Bacteriological studies on mastitic milk in cattle with detection the clinicopathological changes

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Abstract
In the present study, milk and blood samples were collected from mastitic and healthy cows at 5-7 years age from different dairy farms in Ismailia. Milk samples were subjected to bacteriological examination and blood samples to hematological and biochemical examination. Bacteriological examination revealed that, *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa,* coagulase negative *staphylococcus spp*. and Citrobacter species were isolated with the percentage of 29/90 (32.22%), 32/90 (35.56), 20/90 (22.22%), 8/90 (8.89%), 11/90 (12.22%) and 13/90 (14.44%) respectively. Antibacterial susceptibility testing showed that, all isolated bacteria were sensitive to Ceftiofur, Ciprofloxacin and Enrofloxacin, while most of them were resistant to penicillin and amoxicillin. Results of blood analysis showed that, anemia and a significant increase in liver and kidney functions while a notable decrease in total protein, albumin, calcium, zinc and iron with compared to healthy cattle. It is concluded that mastitis may lead to anemia and disturbance in liver and kidney function so application of California mastitis test helpful for detection of infected animals early and treatment.

Key words:
Mastitic, Bacteriological, Hematological and Biochemical.

Introduction

Mastitis is an inflammatory disease condition of udder affecting milk production negatively and having a serious impact on the economy of dairy enterprises. It is considered to be the most costly disease of dairy animals and losses mainly occur through discarded milk, reduction in milk yield, premature culling of animals and replacements **(Halasa *et al*., 2007; Radostits *et al*., 2007 and Hamadani *et al*., 2013).** Bovine mastitis is one of the most problematic diseases and continues to have a major economic impact on the dairy industry throughout the world **(Bachaya *et al*., 2011).** Clinical mastitis during early lactation, markedly and negatively influence the reproductive performance of dairy cows **(Ahmadzadeh *et al.*, 2009 and Yang *et al.,* 2012).** It characterized by physical, chemical and bacteriological changes in milk and pathological changes in glandular tissues **(Ranjan *et al*., 2010).**

The causative organisms of mastitis are categorized as contagious pathogens including *Staphylococcus aureus, Streptococcus agalactiae, and Mycoplasma bovis* or as environmental pathogens such as environmental streptococcus and the enterobacteriaceae **(Hogan and Smith, 1987; Watts, 1988 and Bramley, 1992).** Poor hygienic conditions can lead to *E.coli* mastitis as the udder is infected through teat canal **(Sumathi *et al.*, 2008).** Teat skin opportunistic pathogens (Bacterial pathogens that normally reside on the teat skin) have the ability to create an intramammary infection via ascending infection through the streak canal. Coagulase - negative *Staphylococci* are the most common teat skin opportunistic mastitis pathogens **(Park *et al*., 2012 and Hamadani *et al*., 2013).**

The pattern of mastitis occurrence is signifi-

cantly increasing in (Ca, Na, K, Mg, P, Fe, Zn and Cu) and of enzymes (LDH, GOT and ALP) in both cattle and buffaloes which is a major challenge for field veterinarians and researchers **(Awale *et al.*, 2012).**

This study is designed to determine the prevalence, bacterial causative agents and antimicrobial susceptibility of clinical mastitis in cattle as well as hematological and biochemical alteration in blood of cattle.

Material and methods

Blood and milk samples were taken from cows diagnosed with clinical mastitis and from healthy cows. 100 milk samples of cows from clinical mastitis (90) and healthy (10) cases at 5-7 years age, were received from different dairy farms and animal growers living in and around Ismailia. The samples were cultured on nutrient agar, blood agar and MacConkey agar plates, supporting the growth of various types of bacteria for their study and isolation. The plates then incubated at 37°C for 24-48 hours. The isolated bacteria were identified on the basis of their cultural and morphological characteristics for growth of colonies. Colonies were selected and examined for morphological appearance (colony shape and haemolytic characteristics), gram staining and catalase test. Gram staining and catalase positive cocci were further tested for coagulase and mannitol fermentation as per standard procedure **(Quinn *et al.*, 1994)** to confirm as *staphylococci*. The pure cultures of gram negative bacterial isolates were obtained by subculturing on differential and selective media. The bacterial isolates further subjected to biochemical tests for confirmation (Sugar fermentation, Indole, Methyl Red, Voges-Proskauer, Hydrogen Sulphide, Citrate and Catalase). The 32 E. coli isolates were serotyped based on their somatic (O) antigens (Pro Lab diagnostic co.) according to **(Edwards and Ewing, 1972)** at Animal health research institute, Dokki, Egypt.

Antimicrobial sensitivity test of the isolated bacteria were done on Muller–Hinton medium (Oxoid) using different chemotherapeutic sensitivity discs (Oxoid) namely Ceftiofur, Ciprofloxacin, Enrofloxacin, Gentamycin, Doxycycline, Ampicillin, Chloramphenicol, Erythromycin, Streptomycin, sulphamethaxazole/ trimethoprim, Amoxicillin and penicillin.

20 Blood samples were collected by puncture of the jugular vein, with the addition of heparin as an anticoagulant. The serum blood was separated by centrifugation at 1500 g for 10 minutes and stored at -20ºC for a maximum of 60 days until assayed according to metabolic profile **(Payne *et al*., 1970).**

Hematological studies: Red blood corpuscles (RBCs 10^6 /µl), hemoglobin concentration (Hb, conc.), packed cell volume (PCV %), and Total leucocytic count (W B Cs 10^3 /µl) were estimated **(Jain, 2000).**

Biochemical serum analysis**:** The concentrations of calcium, copper, magnesium, iron, zinc, urea, creatinine, total protein, albumin and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl-transferase (GGT), and alkaline phosphatase (ALP), were all assayed by an automatic analyser Hitachi 912 in hospital Suez Canal University. Enzyme assays were done at 37ºC, and alkaline phosphatase activity was measured at pH 10.5.

Immunological studies: Protein electrophoresis was done using SDS- Polyacrylamide gel electrophoresis according to **(Laemmli, 1970)** in animal health research institute in biochemistry department.

Statistical analysis: The data were statistically analyzed **(Snedecor & Cochran, 1982).**

Results

Out of 90 mastitic milk samples of cows, 113 isolates were obtained from clinical cases of mastitis in and around Ismailia. Major bacterial isolates were *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa,* coagulase negative *staphylococcus spp*. and *Citrobacter* species was 29/90 (32.22%), 32/90 (35.56), 20/90 (22.22%), 8/90 (8.89%), 11/90 (12.22%) and 13/90 (14.44%) respectively(table,1). Twenty from the 32 *E coli* isolates were identified and belonged to different serotypes, namely O114, O55, O78,

O125 and O157 with frequencies of 5, 4, 4, 3 and 4 respectively. However, the remaining 12 *E coli* isolates were untypable.

Antibacterial susceptibility testing (table, 2) showed that all isolated bacteria were sensitive to Ceftiofur, Ciprofloxacin and Enrofloxacin. While most of them were resistant to penicillin and amoxicillin.

Hematological findings of apparent healthy and mastitic cows were reported in Table (3) and biochemical parameters of apparent healthy and mastitic cows in farms were reported in table (4), the data in table 3&4 revealed that a significant decrease in RBCs count, Hb conc, and PCV value in matitic cows as compared with apparently healthy cows. There was leucocytosis, lymphocytosis, monocytosis, and neutropenia, as well as a significant increase in AST, ALT, GGT, ALP, urea, creatinine and copper. Hypoproteineamia, hypoalbumineamia, and significant increase in gamma globulins in addition to significant decrease in serum calcium, zinc, iron.

Table 1. Percentage of distribution and frequency of isolation of different bacterial species from mastitis milk samples

Bacterial Species	No of isolates	Percent	Percentage of isolate/ Total milk sample (90)
E. coli	32/113	28.32	35.56
Staphylococcus aureus	29/113	25.66	32.22
coagulase negative staphylococcus sp.	11/113	9.73	12.22
Proteus vulgaris	20/113	17.7	22.22
Pseudomonas aeruginosa	*8/113*	*7.08*	*8.89*
Citrobacter species	13/113	11.5	14.44
Total	113	100	

Table 2. Sensitivity of isolated bacterial agents against different antimicrobial discs.

Antibiotic disc / Microorganism	Ceftiofur	Cipro-floxacin	Enro-floxacin	Genta-mycin	Doxy-cycline	Chlora mphen icol	Stre pto myc in	Eryt hrom ycin	Sul-phametha xazole / trimethop rim	Am-picill in	Amo xicilli n.	Peni-cillin
Staphylococcus aureus	S	S	S	S	R	I	I	S	S	I	R	R
E.coli	S	S	S	S	R	R	I	R	S	R	R	R
Ps. Aeruginosa	S	S	S	S	R	I	I	R	R	R	R	R
coagulase negative staphylococcus	S	S	S	S	I	I	I	S	I	R	R	R
Proteus vulgaris	S	S	S	S	I	I	I	S	S	I	R	R
Citrobacter species	S	S	S	S	I	I	I	R	R	I	R	R

N.B: Zone diameter limits for quality control of antimicrobial disc diffusion susceptibility test by **NCCLS (2002).**

Table 3. Some hematological parameters in healthy and mastitic cattle.

Parameter / Groups	Apparently healthy cow	Mastitic cow
RBCs (10^6/µl)	4.9 ± 0.11	2.4 ± 0.05**
HB (Gm %)	9.8 ±0.17	5.5 ± 0.07**
PCV (%)	30.0 ± 0.3	18.5 ± 0.36**
WBCs (10^3/ µl)	8.86 ±0.11	11.35 ± 0.1**
Neutrophil (10^3/ µl)	5.0±0.22	3.2 ±0.5**
Lymphocyte (10^3/ µl)	3.5 ± 0.2	6.8 ± 0.13**
Monocyte (10^3/ µl)	0.26 ±0.14	1.2 ± 0.03**
Eosinophil (10^3/ µl)	0.1 ± 0.11	0.15± 0.04

* Significant P< 0.05 ** highly significant P <0.01

Table 4. Serum biochemical parameters in healthy and mastitis cattle.

Groups / Parameters	Healthy cow	Mastitis cow
ALT (u/l)	13.4 ± 0.03	17.54 ± 0.11**
AST (u/l)	107.50±5.50	162.3 ± 3.14 **
ALP(u/l)	48.50±3.50	71.80± 5.70**
GGT(u/l)	18.80±8.90	24.50±1.50**
Urea (mg/dl)	20.25±2.52	24.26±1.58**
Creatinine (mg/dl)	1.1 ± 0.02	1.7 ± 0.01**
T. protein (gm/dl)	8.50±0.70	7.06 ± 0.05**
Albumin (gm/dl)	3.50±0.20	1.30 ± 0.01**
Globulin (gm/dl)	4.95±0.55	5.76 ±1.8**
α– globulin (gm /dl)	1.32 ± 0.58	1.89± 0.3*
β– globulin (gm /dl)	0.85 ± 1.04	0.81± 0.5
l– globulin (gm /dl)	1.8 ± 0.95	2.10 ± 0.6 *
Mg (mg/l)	2.56 ± 0.05	2.55 ± 0.09
Ca (mg/dl)	14.3 ± 0.33	10.8 ±0.5**
Zn(mg/l	1.1 ± 0.1	0.7 ± 0.03*
Fe (mg/l)	3.11 ± 0.3	2.4 ± 0.25**
Cu (mg/l)	0.52 ± 0.01	1.05 ± 0.05*

* Significant P< 0.05 ** highly significant P

Discussion

Mastitis is the one of the most important disease affecting dairy animals **(Anwar *et al*., 2003).** In the present study of clinical mastitis in cattle were originated from the environmental bacteria. *E. coli* and *Staphylococcus aureus* were the most isolates (35.56% and 32.22%). The higher incidence rate of *E. coli* and *Staphylococcus aureus* in mastitic cows were in agreement with the work of **(Maisa and Kawther, 2008; Sumathi *et al*., 2008; Sudhakar *et al*., 2009; and Akram *et al*., 2013 and Azza and Ebtisam, 2013).** The other bacterial isolates were *Proteus vulgaris, Pseudomonas aeruginosa, coagulase negative staphylococcus* and *Citrobacter* species and their incidence in milk samples were 29/90 (32.22%), 32/90 (35.56), 20/90 (22.22%), 8/90 (8.89%), 11/90 (12.22%) and 13/90 (14.44%) respectively. Nearly similar bacterial pathogens were isolated from mastitic milk of dairy cow by (**Anwar *et al*., 2003; Eman *et al*., 2006; Maisa and Kawther, 2008; Baloch *et al*., 2011 and Dar et *al*., 2014). Amira *et al*., (2013)** reported that the level of *E.coli* isolated from mastitis milk in percentage of 18.47%. Also, these results were nearly similar to (**Ahmed and Shimamoto, 2011)** reported that the bacterial species isolated from mastitic cow were *Escherichia coli* (42 isolates), *Klebsiella pneumoniae* (19 isolates), *Enterobacter cloacae* (18 isolates), *Klebsiella oxytoca* (12 isolates), *Citrobacter freundii* (10 isolates), *Proteus mirabilis* (5 isolates), *Proteus vulgaris* (4 isolates), single isolates of *Pseudomonas stutzeri* and *Serratia marcescens.*

The prevalence of Bovine mastitis coagulase-negative *Staphylococcus* spp. was higher in first-lactation heifers than cows and higher immediately after calving than in the remainder of lactation. In the present study coagulase negative *Staphylococcus* spp. was isolated in percentage (12.22%) and this result was agreed with **(park *et al*., 2012 and Hamadani *et al*., 2013)** reported that the percentage of coagulase negative staphylococcus was 32.5 % in mastitic cow**.**

Higher incidence rate of *E. coli* mastitis may be due to poor hygienic conditions of the farm and animal environment as *E. coli* infects the udder via teat canal from the environment **(Sumathi *et al*., 2008).**

In the present work, among the serotyped strains of *E. coli*, there was no predominant serogroup and this result was agreed with **(Linton and Robinson, 1984)** and this emphasized that serotyping of *E. coli* not of high significance in mastitis cases characterization. This observation had been suggested by many researchers **(Morner *et al*., 1998; Bradley and Green, 2000 and El-Mahronki *et al*., 2006)** which made further studied on virulence factors of these *E. coli* serogroups based on epidemiological studies. O157 serotyped *E. coli* strains recovered from clinically mastitic cases and this serogroup is one of the most important shiga toxins *E. coli* that causes severe disease in human and was mentioned by many other authors as a cause of mastitis **(Aly, 2006).** Many of *E. coli* isolates detected in this study belonged to classical E.P.E.C serogroups as O114, O55, O78 and O125 that were also detected by others **(Mosherf, 2004 and Sabry *et al*., 2006)** among serotyped *E. coli* strains from cases of mastitis. Also, these results nearly agreed with **(Amira *et al*., 2013)** who resulted of 63 *E. coli* isolates were serotyped to O157, O153, O55, O18, O114, O126, O26, and O1 and 21 untyped strains.

The antibiotic profile of different bacterial isolates indicated that Ceftiofur, Ciprofloxacin, Enrofloxacin and Gentamycin proved to be the most effective antimicrobials against mastitis causing bacteria in the study (Table-2). Similar antibiotic pattern was reported by **(Sumathi *et al*., 2008; Sudhakar *et al*., 2009; Ahmed and Shimamoto, 2011 and Azza and Ebtisam, 2013)** who reported higher efficacy of Gentamycin, Enrofloxacin and Ciprofloxacin in the mastitis cow. **Dar *et al*., (2014)** reported that the most effective antibiotics for mastitis cows were Enrofloxacin (74.93%), Gentamycin (67.74%), Tetracyclin (57.81%), and Amoxicillin (50.37%) respectively.

Hematological findings of apparent healthy and mastitic cow were reported in Table (3) a significant decrease in RBCs count, Hb conc, and PCV value in mastitic cow indicate anemia correlated with the severity of bacterial infection. These results were in accordance with **(Jain, 2000; Mona *et al*., 2010 and Azza and Ebtisam, 2013).** There was leucocytosis, lymphocytosis, monocytosis and neutropenia. **Jain (2000)** stated that the neutropenia and lymphocytosis is seen in ruminant with peracute and acute sever inflammatory disease include mastitis. These results were agreed with **(Mona *et al*., 2010 and Azza and Ebtisam, 2013).**

In table (4) revealed that a significant increase in AST, ALT, GGT and ALP. These changes may be attributed to hepatic degenerative and necrotic changes due to bacterial infection and toxin **(Kaneko, 1989).** These results were agreed with **(Mona *et al*., 2010 and Azza and Ebtisam, 2013)** reported that the highly significant increases detected in AST values in mastitic cow. However, these changes attributed to stressful conditions.

Our investigation on the biochemical analysis of kidney function recorded significant increase in the level of urea and creatinine in mastitic cows. **Coles (1986)** attributed these results to excessive protein catabolism and present azotaemia. Hypoproteinaemia and hypoalbuminaemia were observed in mastitic cow which might possibly be attributed to renal dysfunction **(coles, 1986).** In addition to, the hypoalbuminaemia which is an important feature of the liver disease **(Kaneko, 1989)** as the liver is the sole site of albumin synthesis. These results were agreed with **(Mona *et al*., 2010 and Azza and Ebtisam, 2013).** Increased globulin and gamma globulin in the blood of mastitic cows indicates an activation of immune response following infection of the mammary gland. These proteins are mainly immunoglobulins that are implicated in udder defense mechanisms **(Tsenkova *et al*., 2001 and Azza and Ebtisam, 2013).**

In table (4) revealed that a significant decrease in serum calcium, zinc, iron and increase in copper as well as non- significant in magnesium levels. This could be due to damage to the mammary glands by pathogens, which is often disrupting the functional complex of the secretory epithelium that essentially impermeable to calcium transport from milk to the blood **(Ebtisam *et al*., 2005).** Mastitis infection was demonstrated to alter milk trace elements e.g. copper, zinc and iron **(Middleton *et al*., 2004).** Similarly, **(Mohamed *et al*., 1999; Ibtisam *et al*., 2006; Maisa and Kawther, 2008 and mona *et al*., 2010)** found that mastitic cow's milk contained slightly less zinc, iron and increased in copper than those from healthy quarters.

It is concluded from present investigation that these bacterial species *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa,* coagulase negative *staphylococcus sp*. and *Citrobacter* species are considered to be the major causal agents of clinical mastitis in cows. Also, it is concluded that the mastitis effect on the general health condition of cattle by producing anemia and disturbance in liver and kidney function accompanied with disturbance in trace element. So, it must be isolated and accurate identification of bacterial cause of mastitis at the first appearance of signs and directly make the sensitivity test before any treatment with periodic blood analysis to discover any infection as soon as possible

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دراسات بكتريولوجية على الحليب فى الابقار المصابة بإلتهاب الضرع مع الكشف عن التغيرات الاكلينيكية

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معمل بحوث صحة الحيوان الإسماعيلية

الملخص العربى

في هذه الدراسة تم تجميع عينات لبن ودم من ماشية (90 مصابة و10 سليمة ظاهريا) من 5 مناطق مختلفة بمحافظة الاسماعيلية .وقد تم تعرض عينات اللبن للفحص البكتيولوجي وعينات الدم لدراسة التغيرات الدموية والبيوكيميائية. وكشف الفحص البكتريولوجي على وجود مسببات بكتيرية وهي كما يلي: المكورات العنقودية الذهبية، الميكروب القولوني، البروتيس الاعتيادية، الزائفة الزنجارية، المكورات العنقودية السلبية. والأنواع الليمونية ومعدل الإصابة بها في عينات الحليب وكان 29/90 (32.22٪)، 90/32 (35.56)، 20/90 (22.22٪)، 8/90 (8.89٪)، 11/90 (12.22٪) و 13 / 90 (14.44٪) على التوالي. وأظهرت نتيجة اختبار الحساسية ان جميع البكتيريا المعزولة كانت حساسة الى سيفيوفور وسيبروفلوكساسين وانروفلوكساسين. وكان معظمم مقاوم للبنسلين وأموكسيسيلين. كما اظهرت تحاليل الدم عن وجود انيميا وزيادة كبيرة في وظائف الكبد والكلى مع انخفاض ملحوظ في البروتين الكلى والالبيومين والكالسيوم والزنك والحديد بالمقارنة بالايقار السليمة. ومن هذه الدراسة استنتجنا أن التهاب الضرع ت يؤدى الى انيميا واضطرابات في وظائف الكبد والكلى لذلك يجب أن يتم عمل اختبار الكاليفورنيا لاكتشاف الحيوانات المصابة بالتهاب الضرع مبكرا وعلاجة .