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Biochemical Parameters in Milk Triggered by Bacterial Pathogens Causing Subclinical Mastitis

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Abstract

SUBCLINICAL MASTITIS is characterized by inflammation in the udder without any signs of clinical illness. Udder inflammation is mainly associated with major alterations in biochemical milk components, which may be identified as indicators for pathogenic infections. To identify key biochemical changes in milk relevant to early detection of subclinical mastitis, milk samples were collected from 10 healthy cows and 50 cows diagnosed with subclinical mastitis based on the California Mastitis Test, followed by analysis of bacterial contents and biochemical components. The bacteriological analysis revealed that S. aureus was the main pathogen associated with subclinical cases (32%) either as a single infection or mixed with E. coli (4%) or Klebsiella spp,. (8%). Single bacterial infections by E. coli, Klebsiella spp., and Enterobacter spp. were detected in 7%, 7% and 3% of the subclinical mastitis cases, respectively. Biochemical composition analysis of bacteriologically positive milk samples versus healthy samples showed a significant decrease in important electrolytes (Ca, P, K, Mg) and total proteins, in addition to a significant increase in blood electrolytes (Na & Cl), and C-reactive proteins (CRP). The result showed no significant difference in all parameters except for CRP between single and mixed bacterial infections. Albumin concentrations showed no significant changes in all tested milk samples, while vitamin E & A concentrations showed variation among the tested milk samples. In conclusion, CRP as well as total protein, blood and important electrolytes, have potential as biomarkers for subclinical mastitis, suggesting that they could be integrated as practical tests to monitor dairy herd health and early detection of subclinical mastitis..

Keywords: S. aureus, subclinical mastitis, CRP, electrolytes, cows, biochemical parameters.

Introduction

Bovine mastitis or udder inflammation is the most common disease diagnosed on dairy farms worldwide. It is considered a serious disease that affects dairy cows and causes huge economic losses in the dairy industry [1-4]. Udder inflammation is often caused by multiple etiological agents, including bacteria, viruses, or fungi5. Bacterial mastitis is highly important due to its physiological complications and economic consequences. Various species of both Gram positive (*staphylococci* and *streptococci*), and Gram negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) are responsible for the development of bovine mastitis6-8. According to the National Mastitis Council, approximately 200 microorganisms may cause mastitis in dairy cows [9]. Infectious pathogens targeting the udder induce various changes in the biochemical components of milk. These changes are mediated by the severity of infection, virulence of the causative agents, and extent of secondary physiological disturbance, which impair the normal function of the mammary gland [1,5]. A clear correlation has been detected between udder infections and significant changes in milk from cows diagnosed with mastitis compared to normal milk5.

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Udder inflammations are classified into clinical or subclinical forms. Cows with clinical mastitis exhibit systemic symptoms associated with varying degrees of changes in udder tissue and produce abnormal milk with visible changes such as the presence of blood, discoloration, or lumps [10- 11]. On the other hand, the subclinical form of mastitis does not show any changes in the milk or udder tissues but is associated with extravasation of inflammatory cells into the milk, which increases the number of somatic cells [12].

Early diagnosis is crucial to the control of mastitis and is primarily based on the California Mastitis Test (CMT); which depends on the interaction between DNA from inflammatory cells present in milk and specific reagents, leading to the formation of a gelatinous substance with a consistency that allows self-analysis of the amount of cells in the milk sample [13]. Alternatively, the number of cells in milk can be analyzed quantitatively via the somatic cell count test (SCC). However, SCC can be affected by the degree of infection, lactation state, age, breed, and milk transport 4. Key biochemical changes in milk can be used as indicators for early diagnosis of subclinical mastitis and reducing potential economic losses associated with mastitis in the dairy industry.

Therefore, the aim here is to identify milk biomarkers relevant to subclinical mastitis by studying the biochemical composition of milk from cows with subclinical mastitis and its relationship to the bacterial pathogens involved.

Material and Methods

Sampling: Milk samples (50 mL) were collected aseptically from 10 healthy dairy cows, and 50 cows tested positive using CMT in Fayoum Governorate immediately before routine daily milking. The CMT was performed as described by Kandiwa et al. [14]. Precisely, the milk from each quarter is drawn into separate cups of a four-cup plastic paddle. The paddle is then tilted to equalize the amounts of milk in the cups by approximately 1/2 teaspoon each, then adding 1/2 teaspoon of the test reagent to each cup. Rotate the paddle to mix, and observe changes in color and gel formation within 10 to 15 seconds after mixing. Results were interpreted referring to the change in color and grade of gel formation as negative, trace, 1+, 2+, and 3+ as described by Kibebew et al. [15].The udder of each cow was first washed with a dilute solution of povidone-iodine (El-Nile Co., Egypt), dried with paper towels, then the teats were swabbed with 70% ethyl alcohol and allowed to air dry for 1 min. The milk samples were collected in a plastic Falcon tubes, and transferred immediately to the laboratory in iced insulated boxes. All samples were maintained at −10°C until analyzed for biochemical composition within 24-48 hrs.

Bacterial isolation and biochemical characterization: The milk samples were enriched on nutrient broth (Himedia, Mumbia, India, M002-500G) overnight, and then a loopful from each culture was streaked onto either MaCconkey (MAC; Oxoid, Manchester, UK, CM0115) or Mannitol salt agar (MSA; Himedia, Mumbia, India, M118-500G). The lactose fermenter colonies (pink colonies) were then streaked on Eosin Methylene Blue (EMB Agar,Levine; Himedia, Mumbia, India,M022-500G) to differentiate E. coli and Klebsiella or Enterobacter from other Enterobacteriace, while the yellow colonies that appeared on MSA were then transferred to Baird Barker media (Himedia, Mumbia, India, M043-500G). The suspected colonies were further subjected to biochemical tests as described by Guentezel et al. [16].

Biochemical analysis of milk.

The concentration of sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) in milk samples was determined with an atomic absorp¬tion spectrophotometer (B3003, Perkin Elmer-AAS, MA, USA). Samples were prepared according to the procedures described in the technical manual of the AAS. The data obtained were expressed as mg/dL. Milk phosphorus (P) determination was performed according to the technique of Gliszczyńska-Świgło and Rybicka [17], and chloride (Cl) was performed according to Jaudzems et al. [18]. The total protein and albumin in milk were measured according to the method described by Buzanovskii [19]. The C-reactive protein concentration was measured following the manufacturer's instruction using the immune-assay based fluorescent kits (QAYEE-BIO, Life Science,Co.,Ltd., Daegu, South Korea). Vitamins E & A were also determined using high-performance liquid chromatography (HPLC) as described by Salo-Väänänen, P., et al [20].

Statistical analysis

SPSS software was used to analyze the data using One-way ANOVA test and $P<0.05$ was considered statistically significant. Plotting the data on Origin Software expressed as mean ± SD.

Results and Discussion

Mastitis is a major infection associated with a negative economic impact on the dairy industry. Most dairy cows with subclinical mastitis do not produce characteristic symptoms of mastitis but are persistent shedders of zoonotic bacteria in milk. Early detection of mastitis is crucial to facilitate treatment to minimize economic losses in dairy farms, and to prevent zoonotic transmission. Udder infection is associated with major changes in milk components, which may be identified as indicators for pathogenic infections. To elucidate this, 10 milk samples from CMT- negative cows and 50 milk samples from cows with subclinical mastitis diagnosed as positive for CMT were collected.

All collected samples were analysed bacteriologically on MAC and EMB. The pink colonies on MAC and green metallic sheen colonies with black center on EMB were suspected to be E. coli, the mucoid pink colonies on MAC and EMB were suspected to be Klebsiella spp., while pink buff colonies were suspected to be Enterobacter spp. The biochemical characterization confirmed the identification of all E. coli isolates, which were oxidative fermentative on glucose fermentation tests and produced positive reactions with indole, catalase and methyl red while they were negative with oxidase and urease. All Klebsiella spp. isolates showed an oxidative fermentation reaction, and were positive for catalase and urease while negative for motility test, methyl red, oxidase and indole. The Enterobacter species isolates showed citrate, catalase, urease, Voges–Proskauer and motility tests positive but were negative for oxidase, methyl red and indole or H2S.

Out of 50 milk samples, 7 (14%), 3 (6%), 7 (14%), and 16 (32%) tested bacteriologically and biochemically positive for *E. coli, Enterobacter spp., Klebsiella spp.,* and *S. aureus*, respectively. The recovery rate of *S. aureus* in examined cows is significantly higher than the other detected bacterial pathogens. Typically, previous studies have shown that *S. aureus* can be isolated in a significant percentage of mastitis cases, often ranging from 15% to 50% in various regions[21]. The prevalence of S. aureus in this study is slightly lower than previous studies, which may be explained by differences in geographical distribution, immunological status, as well as biosecurity practices of the study areas in comparison to other studies [22]. Co-infections of S. aureus alongside other bacterial partners, such as *E. coli, Streptococcus agalactiae, Klebsiella pneumoniae and Streptococcus uberis* have been frequently detected in bovine mastitis. Co-infections can complicate treatment and affect the overall outcomes of mastitis cases. As shown in table1, 8% of subclinical mastitis cases were caused by co-infection of *S. aureus* with *Klebsiella spp*. Our result also detected co-infection of S. aureus accompanied with *Enterobacter spp* or *E. coli* in 6% and 4% of subclinical mastitis cases respectively. Similarly, Enterobacter species have been isolated from 6.67% of bovine mastitis cases as a single infection or as part of mixed infections with *S. aureus, E. coli*, *Bacillus spp.* and *Pseudomonas aeruginosa* [23].

A higher recovery percentage of K. pneumoniae (59.5%) from clinical mastitis cases has been reported in Brazil, while co-infection with *S. aureus* and *S. agalactiae* was detected in 32.4% of cases24.

Mixed infections with bacteria like, *E. coli, Klebsiella pneumoniae* and *Enterobacter spp.* were noted in some cases, particularly in dairy farms with poor hygienic environments. A study in India reported simultaneous occurrence of methicillin-resistant *S. aureus, S. epidermidis,* and extended spectrum β-lactamase producing (ESBL*) E. coli* in cases with subclinical mastitis and clinical mastitis [25].

Mastitis caused by bacterial pathogens is the most frequently reported in dairies. Its serious effect doesn't only include milk yield reduction but also alters the milk chemical composition26. The level of these changes was influenced by the causative agent. Thus, all milk samples that are bacteriologically positive for single or mixed bacterial infections were subjected to analysis of the biochemical parameters. As shown in Table 2, multiple parameters are measured in cow's milk including CRP, vitamins (E & A), blood electrolytes (Na & Cl), important electrolytes (Ca, P, Mg & K), albumin, and total proteins. Blood electrolytes of the bacteriologically positive samples showed high increase compared to the bacteriologically negative samples (Fig 1 A). The highest blood electrolytes levels were reported in single infection cases caused by *E. coli or S .aureus* and mixed infections cases caused by *S. aureus* along with *E. coli.* This indicates the significant implications of both *E. coli* and *S. aureus* in the severity of udder inflammation. Batavani et al. reported that milk from subclinical mastitis cows showed elevated levels of sodium and chloride [26]. They also reported lowered contents of calcium, potassium and inorganic phosphorous. The concentration of blood electrolytes is increased in milk from infected cows due to the damaged epithelial cells caused by mastitis pathogens that weaken the milk/blood barrier, and consequently, milk and blood components pass through. The impaired blood/milk barrier also causes a loss of important milk electrolytes [27-28]. In agreement, our data revealed a significant reduction (p value <0.05)in the concentration of important electrolytes in milk in subclinical cases due to either single or mixed bacterial infection compared to healthy cases. No significant difference was found between the bacteriologically positive milk samples with respect to the pathogen in question (Figure 1B). Likewise, clinical and subclinical mastitis in Egyptian cattle was found to be associated with increased salt concentrations in the blood and decreased important salts in the milk1.

Vitamins such as A and E play critical roles in udder health and the immunological function of dairy cattle. Vitamin A is vital for maintaining epithelial integrity and immune activity, while Vitamin E, especially in combination with selenium, acts as an antioxidant that protects cells from damage during inflammation1. Adequate levels of these vitamins in cow feed are important to maintain udder health, which is crucial for mastitis prevention. The analysis of milk biochemical composition showed that vitamin E was significantly decreased in milk positive for *E. coli, Klebsiella spp, Enterobacter* and mixed infection of *S. aureus and Klebsiella*. Vitamin

A was also reduced in milk positive for E. coli and Enterobacter subclinical infections. As shown in Figure 1C, not all cases of subclinical mastitis had a significant reduction in milk vitamin levels, suggesting that they cannot serve as biomarkers of mastitis. However, low levels of vitamins indicate the negative impact of mastitis on milk quality.

Furthermore, albumin concentration showed no significant changes among all milk samples (Fig2A). As detected by Shaheen et al., the subclinical cows also did not exhibit significant differences in albumin, protein and milk fat when compared with normal lactating cows [29]. Conversely, measurement of total protein in the current (Fig2A) and previous studies revealed that total protein was significantly reduced in subclinical infected milk compared with healthy milk samples1.

Acute-phase proteins, such as CRP are produced by the liver in response to cytokines released during immune activation. In both humans and animals, mononuclear cells such as monocytes and macrophages respond to infection by secreting cytokines, which in turn stimulates the liver to rapidly manufacture large amounts of CRP [30]. CRP levels rise in response to acute and chronic inflammation, often before clinical signs of disease appear. This makes CRP a powerful tool for early detection of diseases. In dairy cows, early detection of infections, such as mastitis, can significantly reduce the spread of pathogens and limit economic losses associated with the disease. Our research has demonstrated that CRP concentration was significantly ($P<0.05$) higher in the bacteriologically positive cows compared with the bacteriologically-negative cows. Nevertheless, CRP levels were significantly higher in the milk of cows bacteriologically positive for mixed infection compared to those positive for a single bacterial pathogen ($P<0.05$). Consistently, Lee et al identified CRP as a potential biomarker in serum and milk for early surveillance of herd health status [31]. The high elevation in milk from cows with subclinical mastitis compared to healthy cows indicates the potential relevance of CRP in milk as a biomarker of subclinical mastitis. As confirmed by Ali et al., CRP can be measured in milk with a high specificity and sensitivity to diagnose udder inflammation [32].

Elevated concentrations of CRP, total proteins and blood electrolytes in milk along with decreased important electrolytes have been correlated positively to high SCC which has traditionally been used for mastitis detection. This emphasizes the potential role of these parameters in the early detection of mastitis, even in the absence of clinical signs.

Using periodic CRP and blood electrolytes measurements along with total protein, veterinarians can identify sick animals before symptoms appear, allowing for faster interventions such as isolation, treatment or preventive measures. However, the CRP level alone can be used to monitor udder health and detect subclinical mastitis before a significant decline in milk production occurs. Thus, our data and previous studies [31-34] recommend integrating CRP measurement into routine milk examinations to verify udder health in dairy cows.

Conclusion

Subclinical mastitis is inflammation of the udder with no clinical signs of illness, causing significant implications for milk production and animal welfare. The current study reported alteration of biochemical parameters, including elevated levels of blood electrolytes and CRP, along with reduced important electrolytes and total proteins in milk from subclinical mastitis. Previous studies and our data indicate that CRP, as well as total protein, blood, and important electrolytes, have potential as biomarkers for subclinical mastitis, suggesting that they could be adopted in practical applications to improve the management of dairy herd health. This research suggests that veterinary professionals should consider integrating these test parameters (CRP, total protein, blood, and important electrolytes) alongside other diagnostic measures to develop comprehensive herd health assessment methods.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

Not applicable

TABLE 1. Isolation rate of bacterial pathogens from subclinical mastitis cases (n=50)

Bacterial strains	*S. aureus*	*Klebsiella spp.*	*Enterobacter spp.*	*E. coli*	*S. aureus & E. coli*	*S. aureus & Klebsiella spp.*
Milk samples (n:50)	*16 (32%)*	7(14%)	3 (6%)	7 (14%)	2 (4%)	4 (8%)

TABLE 2. The biochemical analysis of milk from CMT –negative (n:10) and CMT- positive cows (n:50).

Milk samples	Parameters		Vitamins		Blood electrolytes		Important electrolytes				Alb	T.P
	Bacterial strains		Vit E	Vit A	Na	Cl	M9	P	K	Ca		
CMT -Ve milk	No bacteria	1.14±0.0 9a	13±0.6	34.02±0.8a	33.1±0.06a	103.7±1.8a	8.9±0.09[a]	42.3±0.2a	153.9±2.72a	5.2±0.01a	0.244±0.026	1.51±0.03a
CMT +Ve milk	*E. coli*	35±1.73b	8.3±2.4b	28.3± 1.013b	64.1±2.6b	176.5±3.08b	7.1±0.06[b]	38.5±0.7b	144±2.08b	4.35±0.15	0.216±0.033	1.92±0.015b
	S. ureus	33.5± 0.6 b	10.3±1.3	30.6±1	65±0.7b	183.16±0.66b	7±0.07[b]	34.6±0.33b	144±0.57	3.9±0.14b	0.245±0.031	1.91±0.041b
	Klebsiell a spp.	30.67 ±2 b	8.3±0.6 b	32±1	53±5b	136.6±1.33b	7.55±0.23[b]	39.6±0.33b	143.3.5±1.6b	4.6±0.2	0.203±0.006	1.89±0.01b
	Enterobacter spp.	36b	6.3±0.3b	25±2.6b	62.3±1.6b	113.3±1.6b	7.2±0.06[b]	38±0b	141.6±0.6b	4.1±0.13	0.21±0.01	1.865±0.01b
	S. aureus & E. coli	39± 1.052 b	10±2	31.3±0.7	67.3±0.3b	183.6±2.68b	7.2±0[b]	36.6±0.33b	143.3±1..33b	4.1±0.36	0.25±0.03	1.935±0.02b
	S. aureus & Klebsiella spp.	37g ±0.046b	11±0.5	30.1±1.49	64.3±2.1b	119.1±0.68b	7.15±.0.0[2b]	34±1b	145.5±1.04b	4.45±0.15	0.21±0.088	1.87±0.01b

* Significant using ANOVA test at $p<0.05$. Different letters in the same column are significantly different. Na=Sodium, Cl=Chloride, Ca=Calcium, p=Phosphorus, Mg=Magnesium, K=Potassium

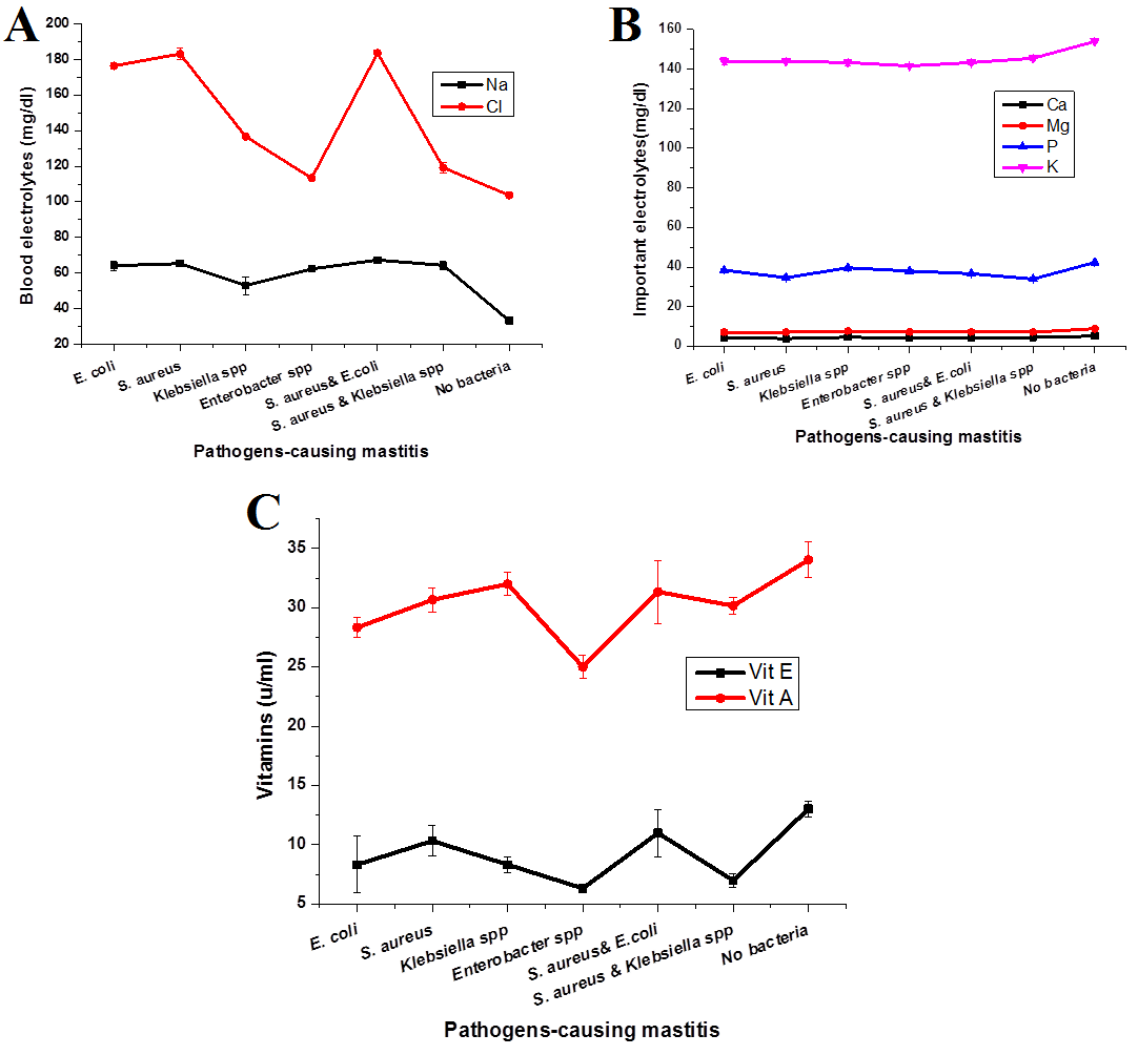

Fig. 1. Biochemical parameters of cow milk positive for single bacterial pathogens (*E. coli, S. aureus, Klebsiella spp.,* and *Enterobacter spp.)* and mixed infections (*S. aureus & E. coli or S. aureus & Klensiella spp*.) versus bacteria negative milk. A: blood electrolytes, B: important electrolytes, and C: vitamins.

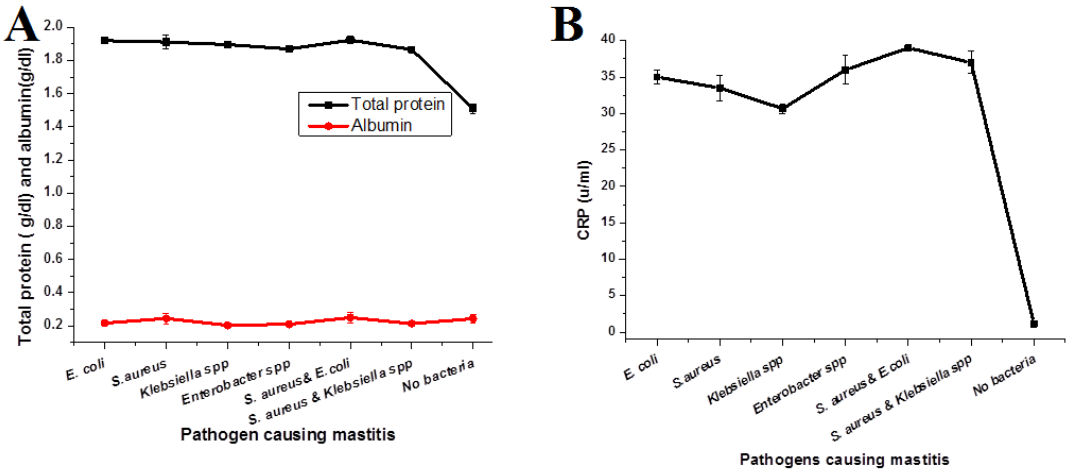

Fig. 2. Biochemical parameters of cow milk positive for single bacterial pathogens (*E. coli, S. aureus, Klebsiella spp., and Enterobacter spp.*) and mixed infections (*S. aureus & E. coli or S. aureus & Klensiella spp*.) versus bacteria negative milk. A: Total protein & Albumin and B: CRP.

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المشخصات البيوكيميائية في الحليب الناجمة عن الأمراض البكتيرية المسببة لالتهاب الضرع تحت السريري

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الملخص

يتميز التهاب الضرع تحت الإكلينيكي بالتهاب في الضرع دون ظهور أي علامات مرضية سريرية. يرتبط التهاب الضرع بشكل رئيسي بالتغيرات الكبيرة في مكونات الحليب البيوكيميائية، والتي يمكن تحديدها كمؤشرات للعدوى المسببة للأمراض. لتحديد التغيرات البيوكيميائية الرئيسية في الحليب ذات الصلة بالكشف المبكر عن التهاب الضرع تحت الإكلينيكي، تم جمع عينات الحليب من 10 أبقار سليمة و50 بقرة تم تشخيص إصابتها بالتهاب الضرع تحت الإكلينيكي بناءً على اختبار كاليفورنيا لالتهاب الضرع، متبوعًا بتحليل المحتويات البكتيرية والمكونات البيوكيميائية. أظهر التحليل البكتريولوجي أن S. aureus هي العامل الممرض الرئيسي المرتبط بالحالات تحت السريرية (32%) إما كعدوى منفردة أو مصحوبه ب E. coli4% أو Klebsiella spp. 8 %. الالتهابات البكتيرية المفردة بواسطة E. coli و Klebsiella spp و Enterobacter spp. تم الكشف عنها في 7%، 7% و 3% من حالات التهاب الضرع تحت السريري، على التوالي. أظهر تحليل التركيب البيوكيميائي لعينات الحليب الموجبة بكتريولوجياً مقابل العينات السليمة انخفاضاً معنوياً في الأملاح المهمة (Ca, P, K, Mg) والبروتينات الكلية، بالإضافة إلى زيادة معنوية في أملاح الدم (Na & Cl)، و بروتينات (CRP). أظهرت النتيجة عدم وجود فرق كبير في جميع العوامل باستثناء CRP بين الالتهابات البكتيرية المفردة والمختلطة. لم تظهر أي تغيرات معنوية في تراكيز الألبومين في جميع عينات الحليب المختبرة، في حين أظهرت تراكيز فيتامين E و A تبايناً بين عينات الحليب المختبرة. . في الختام، فإن بروتين CRP وكذلك البروتين الكلي والدم والمعادن المهمة، لها إمكانات كمؤشرات حيوية لالتهاب الضرع تحت الإكلينيكي، مما يشير إلى أنه يمكن دمجها كاختبارات عملية لمراقبة صحة قطيع الألبان والكشف المبكر عن التهاب الضرع تحت الإكلينيكي.

الكلمات الدالة: المكورات العنقودية الذهبية، التهاب الضرع تحت السريري، CRP، أملاح ، الأبقار، المشخصات البيوكيميائية.