Biochemical study of DNA markers for Bacterial infection in bovine mastitis

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ABSTRACT

Mastitis is a multifactorial disease and very difficult to control. It results from injury, chemical irritation and infection caused by different bacterial species**.** Mastitis remains one of the most common economic problems of dairy industry worldwide as it is the most expensive disease of dairy animals resulting in the reduction of milk production and quality. These expenses in terms of reduction of production, discarding milk, drug therapy, veterinarian charges, culling of incurable animals and extrause of labor. Analysis of bacteriological examination of milk samples was made to identify the main etiological agents involved in the disease. The organisms were identified on the basis of their cultural, staining characteristics, biochemical reactions .and molecular detection. Milk sample of 23 cows, which were positive for California mastitis test, cultured for microbiological examination in the study period. Two bacterial species were isolated, Staphylococcus aureus (Staph. aureus) and *E. coli* bacterial isolates. The predominant isolated bacteria were *Staph. aureus* with isolation rate of 37.77% however *E. coli* was isolated with isolation rate of 13.33%). Serum alkaline phosphatase (ALP) enzyme and calcium levels were highly significant decreased while C-Reactive protein (CRP) titre and phosphorus levels were highly significant increased. Lactate dehydrogenase enzyme(LDH), Aspartate aminotransferase enzyme(AST), Gamma-glutamyl transferase enzyme(GGT), Albumin, sodium, potassium and chloride levels were non-significantly changed in serum of mastitic cows compared to healthy ones. While LDH, ALP and phosphorus levels were highly significant increase in milk of mastitic cows compared to that of healthy ones. However, the calcium level was high significantly decreased in mastitic cows compared to healthy ones. Molecular detection of *Staph. aureus* and *E. coli* isolates by PCR. The expected sizes of PCR products of *Staph. aureus* was (984bp), while that for *E. coli* were (662bp).

Key Words: Bovine mastitis, bacteriological examination, LDH, ALP enzymes, PCR.

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1. INTRODUCTION

Mastitis is a multifactorial disease, results from injury, chemical irritation and infection caused by different bacterial species. Mastitis is most expensive disease of dairy animals resulting in the reduction of milk production and quality. These expenses in terms of reduction of production, discarding milk, drug therapy, veterinarian charges, premature culling, and extra use of labor (Anonymous, 1998). Bovine mastitis is the inflammation of the parenchyma cells of the mammary glands of cattle, buffalo and other animals (Radostit et al., 1996) associated with microbial infections (Schroeder, 1997) and physiological changes (Shouky and Shabana, 1997). Mastitis is caused by a group of infective and potentially pathogenic bacteria (Bezek and Hull, 1995), viruses (Wallenberg et al., 2003), fungi and algae (Radostit et al., 1996). Mastitis is mainly economical and the most evident costs are reduced milk yield, veterinary costs and the disposal of milk (Kossaibati and Esslemont, 2007). Examples of more indirect costs are reduced fertility, increased work load for the farmer and reduced quality of milk that aggravate the making of cheese and yogurt (Kossaibati and Esslemont, 2007). The bacterial agents responsible to cause inflammation of udder are classified as either contagious or environmental, based upon their primary reservoir and mode of transmission. *Staphylococcus aureus and Streptococcus dysaglactiae* are recognized as contagious bacterial species, commonly transmitted among dairy animals through contact with infected milk. The pathogens reside in environment are of 2 types, one is *Coliforms (Escherichia coli, Klebsiella*) and other is Streptococcal species other than *Streptococcus dysgalactiae* entering into the udder between milkings, when teats are exposed to mud, manure, and dirty bedding materials (Anonymous, 1998). For years, the use of different enzymes in

milk as biomarkers to identify mastitis has attracted attention and it has been shown that measuring enzyme activities in milk has a diagnostic potential for detection of mastitis. The concentrations of some milk enzymes such as lactate dehydrogenase and alkaline phosphatase increase during inflammation of mammary glands and the enzymes have the potential to be used as a screening test for detection of subclinical mastitis. (Babaei H et al., 2007; Batavani et al., 2007; Ibtisam El Zubeir et al., 2005).

The aim of the present study: Estimation the biochemical parameter changes in both serum and milk samples which can be used as early and less expensive diagnostic tools and prognostic markers in mastitic cases. Using PCR "Polymerase Chain Reaction" technique for confirmation of pathogens causing mastitis from isolated colonies using specific primers. Early diagnosis of mastitis is essential for reduction of production losses and for enhancing the prospects of recovery. Also, the identification of sub clinically infected gland is urgently required for successful control of mastitis in dairy animals (Ahmed et al., 2008).

2. MATERIALS AND METHODS

2.1. Animals:

The current work was carried out on 45 dairy cattle of ages ranged from 3-7 years obtained from some private farms of dairy cattle in Kalyobia Governorate during the period from 2014-2015.

2.2. Samples:

2.2.1. Blood samples

Blood samples were collected from the clinically suspected animals in plastic test tubes without anticoagulant and serum separated by centrifugation for 10 minutes then was transported in very sterilized container for biochemical examination such as serum albumin, calcium concentration in serum and milk, CRP titre in serum, ALP in serum and milk,, LDH in serum and milk**,** Phosphorus level in serum and milk, serum Gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), serum Potassium and sodium level and serum chloride level.

2.2.2. Milk Samples

A total of 45 milk samples (50 ml each) collected aseptically in sterile McCarteney bottles were positive to CMT and used in this study. Each sample was divided into two parts, each part put in a sterile McCartney bottles. One was incubated at 24 hrs for bacteriological examination, the second part for detection of biochemical analysis.

2.3. Polymerase chain reaction (PCR) for identification of Staph. aureus:

Amplify the isolated DNA samples using taq green PCR master mix kit following the manufacturer protocol (Promega) and a Biorad thermocycler (Biorad, USA). PCR reaction kit (Taq Green PCR master mix, Promega).

Procedure: Two pairs of PCR primers were used based on the published nucleotide sequence information of the *Staphylococcus aureus genes* (GenBank accession *S. aureusX68425*). Forward and reverse primers sequence for *Staphylococcus aureus genes*, size of PCR amplicon (984 bp) are: Forward primer is 5` AGCGAGTCTGAATAGGGCGTTT 3`. Reverse primer is 5` CCCATCACAGCTCAGCCTTAAC 3`

2.4. Polymerase chain reaction (PCR) for identification of E. coli:

Procedure: The 16S and 23S rRNA gene were screened by uniplex PCR assay using *E. coli* specific primers; Eo 2083 (forward) 5'-GCTTGACACTGAACATTGAG-3' and Eco 2745 (reverse) 5'-GCACTTATCTCTTCCGCATT-3'. To ensure primer sequence is unique for the template sequence; we checked similarity to other known sequences with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Primers were dissolved in nuclease-free water to obtain 50 – 100 ml concentration.

2.5. California Mastitis test:

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

2.6. Bacteriological Examination:

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

2.7. Statistical analysis:

The Statistical analysis was carried out using ANOVA with two factors under significance level of 0.05 for the whole results using SPSS (ver. 22). Data were treated as complete randomization design according to (Steel et al., 1997). Multiple comparisons were carried out applying LSD.

3. RESULTS

3.1. Results of bacterial isolation from milk samples:

Analysis of bacteriological examination of milk samples was made to identify the main etiological agents involved in the disease. The organisms were identified on the basis of their cultural, staining characteristics and biochemical reactions. Milk sample of 23 cows, which were positive for CMT, cultured for microbiological examination in the study period. Two bacterial species are isolated, Staphylococcus aureus (Staph. aureus) and *E. coli* bacterial isolates. The bacterial isolation rate was shown on (Table. 1). The predominant isolated bacteria were *Staph. aureus* with isolation rate of 37.77% but *E. coli* was isolated with isolation rate of 13.33%).

3.2. Biochemical analysis of enzymes and minerals in serum and milk:

Table (2) showed that ALP enzyme activity and calcium levels were high significantly decreased while CRP titre and phosphorus were high significantly increased. LDH, AST, GGT, Albumin, sodium, potassium and chloride levels were non-significantly changed in mastitic cows compared to healthy ones. Table (3) showed that LDH, ALP enzyme activities and phosphorus levels were high significantly increased in milk of mastitic cows. However, the calcium level was high significantly decreased in milk of mastitic cows compared to healthy ones.

3.3. Molecular detection of Staph aureus and E. coli isolates:

Table (1): Bacterial species isolated during the study period

Bacterial species	Total number of isolates	% of isolates
Staph. aureus	17	37.77%
E. coli	6	13.33%
total	23	51.1%

Table (2): Changes in various serum enzymes (U/L) and minerals (mg/dl) (Mean±SD) of mastitic and healthy animal groups.

Parameter	Healthy animal (control group)	Mastitic animal	
		Staph. aureus positive group	*E. coli* positive group
LDH(U/L)	1176.18±67.72	1182.06±87.75	1027.5±99.69
CRP(mg/L)	0.28±0.05	0.82±0.05***	0.60±0.15**
ALP(U/L)	44.59±5.2	39.52±4.19**	41.65±11.7*
AST(U/L)	92.67±7.83	86.82±10.75	114.17±12.77
GGT(U/L)	15.20±0.64	15.61±0.89	13.03±1.93
Albumin(g/dl)	4.22±0.09	4.09±0.06	4.01±0.12
Calcium (mg/dl)	9.73±0.09	9.35±0.10*	9.17±0.21*
Phosphorus (mg/dl)	5.27±0.26	5.54±0.30*	5.92±0.65*
Sodium (mmol/L)	139.49±0.72	138.72±3.03	140.98±2.76
Potassium (mmol/L)	4.48±0.16	5.03±0.20	5.04±0.31
Chloride (mmol/L)	103.25±2.22	108.44±2.63	103.45±1.49

Table (3): Changes in various milk enzymes (U/L) and minerals (mg/dl). (Mean±SD) of mastitic and healthy animal groups.

Parameter	Healthy animal	Mastitic animal	
		Staph. aureus positive group	*E. coli* positive group
LDH	147.18±8.27	1465.88±156.59***	1416.75±265.26***
ALP	121.81±13.46	1032.06±58.32***	718±82.98***
Calcium	91.02±0.69	77.17±0.42***	77.83±1.18***
Phosphorus	38.15±3.49	45.28±3.04**	44.5±9.37**

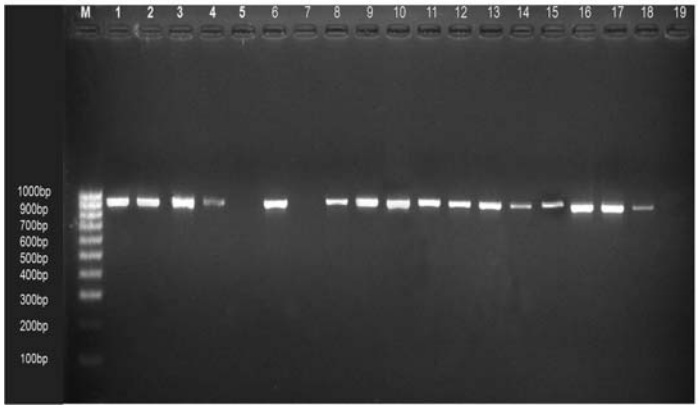

Fig.(1): Ethidium bromide stained 2 % agarose gel of PCR products showed S. aureus +ve samples of 984bp PCR products from bacterial culture from milk samples (lanes 1-4, 6, 8-17), –ve samples (lanes 5and 7), +ve control (lane 18) and –ve control (lane 19). M, represents a 100bp DNA ladder as a size standard.

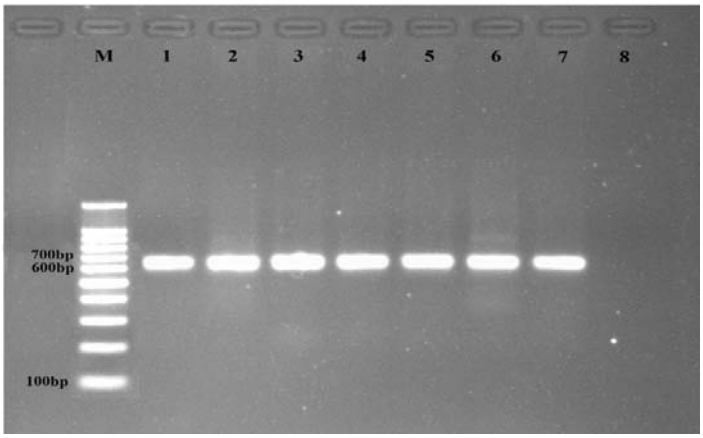

Fig. (2): Ethidium bromide stained gel electrophoresis shows the size of the PCR products of *E. coli* 16S and 23S rRNA genes (lanes 1-6); lane7, positive control; lane 8, negative control; M, 100bp marker (ladder).

3.3.1. Detection of Staph aureus isolates by polymerase chain reaction (PCR):

Two pairs of PCR primers were used based on the published nucleotide sequence information of e *Staph. aureus* genes (GenBank accession S. aureus X68425). Presence of the expected sizes (984 bp) of PCR products was regarded as a positive result for the existence of *Staph. aureus* (Fig. 1).

3.3.2. Detection of E. coli isolates by (PCR):

The extracted DNA was used as a template for PCR to amplify the 16S and 23S rRNA gene. The size of the gel electrophoresis bands was compared

with the bands of the DNA molecular marker. Presence of the expected sizes (662bp) of 16S and 23S rRNA was regarded as a positive result for the existence of *E. coli* (Fig. 2)

4. DISCUSSION

The bacterial agents responsible to cause inflammation of udder are classified as either contagious or environmental, based upon their primary reservoir and mode of transmission. The predominant isolated bacteria were *Staph. aureus* with isolation rate of 37.77% but *E. coli* was isolated with isolation rate of 13.33%). This result was similar to that reported by Mohamed et al. (2013) who found that the incidence of subclinical mastitis in buffaloes depending on the bacterial cultivation was the highest in S. aureus (31.1%), *E. coli* infection (13.9%), followed by, Enterococcus spp. (8.9%), *S. dysgalactiae* (5.6%) and *S. agalactiae* (4.4%) and Klebsiella spp. (2.2%). This also comes in agreement with El-Khodery and Osman (2008) who reported that the bacteriological examination of buffalo's milk samples with acute mastitis revealed that coliform bacteria was the most common pathogen followed by *S. aureus* then *S. uberis*, and *S. agalactiae*. This also the results came to some extent with Ahmed et al. (2008) who reported high incidence of bacteria isolated from milk samples of Egyptian buffalo-cows suffering from sub-clinical mastitis where the most prevalent isolates were *E. coli* (94.99%), *S. epidermidis* (78.33%), C. bovis (55%), *Klebsiella spp*. (51.67%), *S. uberis* (46/67%), *S. aureus* (33.33%) and *S. agalactiae* (31.67%).

Regarding the biochemical analysis of serum enzymes and minerals table (2) shows that ALP enzyme activity was high significantly decreased while CRP enzyme activity was high significantly increased in mastitic cows compared with healthy ones. Calcium level was significantly decreased while phosphorus level was significantly increased in infected cows compared to healthy ones. However, the LDH, AST, GGT, Albumin, sodium, potassium and chloride levels were non-significantly changed in mastitic cows compared to healthy ones.

Alkaline phosphatase level in serum of mastitic cows was high significantly decreased comparing with healthy ones, this result agrees with Babaei H et al. (2007) who reported that, since the blood-milk barrier is damaged, so it is also possible that the blood ALP may be transferred to milk. Contrarily, this result disagrees with Matei et al. (2010) who reported that ALP activity increased in cows diagnosed with subclinical mastitis. There are many experimental studies that indicate an increase of serum alkaline phosphatase from mastitis cows, which may suggest that this enzyme plays a role in the pathogenesis of the disease (Vangroenweghe, 2004).

Serum CRP titre was high significantly increased. This result was similar to Tsenkova et al. (2001) who reported that, increased proteins in the blood of mastitic cows indicate an activation of immune response following infection of the mammary gland. These proteins are mainly serum immunoglobulins that are implicated in udder defense mechanisms. Also Pandey et al. (2005) mentioned that, immunoglobulin plays an important role in host immunity and inflammation, moreover a correlation between total serum protein and somatic cells count in milk was recorded.

The LDH activity in the serum samples was non-significantly changed in mastitic cows compared to healthy ones. This result agrees with that reported by Shahabeddin et al. (2013) who indicate that serum LDH, AST and GGT, had a low clinical accuracy for detection of subclinical mastitis and therefore, could not be used as a reliable marker for study of udder inflammation.

In the present study, serum calcium level was significantly decreased in mastitic cows compared to healthy ones. This result agrees with Bogin and Ziv (1973) and Coulon et al. (2002) who reported that calcium was reduced during intramammary infections. While, this result disagrees with (Doornenbal et al., 1988) who reported that Calcium level found in infected cows was 12.46±4.00mg/dl and 11.54±1.87mg/dl in non-infected cows. Lower percentages in lactating cows may be due to calcium losses during milk production.

Regarding the phosphorus level in serum of mastitic cows was significantly increased compared to the healthy ones. This result agrees with (Dwivedi et al., 2004) who reported that There was an increase in phosphorus level in infected cows 5.78±0.98 mg/dl whereas the normal cows having 4.98±0.53 mg/dl. The variation in phosphorus of the cow of two group was statistically significant (P<0.05) which could be attributed to its more secretion in milk, due to injury to the udder wall, thus more loss in milk. While this result disagrees with Bogin and Ziv (1973) and Coulon et al. (2002) who reported that phosphorus was decreased during inframammary inflammation.

Although CMT is a reliable, easy, rapid and cheap tool helping in diagnosis and still the gold-standard screening test for high somatic cell count(SCC) as it directs attention to individual mammary quarter that is secreting milk of high

(SCC) (Abdel-Rady and Sayed, 2009; Leslie et al., 2002). It is not suitable in early lactation so the measurement of enzyme activity appears to be suitable diagnostic method for identifying subclinical mastitis (SCM) in early lactation or in dry period (Babaei H et al., 2007).

Regarding the biochemical analysis of milk enzymes and minerals Analysis of data revealed that, LDH, ALP enzyme activities were high significantly increased while calcium levels were high significantly decreased in milk. However, the phosphorus level was high significantly increased in mastitic cows compared to healthy ones Table (3).

The increase in milk enzymes including lactate dehydrogenase and alkaline phosphatase in mastitic animals may be linked with tissue damage occurring in mammary tissue and is very much an expected change (Batavani et al., 2003). These results were similar to that of Babaei H et al. (2007) and Ibrahim et al. (2011) who recorded such changes in ewes and cattle.

The increased levels of various enzymes in milk occur mainly due to increased permeability of microcirculatory vessels in inflamed areas along with leakage from degenerated/necrotic parenchymal cells and leukocytes (Hussain et al., 2012). Also Chagunda et al. (2005) developed a statistical model for the detection of mastitis based on LDH activity. Sensitivity and specificity were 76.5 and 97.7%, respectively for diagnosing clinical mastitis. The higher level of LDH in mastitic milk than blood serum showed that blood serum was not the sole source of this enzyme in mastitic milk and it was probably liberated from disintegrated leukocytes and the parenchymal cells of the udder (Kato et al., 1989).

The mean LDH activity was significantly higher in milk from inflamed SCM quarters than in normal one; no significant difference was in blood enzyme values (Table 2 & 3). The origin of LDH in mastitic milk is attributed to leucocytes (Kato et al., 1989) and also epithelial cells from the udder (Zank and Schlatterer, 1998).

Bogin and Ziv (1973) have suggested that LDH in milk was a sensitive indicator of epithelial cell damage and subsequently proposed that LDH originated mainly from the damaged udder epithelial cells and from the elevated numbers of leucocytes. In the context of milk lactate being a potential diagnostic measurement for mastitis, Mayer et al. (1988) reported that milk oxygen concentrations were reduced during mastitis suggesting that, given a favorable supply of substrates, the metabolism of somatic cells in milk and/or the mammary epithelium may become anaerobic leading to release of lactate into milk (Davis et al., 2004).

Mackie et al. (1977) found a relatively slow but substantial increase in lactate in milk during mammary involution with concentrations reaching approximately 5 mM, 5 days after the last milking. Kato et al. (1989) reported highest LDH activity in milk samples infected with S. aureus. Lower LDH activities were found in samples being positive for C. bovis and CNS. Higher LDH activity in milk serum of inflamed udders has been previously reported in cows (Grun et al., 1992; Kovac and Beseda, 1975) and sheep (Batavani et al., 2003; Nizamliglu and Erganis, 1991).

Regarding the calcium levels were high significantly decreased in milk of mastitic cows compared to healthy ones Table (3). These results agree with Ahmad T et al. (2007) and Hussain et al. (2012) who reported that, there was a decrease in the level of calcium. However, these results disagree with Zannatul et al. (2015) who reported that**, c**alcium level increased in infected cows compared to non-infected cows. Lower percentages in lactating cows may be due to calcium losses during milk production. In older animal, there was a decreased need for calcium and phosphorus for this purpose and this was why lower calcium level in blood levels of cows (Doornenbal et al., 1988).

Regarding the phosphorus level was high significantly increased in mastitic cows compared to healthy ones. These results agree with the observation of Dwivedi et al. (2004) and Zannatul et al., (2015) who reported that, there was an increase in phosphorus level in infected cows which could be attributed to its more secretion in milk, due to injury to the udder wall, thus more loss in milk. However, these results disagree with Ahmed et al., (2007; and Hussain et al., (2012) who reported that, there was a decrease phosphorus in milk samples from mastitic animals.

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