test (WST)

The WST was carried out according to Coles, 1986 (17) by mixing 2 drops of 4 percent sodium hydroxide with 5 drops of milk on a dark glass plate, stirring for 20 seconds and noting the degree of coagulation . Results were recorded according to the following table (Table-2) (17).

Score	reaction	No WBC/ml milk
-	Milky opaque mixture.	Under $5x10^5$
±	Milky, fine coagulated particles.	$5x10^5 – 1.5x\ 10^6$
1+	Less opaque mixture, still milky with larger coagulated particles.	$1\text{-}2x\ 10^6$
2++	More watery mixture with coagulated large particles, fine threads and strings on rapid stirring.	➢ $2x\ 10^6$
3+++	Back ground is very watery, whey- like with large masses of coagulated materials with strings and threads	Several millions
4++++	Milk thicken shortly after addition of reagent forming gummy mass and tenacious coagulum	Extremely high WBC count

Table- 2. Reaction of the WST and expected total leukocyte count with each reaction.

The Chloride test was conducted according to Coles, 1986 (17). Briefly, to 5 ml of 0.1341% AgNO3 solution, two drops of 10% K2CrO4 was added. This gives red color. To this mixture, 1 ml of milk was added. The milk containing abnormally high percentage of chloride (> 0.14%), yellow color appeared, indicating a positive sample. Persistent red color (due to formation of silver chromate) indicated a negative sample.

The pH of milk samples were measured using the Bovi Vet. Indicator paper (Fig.3)

Fig. 3- Card test.

(V MED SUPPLY, INC). A couple of drops of milk on the *test* paper recording change in the spot color. Change in colour was correlated with pH according to the manufacturer instructions and as follows; pH 6.6-6.7 pale-green, pH 6.8 moderate green, pH 7.1 green, pH 7.4 dark blue-green.

Results And Discussion:

Subclinical mastitis is important because it is 15 to 40 times more prevalent than its clinical counterpart and usually precedes the clinical form. Moreover, it is of long duration and is difficult to detect. It reduces milk production and adversely affects milk quality (24).

Below; are pictures for reactions of different tests applied in the study.

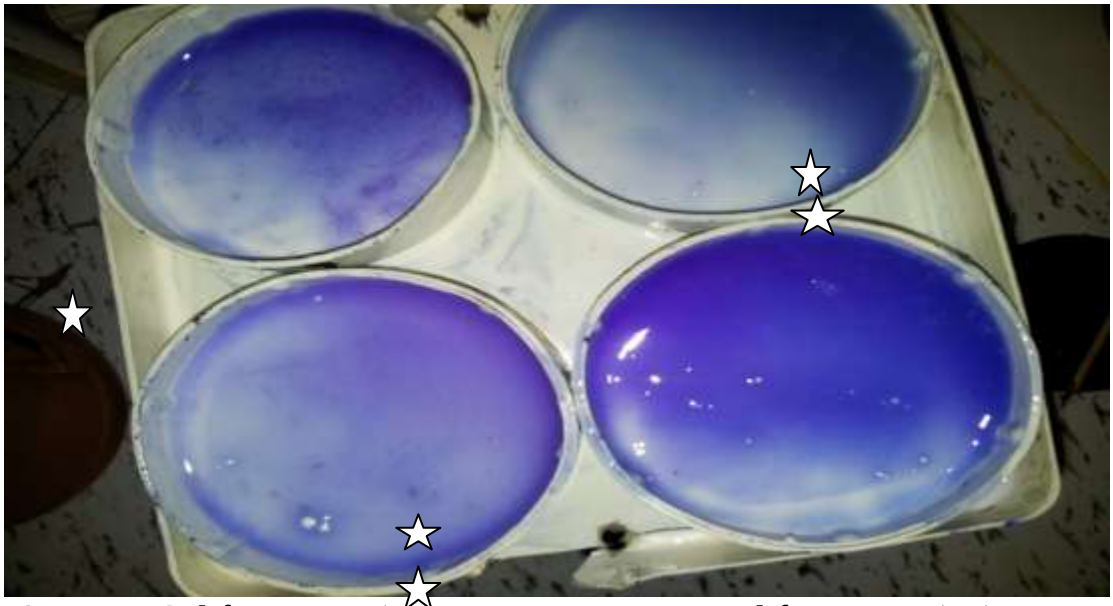

Fig-4- AMT: * Gel formation (+2), ** Precipitate no gel formation (+1).

Fig-5- AMT: *Thick gel formation (+3). ** Negative, no gel or precipitate.

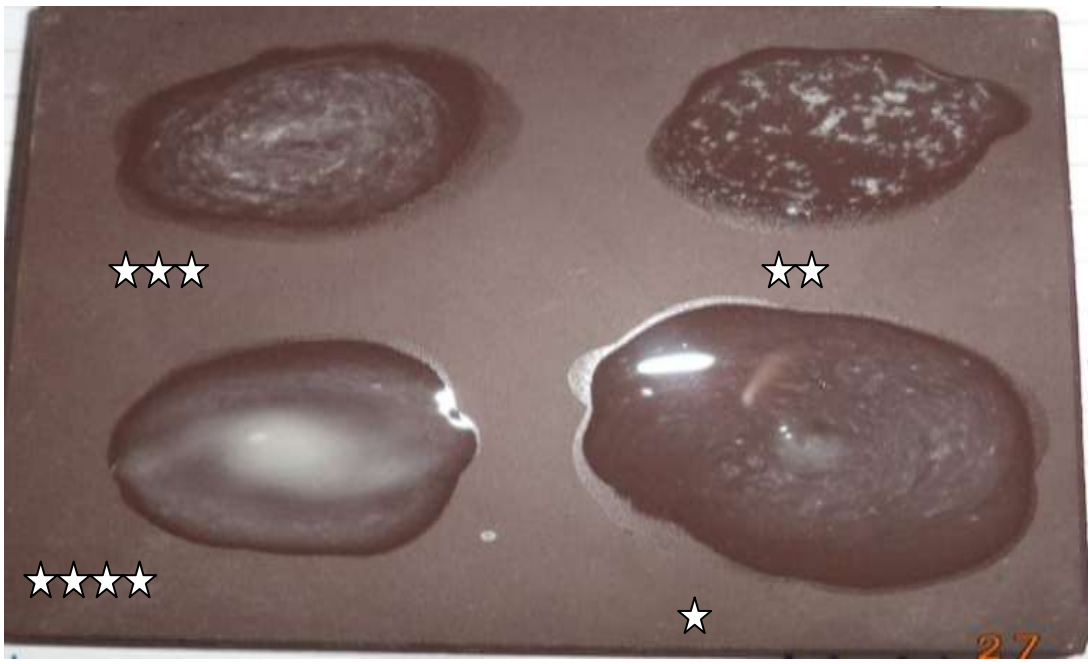

Fig-6- : Different reactions of WST,*+1, **+2, ***+3, ****+4.

Fig- 7- Chloride test: Yellow colour indicating increase in chloride %.

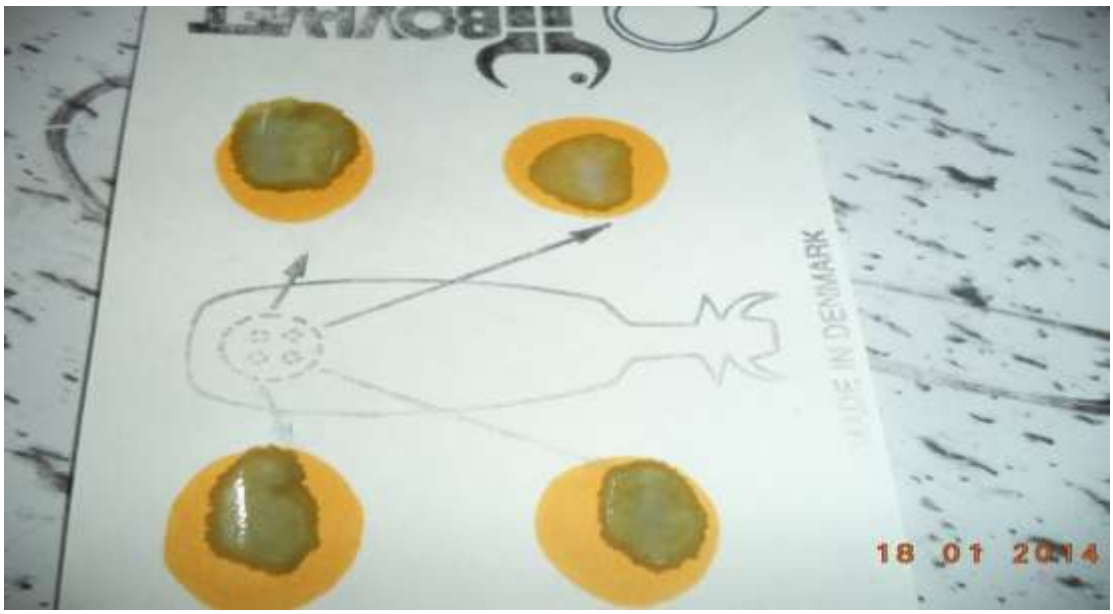

Fig- 8-Card test; all quarters showing slight increase in pH especially the hind left quarter.

Data obtained by applying different test are summarized in table-3.

Case No.	AMT	WST	Chloride test	pH-Card test
1- FL	+2	+2	+	Increased
FR	+2	+3	+	Increased
HL	+2	+1	+	Increased
HR	+2	+1	+	Increased
7- FL	-	-	+	Normal
FR	-	-	+	Normal
HL	+1	+1	+	Increased
HR	+1	+1	+	Increased
8- FL	-	-	+	Normal
FR	-	-	+	Normal
HL	-	-	+	Normal
HR	+1	-	+	Increased
11- FL	+1	+1	+	Increased
FR	+1	+2	+	Increased
HL	-	-	+	Increased
HR	-	-	+	Increased
12- FL	+2	+1	+	Increased
FR	+2	+1	+	Increased
HL	+1	+1	+	Increased
HR	+1	+1	+	Increased
13-- FL	+2	+1	+	Increased
FR	+1	+1	+	Increased
HL	+1	+1	+	Increased
HR	+2	+2	+	Increased
16- FL	+1	+1	+	Normal
FR	-	-	+	Normal
HL	+1	-	+	Normal
HR	+1	-	+	Normal
18- FL	+1	+1	+	Increased
FR	+2	+2	+	Increased

HL	+2	+2	+	Increased
HR	+1	-	+	Increased
19- FL	+1	+1	+	Increased
FR	+3	+3	+	Increased
HL	+1	+1	+	Increased
HR	+3	+4	+	Increased
20- FL	+1	+1	+	Increased
FR	+1	+1	+	Increased
HL	+1	-	+	Increased
HR	+1	-	+	Increased
21- FL	-	-	+	Normal
FR	-	-	+	Normal
HL	-	-	+	Normal
HR	-	-	+	Normal
24- FL	+2	+2	+	Increased
FR	Trace	-	+	Normal
HL	Trace	Trace	+	Increased
HR	Trace	-	+	Increased
25- FL	+1	+1	+	Increased
FR	+1	+1	+	Increased
HL	+1	+1	+	Increased
HR	+1	+1	+	Increased
26- FL	+3	+3	+	Increased
FR	+2	Trace	+	Increased
HL	+2	Trace	+	Increased
HR	+2	+3	+	Increased
27- FL	+2	+3	+	Increased
FR	+2	+3	+	Increased
HL	+1	+2	+	Increased
HR	+1	+2	+	Increased
28- FL	+1	+2	+	Increased
FR	+2	+2	+	Increased
HL	+2	+2	+	Increased
HR	+2	+2	+	Increased
29- FL	+2	+3	+	Increased
FR	+2	+3	+	Increased
HL	+2	+2	+	Increased
HR	+3	+3	+	Increased

Table-3- Results of different laboratory tests applied on milk samples.

From analyzing these data; a complete agreement in the results and scores recorded in the AMT and WST has been detected in 44 milk samples (65%) (Table-3); other results were so close to each other. In Basrah, a study on subclinical mastitis in dairy cattle concluded that CMT gave better results when compared with the Surf mastitis test and WST (25). We found that 83% of samples giving negative reaction to the AMT and WST were of normal pH, we realized that measuring milk pH is a good subclinical mastitis detector.

Total No of milk samples	+ MCT**	+ WST**	+ Chloride test	Increased pH
68	60 (88%)	58 (85%)	68 (100%)	54 (79%)

Table - 4. Number and percentage of milk samples giving positive reaction using the four tests. * AMT: Arial mastitis test. **WST: Whiteside test.

Through observing table No. 4, we can see the close correlation between number of positive reactions with AMT and WST; both tests are known to be used as indirect methods for counting leukocytes in samples of milk in the field and in the laboratory (17). This result runs parallel with that obtained by Harini and Sumathi, 2011 (26), as they detected similar sensitivity of Surf mastitis test (SMT), WST and chloride test. In a study on mastitis in cattle in Sudan; Shallal et al., (27) have isolated large number of important bacterial pathogens from the highly WST positive milk samples, while there was no bacterial growth from WST negative reactors. On the other hand, our result disagrees with that obtained in a Korean study on subclinical mastitis (28) who found that CMT showed better agreement with the direct leukocyte count than did WST. In another study in Pakistan (24); they found a low sensitivity of WST for the diagnosis of subclinical mastitis and they realized that the CMT was the more reliable for detection of subclinical mastitis in this area.

The utility of the Whiteside as a field test was limited by the fact that the reaction was sometimes difficult to observe, and would eventually occur even in normal milk, and the CMT was considered a refined version of the test, with enhanced its sensitivity, and eliminated the confounding effect of milk fat (2). However other studies uncovered stronger positive correlation of both the WST and SFMT with CMT (24).

According to the chloride test; none of the milk samples was of normal chloride concentration, all samples showed increase in the chloride ion. It seems that there is variation in the normal level of chloride ion recorded in normal milk samples; Elango et al., (2010) (29) reported that the normal range of chloride content of healthy animal's milk is 0.08 to 0.14%. While, Sharma et al. (2011) (30) considered that the chloride content of normal milk sample was 0.91%. This may explain the result obtained as the test applied is standardized for normal chloride percentage in the range of 0.08-0.014 (17). This result for the chloride test agreed with that obtained by Guha et al., (10); they considered the chloride test invaluable to diagnose subclinical mastitis in buffaloes in India.

All indirect tests used for the diagnosis of mastitis depend on the compositional changes of milk either because of local effect or because of serum components entering the milk after an increase in vascular permeability of the gland to blood components. Significant changes in ionic composition occur with Na, Cl and bicarbonate levels increasing, K decreasing (with decrease in milk acidity percentage (16). Cows in the first week after calving or in the last stages of lactation were not

included in this study, because they may give strong positive reaction with the previously mentioned, mastitis detecting tests (Radostits et al., 2007).

From this concise study we realized that subclinical mastitis is highly prevalent in cattle raised at various districts of Diyala province. Farmers should be educated and advised through veterinary services about the importance of detecting and treating; apparently normal cows, producing apparently normal milk, affected with subclinical mastitis. To avoid the development of the more severe destructive clinical form of this coasty disease. The use of locally prepared AMT and WST with the imported Card test for milk pH is useful for the detection of subclinical mastitis in the field and in the laboratory.

الخلاصة

جمعت عينات حليب من سبعة عشر بقره حلوب مضربه، طبيعيه سريريا تعود لحقول خاصه في نواحي محافظة ديالى لاجل التحري عن التهاب الضرع تحت السريري. فحصت ثمانيه و ستون من عينات الحليب بفحوصات الايريال مستايتس تيست، الوايت ساید تیست، فحص نسبة الكلورايد و فحص حموضة الحليب لاجل التحري عن وجود التهاب الضرع تحت السريري. وجدت حالات موجبه في 88%،85%،100% و 79% من الحالات باستعمال فحص الايريال مستايتس تيست، الوايت ساید تیست، فحص نسبة الكلورايد و فحص حموضة الحليب، على التوالي. وجد توافق تام بين فحصي الايريال مستايتس تيستو الوايت ساید تیست في 65% من الحالات. و قد اظهر فحص نسبة الكلورايد زياده في نسبة الكلور في كل العينات، يحتاج هذا الفحص الى اعادة تنظيم و الى ان يتم ذلك لا ننصح باستعماله في هذا المضمار. وجدنا ان حوالي 83% من الحالات السالبه بفحصي الايريال مستايتس تيستو الوايت ساید تیست كانت ذات حموضه طبيعيه، ووجدنا ان هذا الفحص جيد للتحري عن التهاب الضرع تحت السريري في الابقار الحلوب. من خلال اجراء هذه الدراسه المختصره وجدنا نسبه عاليه لانتشار مرض التهاب الضرع تحت السريري بين الابقار الحلوب في المنا طق المحيطه بمحافضة ديالى. على الرغم من وجود بعض الاختلافات البسيطه في النتائج فاننا ننصح باستعمال فحصي الايريال مستايتس تيستو الوايت ساید تیست المحضرين محليا وفحص الكارت المستورد لقياس حموظة الحليب في التحري عن الاصابه بالتهاب الضرع تحت السريري.

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References:

1. Shahid, M.; Sabir, N.; Ahmed, I., Wali Khan, R.; Irshad, M; Rizwan, M. and Ahmed, S. (2011). Journal of Agricultural and Biological Science. 6 (11), PP: 18-22.
2. Lightaer,J.K,Miller,GY.,Hueston,W.D.and Dorn ,C.R.(1988).Estimation W. D. and mastitis, using National Animal Health monitoring system and milk somatic calls count data .J. American Vet.Med.Assoooc.,192: 1450-1453 .
3. Pitekala, A. M.; Haveri, S.; Pyorala, M.; Myllys and T Hankanen – Buzalski (2004). Bovine mastitis in Finland 2001 Prevalence, distribution of bacteria and antimicrobial resistance .Dairy sci., 87,pp: 2433-2441.

4. Pyralas and S Tapnoen (2009).Coagulase –negative staphylococci emerging mastitis pathogens. Vet Microbial J.134, pp: 3-8.
5. Barbano.D.M., (1989).Impact of mastitis on dairy product quality and yield Research update .28th Annual meeting national mastitis Council, inc Tampa, Florida .USA.
6. Aiello, S.E. (2010). The Merck's Veterinary Manual. 10th.ed.
7. Watts, J.L.; (1990). Bovine mastitis. In Diagnostic procedures in Veterinary Bacteriology and Mycology, By: Carter, G.; Cole, J. R. Jr. 5th ed.Academic press, Inc., Sandiego. California.
8. Holdaway, R.J.; Holmes, C.W. and Steffert, L.J. (1996). A comparison of indirect methods for diagnosis of subclinical intramammary infection in lactating dairy cows. Aust .J. Diary Tech. 51, pp: 64-71.
9. International Dairy Federation, A monograph of bovine mastitis, 1971.
10. 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-Austral. J. Anim. Sci. 25, pp: 353-360.
11. White, D.; Walmsley, M.; Liew, A.; Claycomb, R. and Mein, G. (2005). Chemical and rheological aspects of gel formation in the California Mastitis Test", Journal of Dairy Research, 72, PP: 115-121.
12. Schalm, O.W. and Noorlander, D.O. (1957) Experiments and observations leading to development of the California mastitis test. J. Am. Vet. Med. Assoc. 130, 199–204
13. Radostits, O.M.; Gay, C.C; Hinchcliff, K.W. and Constable, P.D. (2007). Veterinary Medicine. 10th. ed. Saunders Elsevier- Spain. Pp: 673-728.
14. Whiteside W. H. (1939). Observation on a new test for the presence of mastitis in milk. Canad. Publ. healt. J. 30, p: 40.
15. Murphy, J. M. and Hanson, J. J. (1941). A modified Whiteside test for the detection of chronic bovine mastitis. Cornell Vet., 31, p: 47
16. Schalm O. W., Carrol J. E. and Jain N. C. (1971). Bovine Mastitis. 1st ed. Lea and Febiger, Philadelphia, USA. pp. 132-153.
17. Coles, E. H. (1986). Veterinary Clinical Pathology. 4Th. ed. W.B. Saunders Co. Philadelphia.
18. Marschke R. J. and Kitchen B. J. (1985). Detection of Bovine Mastitis by Bromothymol Blue pH Indicator Test. J. Dairy Sci. 68, pp: 1263-1269.
19. Nangwala, S.W. (1996). Effect of mastitis on quarter milk yield response to improved feeding. Unpublished M. Sc. Dissertation, Sokoine University of Agriculture.
20. Mahmmod, Y. (2013). The Future of PCR Technologies in Diagnosis of Bovine Mastitis Pathogens. Adv Dairy Res, 2, pp:1
21. Koskinen, M.T.; Holopainen, J.; Pyorala, S.; Bredbacka, P.; Pitkala, A.; et al., (2009). Analytical specificity and sensitivity of a real-time PCR assay for identification of bovine mastitis pathogens. J Dairy Sci 92, pp: 952-959.

22. Muhammad. G.; Athar, M.; Shakoor, A.; Khan, M. Z.; Fazal-ur-Rehman and Ahmed, M. T. (1995). Surf field mastitis test: An inexpensive new tool for evaluation of wholesomeness of fresh milk. J. Food. Sci. 5,pp: 91-93
23. Jasper, D.E. 1967 Proceedings of National Mastitis Council
24. Iqbal, M.; Khan, M. A.; Daraz, B. and Siddique, U. (2004): Bacteriology of mastitic milk and in vitro anti bio gram of the isolates. Pakistan Vet. J., 24(4),pp: 161-164.
25. AL-Edany,A. A.; Khudor. M. H. and AL-Mousawi, A. S. (2012). Comparison of three indirect tests for the diagnosis of bovine subclinical mastitis caused by coagulase negative staphylococci with their susceptibility to seven antibiotics. Bas.J.Vet.Res..11(1), pp: 74-83.
26. Harini, H. and Sumathi, B.R (2011). Screening of bovine milk samples for sub-clinical mastitis and antibiogram of bacterial isolates. Veterinary World, 2011,.4(8), pp:358-359.
27. Shallali, M.E.; Salwa, M.E.; Dirdiri, N.I.; Harbi, M.S.M.A. and Shamat, A.M. (1983). A preliminary survey of mastitis and brucellosis in some dairy farms in the Blue Nile Province. Sudan J.Vet. 4, pp: 37-40.
28. Cho, D.Y.; Cheong, C.K. (1970). Comparison of the Modified White Test and the California Mastitis Test for the detection of bovine mastitis. Korean journal of Veterinary Research. 10, (1), pp: 47-51.
29. Elango, A.; Doraisamy, K. A.; Rajarajan, G. and Kumaresan, G. (2010). Bacteriology of sub clinical mastitis and anti bio gram of isolates recovered from cross bred cows. Indian J. Anim. Res., 44 (4), pp: 280 – 284.
30. Sharma, N.; Singh, N. K. and Bhadwal1, M. S. (2011). Relationship of Somatic Cell Count and Mastitis: An Overview. Asian-Aust. J. Anim. Sci., 24(3), pp: 429-438.