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INFLUENCE OF CLINICAL COWS MASTITIS ON MILK PROTEIN FRACTIONS, CERTAIN BLOOD CONSTITUENTS AND BACTERIAL ISOLATES.

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ABSTRACT

The frequent distribution of isolated microorganisms from examined milk samples clears that *Salph. aureus* and *Strept. agalactiae* were the most prevalent isolates (9.38% and 8.50% respectively). Comparing the different rapid tests for diagnosis of mastitis, the peroxidase test accompanied with CMT was used as a rapid screening test for diagnosis of cow mastitis. The metabolic profile of some blood parameters of cows suffering from mastitis showed highly significant changes ($p<0.01$) in case of alanine aminotransferase (ALT) and alkaline phosphatase values, which reflect the severity of mastitis on dairy cow's productivity. Meanwhile, all serum parameters showed no statistic difference between control group and post treated with antibiotics cow, which indicated the reversible of cow productivity. The electrophoresis pattern of casein and whey protein of Friesian cows milk was determined in healthy and in mastitic animals before and after antibiotic treatment. The protein pattern of sick animals was different from that of the healthy group, especially in case of whey proteins. The fractions with slow rate of migration namely κ-casein, immunoglobulins, proteose peptone and bovine serum albumin were found to be more affected in mastitic milk. The degree of changes depends on the disease severity.

Keywords: Cow milk, mastitis, microorganisms, antibiotics, blood profiles, milk protein fractions.

INTRODUCTION

Mastitis is a common disease causing inflammation in the parenchyma of the mammary glands regardless of the cause. It is characterized by physical, chemical and usually bacteriological changes in the milk and by pathological changes in the glandular tissue *(Radostits et al., 1994)*. Few types of bacteria cause the majority of udder infections. Moreover, the common one is contagious mastitis in which bacteria spread from infected quarter to other quarters and cows (*S. agalactiae*, *Staph. aureus* and *c. bovis)*. Meanwhile, environmental mastitis caused by microorganisms may reach teat end from cow's environment (*E.coli, klebsiella species*, *Enterobacter species*, *S. dysgalactiae* and other *streptococcus species*).

So, this needs different control measures for the different groups of microorganisms *(Smith and Hogan, 1993 and Bramley et al., 1996)*. A bovine mastitis cause decline in milk yields about 10-12 %, affects milk quality and composition shortens the productive life of infected cows *(Casado Cimiano, et al., 1988)*. Several methods have been reported for

detecting mastitis, but bacteriological isolation of the causative microorganisms is the most accurate one *(Rossetti, 1993)*. In final analysis, mastitis represents the udder, it was felt that objective measurable changes in protein fractions, that might be a part of or be more closely associated with the abnormalities could be proved as a useful tool in the diagnosis and understanding of the changes in permeability of the host's tissue near the irritant, as well as, the transfer of immune proteins postpartum *(Shimizu et al., 1997)*. The use of antibiotics in controlling mastitis causes, great hazard for public health, meanwhile the holding period for mastitis milk after treatment with antibiotics which ranged 2-5 days causes is of a economic losses due to the discard of the milk containing the antibiotics *(El-Sagheer, 1991)*.

Moreover, control of measure based on milking hygiene and antibiotics have reduced the occurrence of mastitis but numerous intramamary infections still occur, and improved methods are needed to prevent the disease. The elimination of antibiotic sensitive species of microorganisms originally responsible for most cases of the disease has resulted in an increase in the incidence of mastitis caused by partially or completely resistant microbial agents. Therefore, the in vitro sensitivity tests before clinical application would be of great value in choosing the suitable effective antibiotics for treatment of mastitis cases *(Ismail, 1979)*. Moreover, both of mastitis and its treatment with specific antibiotics can affect cows' health, and the quality of their milk *(Hill, 1981)*. Therefore, the aim of this work is to investigate the prevalence of the most common bacteria isolated from lactating cows, to estimate some metabolic profile parameters, which may be affected by clinical mastitis, and treatment with specific antibiotics. Preliminary evaluation of the variations of milk protein fractions by gel electrophoresis of casein and whey proteins of healthy was also investigated, compared with mastitic cows pre and post-treatment with specific antibiotics.

MATERIALS AND METHODS

Total of 32 Holstein-Friesian lactating cows were investigated for clinical mastitis in private farm at Giza Governorate.

Various individual samples were collected from all investigated cows for bacteriological and biochemical studies.

Blood samples:

The blood samples were taken from Jugular vein and the serum was separated and centrifuged at 3000 r.p.m. for 20 minutes and left at 4°C overnight for biochemical analysis. The samples were collected one week before and after treatment with specific antibiotics against mastitis.

Milk samples:

128 individual quarters milk samples were aseptically drawn from each quarter of dairy cows. Collected samples were immediately colded at 4°C and transferred to the laboratory to be examined using rapid screening tests of mastitis.

Casein and whey samples:

The milk was defatted by centrifugation, casein and whey protein fractions were obtained by adjusting the pH of skim milk to 4.6 (25°C) using 0.1 *N* HCL. Casein was collected by centrifugation at 4000 r.p.m. for 30 minutes, then whey was filtered through whatman No.2 filter paper. Sedimented casein was washed three times with distilled water at pH 4.6, after each washing casein was collected by centrifugation at 4000 r.p.m. for 15 minutes. The casein and whey were kept frozen until used.

Diagnostic test kits:

A reagent kits for determination of serum aspertate aminotransferase (AST or GOT) activity, serum alanine aminotransferase (ALT or GPT) activity, serum alkaline phosphatase (Alk.ph.) activity, urea, creatinine and cholesterol were obtained from BioMerieux, France.

Media used for isolation and cultivation of bacterial species:

- **Nutrient agar medium (Oxoid CM3):** It was used for isolation and examination of the obtained colonies.
- **Blood agar medium:** A nutrient agar (oxoid CM3) was used as a basic media + 5–10% defibrinated sheep blood, *(Sojka, 1965)*. It was used as enriched medium for pathogenic bacteria and to demonstrate the different hemolytic activities of isolated microorganisms.
- **MacConkey`s bile salt lactose agar medium (Oxoid CM7):** It was used as a selective medium for the isolation of *Enterobacteriaceae*.
- **Semi solid agar (0.4%):** *(Cruickshank et al., 1975)*.

Antibiotics:

Rilexine 200, L.C® intrammary infusion antibiotic composed of cephalexin 100mg and neomycin sulfate 100mg produce by Virbac Batch No. 741/93 is used after antibiotic sensitivity test to determine drug of choice according to *(Ismail, 1979)*.

Rapid Screening tests for detection of mastitis:

1. Chloride test: The test was carried out according to *(Ling, 1963)*.

2. Bromthymol peroxidase test: According to *Radermacher and Jiruska (1967)*. The test solution is prepared by mixing 25 parts of 9% hydrogen peroxide to 5 part of 1% bromthymol solution in diluted ethanol (1.5). Five ml of milk is mixed gently with 2.5 ml of the reagent, and the result is read within 20 second as follows.

Negative: No foam, yellowish green colour.

Positive: About 1 ml of fine foam, greenish yellow colour of liquid portion.

++, +++, ++++: 2.5-10 ml of foamy liquid portion of greenish, dark green and greenish blue colour respectively.

3. Gel test: California Mastitis tests (CMT):

Recommendation according to APHA (1985). Normal, trace, a, b or c reaction equate to mean cow cell count of 100 000, 300 000, 900 000, 2700 000 and 8100 000 respectively.

Bacteriological examination:

The milk samples were incubated aerobically at 37°C for 24 hour then centrifuged at 3000 r.p.m. for 20 minutes, *(Breed et al., 1957)*. The cream and supernatant fluid were discarded. A loopfull from the sediment was streaked on to the surface of blood agar, nutrient agar, MacConkey`s

agar and Edward's media. The inoculated plates were incubated at 37°C for 24-48 hours and examined for bacteriological growth. Suspected colonies appearing on different media were subcultured, purified and preserved in semisolid agar for further identification. Pure colonies were described for their morphological characters, colonial appearance. Suspected colonies were picked up and examined microscopically by Gram's stain to observe the morphological arrangement and staining reaction before transfer to semisolid media for further investigations. Pure culture of isolates was identified biochemically according to *Carter and Cole (1990)* and *Koneman et al. (1992).*

Determination of AST and ALT were carried out by test kits according to the method of *Reitman and Frankel (1957),* serum alkaline phosphates according to the method of *kind and King (1954),* serum urea according to *Patton and Cruoch (1977),* serum cholesterol according to *Trinder, P. (1969),* and total protein according to *Henry et al. (1974).*

The milk samples from each affected cow were used for separating casein and whey. Electrophoresis patterns of casein and whey proteins fractions in milk were determined before and after treatment with specific antibiotics by polyacrylamide slab gel electrophoresis (PAGE) as described by *Melachouris (1969).*

Data were statistically analyzed using student's t-test at confidence limit of 95% ($p < 0.05$) and 99% ($p < 0.01$) according to *Snedecor and Cochran (1980).*

RESULTS AND DISCUSSION

Bacteriological isolates:

Results obtained in Table (1) revealed that 18 (56.25%) out of 32 cows examined were suffering from mastitis. Meanwhile, examination of 128 quarters of these animals pointed out that 43 (33.59%) quarters were infected with mastitis. Table (2) showed that 4 mastitic cows with one infected quarters (9.30%), 6 with two infected quarters 12 (27.91%), 5 with three infected quarters 15 (34.88%), 3 with four infected quarters 12 (27.91%). The obtained findings are similar to that of *Abdel-Karim and El-Ashmawy (1979); Motie, et al. (1985) and El-Kholy et al. (1994).*

Table (1): The incidence of clinical mastitis among examined cows and quarters.

	Total number examined	Mastitic cases	
		No.	%
Cow	32	18	56.25
Quarter samples	128	43	33.59

No. : positive number.

Table (2): The incidence of mastitic cows in relation to the number of quarters.

Number of quarter affected	Mastitic cases	
	No.	%
One quarter	4	9.30
Two quarters	12	27.91
Three quarters	15	34.88
Four quarters	12	27.91
Total	43	100

No.: positive quarter.

The results of incidence of isolated bacterial species in the examined milk samples were recorded in Table (3), which showed that *staphylococcus aureus* and *streptococcus agalactiae* were the most prevalent isolates (9.38% and 8.59 % respectively). *Pseudomonas aeruginosa* and *strept. Pyogenes* could be detected with 3.12, while *Klebsiella Pneumonae* and *S. epidermidis* could be detected in 2.34%. Moreover, each of *E.Coli, Strept. Dysagalactiae* and *proteus spp.* were isolated from 1.56 % of examined samples. These findings, in clinical mastitis are approximately of similar to those incidences recorded by *El-Gindy et al. (1964); Kotowaski (1991); Lipuzic (1995)* as well as *Lafi and Hilat (1999)*, who reported that the major cause of mastitis is *S. agalactiae* which is the most prevalent isolates. Furthermore, *P. aeruginosa* and *Strept. pyogens* were recovered from the examined samples in an incidence of 3.12%. The obtained results nearly similar to that reported by *Edward (1980)* as well as *Shalaby and Salem (2001)* for *P. aeruginosa*. Our results revealed the incidence of both *E.coli* and *Klebsiela* (1.56 % and 2.34 %, respectively). Morecver, some authors indicated that the so-called "environmental mastitis" pathogens were *E.Coli, K pneumoniae, S. dysgalactiae and S.pyogenes. Smith et al. (1985); Guterbock et al. (1993)* and *Shpigel et al. (1998)* recorded incidences of 62%, 63% and 78.89% respectively for the so called environmental microorganisms incriminated in mastitis. The variable incidences might be attributed to the difference in pathogenic activities of the organisms, the immunity status of infected cows, and to the hygienic measure and way of husbandry of milking cows, as well as, the technique and steps of milking procedures. From the public health point of view, the presence of these microorganisms, acts as a hazard for milk consumers increasing the incidence of disease and food poisoning as well as milk deterioration *(APHA, 1985; Poricenko, 1985* and *James, 1996)*. The comparative statement given in Table (4) showed that the most accurate rapid test in diagnosis of positive bacteriological milk samples was CMT (93.75%) followed by peroxides test (90.62 %) and chloride test (89.06 %).

Comparing to the different rapid tests, it is clear that the highest false positive reactions were detected in CMT (8) and the lowest were detected in chloride test (5), while peroxidase test showed no false positive reaction. These findings are similar to those obtained by *Abdel-Karim and Ashmawy (1979); Mahmoud, (1980) and Poricenko, (1985)*. From the obtained results it can be concluded that peroxidase test accompanied with CMT were rapid screening test for accurate diagnosis of clinical mastitis.

Table (3): Frequency distribution of isolated microorganisms from examined milk samples.

Bacterial species	Frequency	
	No.	%
Grame negative bacteria:		
E.coli	2	1.56
Klebsiella pneumonia	3	2.34
Pseud. aeruginosa	4	3.12
Grame positive bacteria:		
Staph. aureus	12	9.38
Strept. agalactiae	11	8.59
Strept. pyogenes	4	3.12
Strept. dysgalactiae	2	1.56
Proteus spp.	2	1.56
S. epidermidis	3	2.34
Total	**43**	**33.59**

% : was calculated according to the number of the examined samples (n=128).

Table (4): Comparative statement showing the percentage agreement diagnostic tests taking bacteriological methods as standard.

Tests	Total number of samples	Bacteriological		Test reaction				Accuracy (%)
		+ve	-ve	True +ve	False +ve	True -ve	False -ve	
Chloride	128	43	85	38	5	76	9	89.06
Peroxidase	128	43	85	36	-	80	12	90.62
CMT	128	43	85	43	8	77	-	93.75

CMT: California mastitis test.

$$\text{Accuracy \%} = \frac{\text{True +ve} + \text{True –ve}}{\text{Total number of examined cases}} \times 100$$

Metabolic profiles tests:

It is based on concept that the laboratory measurement of certain components of blood will reflect the productivity of dairy cows. As shown in Table (5) illustrating some serum biochemical parameters. It is clear that all parameters (AST, ALT, alkaline phosphatase, urea, cholesterol and total protein) have a higher values in mastitic cases than control group but the post treatment by antibiotics, all the values were similar as the control group values. These results agreed with those obtained by *Pavne et al. (1970); Ingraham and Kappel (1988); Tornquist and Van Saun (1999).* In case of ALT, the values were 93.5, 209.3, and 109.3 U/L for control, mastitis pre and post treated, respectively. Concerning the activity of ALT, the data in table (5) show that the values were 57.0, 161.1 and 61.1 U/L for control, mastitis pre and post treated respectively. Also Alk.ph. values were 107, 258.9 and 158.9 U/L for control, mastitis pre and post treated respectively. The results of control mastitis and the treated cows for urea, cholesterol and total protein contents in the blood sera were 15.47, 29.93, and 19.93; 110.23, 217.35, and 117.35; 8.00, 19.80, and 9.2 mg/dl respectively. Statistically, the obtained highly significant ($p<0.01$) difference between control group and mastitis pre treatment in case of ALT and alkaline phosphates values reflects the severity of mastitis on cow's productivity. On the other hand, all the serum AST, ALT, alkaline phosphatase, urea, cholesterol and total protein

show no statistical differences between control group and mastitis post treatment, this means that the cow treatment with antibiotic causes reversible of productivity (*Payne et al., 1970).*

Table (5): Effect of antibiotic treatment on some blood constituents of mastitic cows.

Parameters	Control		Cows with clinical mastitis			
			Pre treatment		Post treatment	
	×	± SD	×	± SD	×	± SD
(AST) U/L	93.50	1.27	209.30	3.69	109.30	1.64
(ALT) U/L	57.00	2.52	161.10	1.36	61.10	2.22
(Alk. ph.) U/L	107.00	4.04	258.90	3.27	158.90	1.81
Urea (mg/dl)	15.47	2.47	29.93	1.03	19.93	0.98
Cholesterol (mg/dl)	110.23	1.68	217.35	3.67	117.35	2.11
Total protein (mg/dl)	8.00	1.78	19.80	1.50	9.20	1.20

× : Means of 3 samples from 12 animals. SD: Standard deviation.

Electrophoretic patterns of milk protein fractions:

PAGE patterns of casein in normal and clinical mastitis milk samples before and after treatments by special antibiotics are shown in Fig. (1). It can be seen that normal milk samples resolved into three defined protein bands, namely starting from the origin (negative charge) κ-casein, β-casein and α_s-casein, respectively. On the other hand, the mastitis samples pre treatment resolved into the same pervicus bands with some changes of bands intensity, in which an increase the κ-casein fraction and decrease α_s- and β-caseins fractions were detected. While the mastitis samples post treatment revealed the same previous bands of mastitic samples pre treatment with little changes of bands intensity which, decrease κ-casein fraction.

The pattern of whey proteins is represented in Fig. (2). It is clear that β-lactoglobulin (β-Lg) and α- lactalbumin (α-La) were of low intensity, but bovine serum albumin (BSA), immunoglobulins (Igs) and proteose peptone (pp) were of high intensity in mastitis pre treatment comparable to the normal one or the mastitis post treatment. It must be underlined that, especially in case of acute mastitis (c), the Igs and pp increase strongly. In clinical mastitis pre treatment the α-La has been highly increased probably because it plays an important role as regulatory protein in biosynthesis of milk glycoproteins or proteoglycans.

From these results it can be concluded that in cows with mastitis the electrophoretic pattern of milk proteins is modified. The greatest changes are undergone by the protein fractions with the slow rate of migration towards the anode, namely κ-casein, Igs, pp and BSA, which cross the cellular membrane of acini. The degree of these modifications depends on the disease gravity. This fact can be explained by the impairment of the cellular membrane under the microbial action. Similar trends were found by *Lopez, et al. (1962); Nagasowa and Tanahashi (1963); Kisza and Sobino (1963); Nakanishi et al. (1966); Buruiana, et al. (1979) & (1981)* as well as *Hamzawi et al. (1991).*The results obtained suggest that the electrophoretic analysis is a recommendable method for diagnosis a serious and harmful affection of the udder.

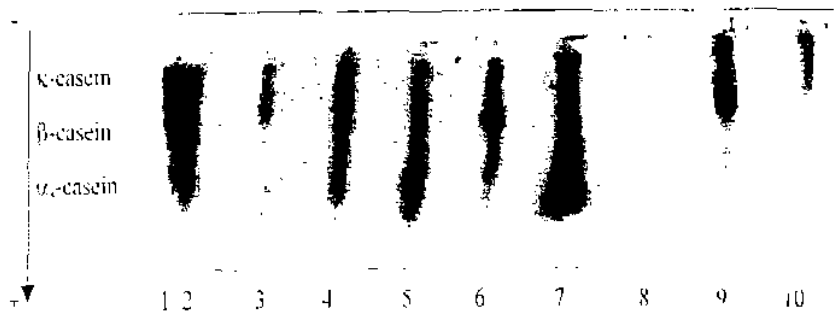

Fig. (1): Polyacrylamide slab electropherograms of casein from cows with normal and clinical mastitis pre and post treatment by antibiotics

Where (3, 8, 10) : Healthy cow
1(b), 2(a), 7(c), 9(b) : clinical mastitis pre treatment
(4, 5, 6) : clinical mastitis post treatment.

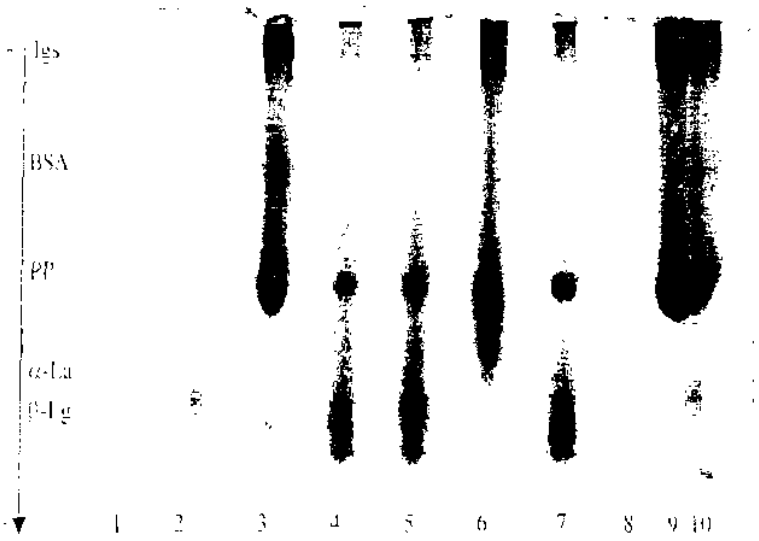

Fig. (2): Polyacrylamide slab electropherograms of whey proteins from cows with normal and clinical mastitis pre and post treatment by antibiotics

Where (1, 2, 8) : Healthy cow.
3(b), 6(b), 9(c), 10(a) : clinical mastitis pre treatment
(4, 5, 6) : clinical mastitis post treatment

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تأثير إلتهاب الضرع الإكلينيكى على مشتقات بروتين اللبـــن وبعـض مكونـات الـدم والبكتيريا المعزولة

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قسم التكاثر الحيوانى والتلقيح الصناعى[1] ، الألبان[2] ، الملوثات وسموم الغذاء[3] ،
الطفيليات وأمراض الحيوان[4] ، المركز القومى للبحوث- الدقى- القاهرة

تم عزل البكتيريا من عينات اللبن المصابه بإلتهاب الضرع الحاد ولوحظ أن الميكروبـــات المسائده هـــى *Staph. aureus* and *Strept. agalactiae* ، وكان أفضل الإختبـــارات الســـريعه لتشـــخيص المـــرض هـــما إختبار البيروكسينيز ، CMT معا.

كانت دلالات مكونات الدم (بعض وظائف الكبد والكلى) المغذره فى الحيوانات المصابه ذات معنويه مرتفعه عند (٠.٠١) فى حالة ALT ، Alk.ph. وذلك عند المقارنه بعينات الدم الغير مصابه (الكونترول). بينمـــا كـــانت التغيرات فى وظائف الكبد والكلى بعد العلاج بالمضادات الحيويه غير معنويه وذلك عند المقارنه بعينـــات الـــدم الغـــير مصابه. وأخيرا تم دراسة تأثير إلتهاب الضرع الحاد على توزيع بروتينات اللبن فى كل من الكازيين والشرش، وقـــد أستخدم طريقة الفصل الكهربى على جل الأكريلاميد حيث لوحظ إرتفاع البروتينات ذات الوزن الجزيئـــى العـــالى ذات معدل الهجره الأبطأ خصوصا الكابا كازين ، البروتينات المناعيه ، البروتيوز ببتون وأخيرا ألبيوم الســـيرم فـــى اللبـــن المصـــاب عن مثيله الغير مصـــاب ، وتعتمد هذه التغيرات على حدة وكثافة المرض.