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Bloody Milk in Buffalo Cows: Diagnosis and Trials for Treatment

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Abstract

The present study was conducted on 80 composite milk samples collected from dairy buffaloes secreting bloody milk from all four quarters without any inflammatory signs on mammary gland, systemic reaction or decrease in milk yield at Sharkia Governorate. Somatic cell count (SCC) revealed that 46 samples (57.5%) have SCC range between 200,000 to 250,000 cell/mL, while, 34 samples (42.5%) have SCC below 200,000 cell/mL. California mastitis test (CMT) was negative for 65 out of 80 (81.3%) and positive in 15 out of 80 (18.7%). Bacteriological examination revealed that 56 out of 80 samples (70%) were bacteriologically positive and 24 (30%) were bacteriologically negative. Coagulase positive *Staphylococcus aureus* were identified in 14 out of 56 (25%), however, 42 samples out of 56 (75%) were contaminated with coagulase negative *Staphylococci* (CNS), 20 of them had SCC less than 200,000 X 10^3. All Coagulase positive *S. aureus* were isolated from milk of SCC between 200 X 10^3 to 250 X 10^3 cell/mL. Antibiotic sensitivity test revealed that Gentamycin, Amoxycillin + Clavuylinic acid and Enrofloxacin were the most effective antibiotics on both *S. aureus* and CNS. Group 1, 2 and 3 (bacteriologically positive cases) were treated with Gentamycin, Amoxicillin + Clavulenic or Enrofloxacin in addition to coagulant (Amri-K) showed cure rate of 80%, 80% and 60%, respectively. Group 4 that contained animals with negative bacteriological culture were treated by coagulant (Amri-K) only, showed cure rate of 60%. However, the return rate of the disease was 0, 20, 40 and 40%, respectively. Biochemical and hematological parameters showed non-significant differences between bloody milk and healthy control dairy buffaloes. This study concluded that either coagulase positive or coagulase negative *S. aureus* is incriminated with the bloody milk syndrome in dairy buffaloes in Egypt, however, Gentamycin in addition to coagulant (Amri-k) is the best treatment.

Keywords: Bloody Milk, Buffalo Cows, SCC, *S. aureus*, Amri-K.

Introduction

Blood tinge or haematogalactia in milk could be attributed to injury in capillaries of mammary glands. Sometimes this situation becomes worse when brown chocolate colour (Haematogalactia) secretions are voided from lactating female buffaloes instead of milk [1]. Somatic cell count (SCC) of milk is the actual index of quarter intra-mammary infection, two types of cells, namely, sloughed epithelial cells from the udder cell population and leukocytes from the blood can be detected [2]. The epithelial cells are present in the normal milk as a result of normal breakdown and repair process, while, leukocytes enter in milk from blood, being attracted by chemical substances released from injured mammary tissue. Most of somatic cells are leukocytes, which include macrophages, lymphocytes and neutrophils. Epithelial cells range from 0 – 7 % of SCC but the main increase in SCC occurs due to the influx of neutrophils into the milk. The level of somatic cell increases with the severity of mastitis [2].

Presence of somatic cells (Leukocytes) in milk indicates the disease combating response in animals and is the actual index of level of

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inflammation in mammary gland quarters. In composite milk samples (from all four quarters), SCC of less than 200,000 cells/mL is used as an indication of infection [3]**.** A positive diagnosis of mastitis should fulfill two criteria, a positive bacteriological test and an inflammatory cellular change [4]**.** Milk from uninfected quarters displays little change in SCC as number of lactations increases. Somatic cell count of milk from uninfected quarters rose from 83,000 cell/mL at 35 days postpartum to 160,000 cell/mL by day 285. Somatic cell counts in milk samples from individual animals can be performed using California Mastitis Test (CMT), in which the reagent reacts with genetic material of somatic cells present in milk to form a gel. For reliable results, tests should be conducted just before milking after stimulating milk letdown and discarding the foremilk [5].

The most frequently isolated bacteria from milk samples of mastitis in previous studies were coagulase negative *Staphylococcus* (CNS) followed by *Corynebacterium* spp. and *Streptococcus* spp. [6]. The aim of the present work is to investigate the bloody milk problem in dairy buffaloes in Egypt. Bacteriological and biochemical examination was conducted to identify the causal agents with some trials of treatment after antibiotic sensitivity testing.

Material and Methods

Animals

The present study was carried out on 80 buffaloes, reared individually from private Buffalo farms at Sharkia Governorate during the period between December 2013 and April 2015. There was no elevation in the body temperature and animal appeared with healthy udder. The secretions of the udder were normal milky color but the owners' complaints were a reddish layer float above the surface of the milk after overnight period or slightly bloody milk at milking without any signs of inflammation or decrease milk production. Cases of physiological bloody milk that occur once after parturition were excluded.

Samples

A total of 80 composite milk samples (pool of the four quarts) of 50 mL were collected aseptically from each animal and placed in sterile screw capped bottles [7]. After dry cleaning of the udder, discharging of the first milk squirts, drying of teats thoroughly with an individual towel, apex disinfection with gauze and alcohol 70% were carried out. California mastitis test (CMT) was performed in the field as previously described by Schalm *et al.* [8]**.** The samples were transported in an ice box as soon as possible to the laboratory for further examination. Portion of the milk samples was used for Somatic cell count (SCC) and the other portion for bacteriological examination.

Somatic Cells Count (SCC)

Somatic cells count (SCC) was determined as soon as possible at Animal Health Research Institute, Zagazig Lab using somatic cell counter (MT05, manufactured by PISOFT, SLOVAK REPUBLIC).

Preparation and bacteriological examination of milk samples

Ten milliliters of each milk sample were centrifuged at 3000 rpm for 15 min, and the sediment was subjected to bacteriological examination. Intact erythrocytes were detected microscopically. One hundred microliters of milk samples were streaked directly on to MacConkey and 5% sheep blood agar plates [9]. The plates were incubated aerobically at 37^{o}C for 24 to 48 h. Subcultures of the resulting growth was made on Mannitol Salt Agar for purification of the isolates and identification on the basis of Gram's reaction, morphological findings, colony characteristics and biochemical reactions. *Staphylococcus* species were identified on the basis of catalase, type of haemolysis on blood agar and rabbit plasma coagulase [10].

Antibiotic sensitivity test

The isolated coagulase positive *Staphylococcus aureus* and coagulase negative *Staphylococci* were subjected to antimicrobial susceptibility test by disc diffusion method [10]**.** Sensitivity was measured against 10 antimicrobials including Enrofloxacin (5 mg),

Gentamycin (10 mg), Ciprofloxacin (5 mg), Sulfamethoxazole+Trimethoprim (25 mg), Tetracycline (30 mg), Amoxycillin+Clavulenic acid (30 mg), Vancomycin (30 mg), Oxytetracycline (1 mg), Flourofenicol (30 mg) and Cefotriaxone (30 mg).

Hematological and biochemical examination

Two blood samples were collected from the jugular vein of five apparently healthy control and five randomly chosen bloody milk buffaloes. The first sample was collected with heparin for hematological examination [11]**.** The second blood sample was collected without anticoagulant for serum separation for biochemical analysis. Serum total protein and albumin were measured by spectrophotometer [12,13], also, serum calcium and inorganic phosphorous were estimated [14,15]. Serum Haptoglobin level was determined by Turbidimeteric methods that described according to the manufacturer's guidelines (Beckman Coulter, Inc., USA**),** while C-reactive protein was also determined (Biosystems S.A. (Spain) & Bio- Med Diagnostics, Egypt).

Treatment regime

Four groups were randomly chosen from bloody milk buffaloes under study to be treated by different sensitive antimicrobials. Animals in Group 1 (n=5) were treated by Gentamycin 10% (I.M injection of 4 mL/100 kg BW, daily, divided into two doses for 3 successive days. Group 2, consisted of five animals, were treated by Amoxicillin+ clavulenic acid (Synulox, Pfizer animal health) by I.M injection of 1 mL/20 kg BW, two doses with 3 days interval. Animals in Group 3 (n=5) were treated by enrofloxacin 10% (Enroflox, El-Nasr, Egypt) by I.M injection of 1 mL/20 kg BW for 3 successive days. Group 4 consisted of five animals that were negative for bacteriological examination, they were treated by Vit. K precursor (Amri-K) as coagulant in a high dose (12 ampules) for five successive days. The first three groups were injected also by coagulant (Amri-k) for five successive days. The cured buffaloes (which did not produce blood in milk after treatment) and recurrent cases (animals produced blood in milk after treatment and curing) were recorded to estimate the cure and return rates.

Statistical analysis

The obtained results for hematological and biochemical parameters are represented as mean ± standard error (S.E.) and results with $P \leq 0.05$ were considered significantly different [16]. The results were statistically analyzed using Student′s t-test. SPSS version 21, IBM Corp., Chicago, IL, USA was used for all analyses.

Results and Discussion

Buffaloes are mostly reared for milk production in Egypt. The decrease in milk production and the presence of blood alone in milk or milk mixed with mucus are of the most important reasons for termination of lactation and unwanted culling of dairy buffaloes [17].

The present study revealed that, centrifugation of the milk samples resulted in sedimentation of erythrocytes in the form of a bead at the bottom of the conical centrifugation tube. Also, microscopical examination of wet milk film from the sediment revealed the presence of intact erythrocytes which exclude the red color of the milk that appears homogenous in the test tube either in case of milk pigment of red color or in case of leptospirosis. However, the milk in leptospirosis, is not expected to contain intact erythrocyte but may be stained red due to the hemoglobin as the disease involves the elaboration of a haemolysin by leptospirae [18].

Table 1: Bacteriological examination of bloody milk samples collected from buffaloes in relation to that of somatic cell counts (SCC) (N=80)

Positive samples for bacteriology (N=56)				Negative samples for bacteriology (N=24)			
SCC<200×10^3		SCC= 200×10^3 -250×10^3		SCC<200×10^3		SCC= 200×10^3 -250×10^3	
No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%
20	25	36	45	14	17.5	10	12.5

The present study revealed that California mastitis test (CMT) was negative in 65 (81.3%) of the examined bloody milk samples. The positive samples by CMT (18.7%) showed a degree of traces or weak positive level according to Schalm *et al.* [8]. The CMT is a reliable, easy, rapid and cheap tool and still the gold-standard screening test for the individual mammary quarter of high somatic cell count [19]. Regarding the results of bacteriological examination of bloody milk samples correlated to SCC, 56 out of 80 (70%) were culturally positive, of which, 36 had SCC of 200,000 to 250,000 cell/mL and 20 samples had SCC below 200,000 cell/mL. On the other hand, 24 out of 80 (30%) were culturally negative, of which, 10 had SCC of 200,000 to 250,000 cell/mL and 14 samples had SCC below 200,000 cell/mL (Table, 1). Nearly similar findings were reported by Chavan *et al.* [20], however, these findings were higher than that of Pankaga *et al.* [21]**.** These differences could be attributed to management and hygienic practices at the farm, also, animal factors such as breed, milk yield, stage of lactation and udder morphology could explain such differences [22]. The SCC test gives only an indication that attention should be made to symptoms because SCC cannot distinguish between leucocytic and epithelial cells and there is a tremendous variation between the number of somatic cells with or without mastitis. Interestingly, cows infected with *S. aureus* do not necessarily have elevated SCC. Only 60 percent of the infected cows with *S. aureus* were found in cows producing milk with SCC greater than 200,000/mL [23].

Table 2: Bacterial species isolated from bloody milk samples of buffaloes in a relation to somatic cell counts (SCC).

SCC (number) (Cell/mL)	Total No. of samples	Bacterial species	Positive samples No.	%
200×10^3 –250×10^3 (36)	56	Coagulase positive *S. aureus*	14	25
		Coagulase negative *Staphylococci*	22	39.29
< 200×10^3 (20)		Coagulase negative *Staphylococci*	20	35.71

The number of somatic cells in milk is generally high in different circumstances such as summer months, the beginning and end of lactation, age, genetic history of the cow and the functional disorder of its reproductive organs [24]. Moreover, a difference of 25% from one day to another can be expected and cows in one herd may react differently to

infection than cows from another herd [25]. Therefore, low cell count does not reflect the true bacteriological status of the udder. Also, the significance of latent mastitis which revealed a negative culture with SCC above 200,000/mL cannot be neglected. Since, some of these cases are likely to convert into the subclinical form and subsequently into clinical mastitis, particularly under unfavorable environmental conditions [26].

Somatic cell counts increased more during summer months from June to August in Holstein cows than in cooler months [21]. Moreover, latent infection also reflects the possibility of teat canal infections serving as a potential source of infection to the milk secretory tissue. Even mammary parenchyma may be damaged due to liberation of bacterial toxins in the infected teat canal .Failure to detect pathogens in such cases might be due to intermittent excretion of the organisms or their disappearance because of spontaneous recovery and this may be ascribed to instantaneous use of antibiotics in the animals by the owners themselves [27,21].

Table 3: Antibiotic sensitivity results of *Staphylococcus* species isolated from bloody milk samples of buffaloes

Antimicrobial agent	Code and potency	Coagulase positive *S. aureus* (14) sensitive samples		Coagulase negative staphylococci (42) sensitive samples	
		No.	%	No.	%
Enrofloxacin	ENR_5	13	92.85	37	88.09
Gentamycin	CN_{10}	14	100	40	95.23
Ciprofloxacin	CIP_5	13	92.85	38	90.47
Sulfamethoxazole+Trimethoprim	SXT_{25}	10	71.42	32	76.19
Tetracycline	TE_{30}	7	50	2	4.76
Amoxycillin+Clavulenic acid	AMC_{30}	14	100	38	90.47
Vancomycin	VA_{30}	13	92.85	36	85.71
Oxytetracycline	OX_1	2	14.29	5	11.9
Flourofenicol	FFC_{30}	2	14.29	2	4.76
Cefotriaxone	CRO_{30}	3	21.42	20	47.61

The bacteriological examination of milk samples revealed that *Staphylococcus* spp. was the only organism isolated from bloody milk samples of buffaloes in the present study (Table 2). From these isolates, 14 out of 56 (25%) were coagulase positive *S. aureus* and all of them were isolated from samples with SCC ranged from 200,000 to 250,000 cell/mL. The remaining 42 out of 56 isolates (75%) were coagulase negative *Staphylococci* (CNS). These results were nearly similar to other reported investigations [28-31]. Out of 38 bacteriologically positive buffalo milk samples in India, a total of 44 isolates were identified, of which, 15.9% were coagulase positive *S. aureus* and 47.7% were CNS [21]. In Iran, 38.9% of the examined milk samples were contaminated with CNS [32]. However, in India, 13.7% CNS were isolated from milk of subclinical mastitic cows [33]. The higher susceptibility of milking buffaloes to pathogens could be due to several reasons such as; unhygienic milking places, close contact between healthy and diseased animals in common grazing and wallowing places, unhygienic milking procedures, exposure of teats to injury with inverted thumbs and unweaned calves, pulling and hitting the udder resulting in injury and infection [34].

Table 4: Erythrogram and Leukogram (Mean values±S.E) of healthy and bloody milk buffaloes (N=5)

Parameters	Healthy control buffaloes Group (I)	Bloody milk buffaloes Group (II)
RBCS ($X10^6$/µL)	6.90±0.15	6.56± 0.06
Hb (g/dl)	11.24± 0.43	10.46± 0.24
PCV (%)	31.70± 0.60	30.30± 0.46
MCV (FL)	46.01± 0.97	46.20± 0.51
MCH (Pg)	16.28± 0.36	15.95± 0.33
MCHC (g/dl)	35.42± 0.91	34.51± 0.49
Platelets (count/ µL)	354 ± 31.24	293 ± 17.64
TLC ($X10^3$/µL)	7.87 ± 0.38	8.13 ± 0.43
Neutrophil (%)	39.75 ±0.85	41.25 ±1.11
Lymphocyte (%)	55 ± 0.71	53.25± 1.18
Eosinophil (%)	1.75 ±0.25	1.25 ± 0.25
Monocyte (%)	3.5 ±0.50	4.25 ± 0.25
Basophile (%)	0.00	0.00

***group(II)was contained 4 groups and group (I) was a healthy control. *values did not show any significant differences at p< 0.05**

Regarding the antibiotic sensitivity test of the obtained isolates the results in Table (3) revealed that, Gentamycin and Amoxycillin+Clavulenic acid were the most effective antibiotics on coagulase positive *S. aureus* (100%, each) followed by Enrofloxacin, Ciprofloxacin and Vancomycin (92.85% for each), however, Gentamycin was the best effective antibiotic (95.23%) on coagulase negative *Stapylococci* followed by Amoxycillin+Clavulenic acid and Ciprofloxacin (90.5%), Enrofloxacin (88.1%) and Vancomycin (85.7%). On the other hand, Tetracycline, Oxytetracycline, Flourofenicol and Cefotriaxone were the lowest effective antibiotics on both coagulase positive *S. aureus* and CNS. The current study agrees with Rossetti [35] who found that 100% of *S. aureus*, the most commonly isolated pathogen from mastitis, were sensitive to Gentamycin. Similarly, Gentamycin and Enrofloxacin were reported to be the most effective drugs against *S. aureus* [36]. Recently a higher susceptibility of *S. aureus* and *Escherichia coli* to Amoxycillin+Clavulenic acid and Enrofloxacin was also reported [30]**.** *S. aureus* showed a higher susceptibility to Cotrimoxazole (100%) and Oxytetracycline [27]**.**

The efficacy of treating buffaloes with bloody milk revealed that, Groups 1, 2 and 3 (bacteriologically positive cases) were treated with Gentamycin, Amoxicillin+Clavulenic or Enrofloxacin in addition to coagulant (Amri-K) and showed cure rates of 80%, 80% and 60%, respectively. Group 4 that contained animals with negative bacteriological culture were treated by coagulant (Amri-K) showed recovery of 60% of the animals. However, the recurrence of bloody milk in the treated animals was with the percentages of zero, 20, 40 and 40% in the four groups, respectively. Treatment of buffaloes suffering from bloody milk syndrome with Gentamycin 10% in addition to coagulant induced the best results. The cure rate in this study ranged between 60-80% which is higher than that recorded in ten Dutch herds (34%) treated from subclinical *S. aureus* mastitis [37]. All treated cases in our study were in the lactation period and the successful treatment during lactation is greater if detected and treated early, whereas, the response is lower when treating chronic infections [38]. The intermittent changing pattern of antibiotic susceptibility against *Stapylococci* could be attributed to the misuse of different antibiotics resulting in development of resistance [27].

Table 5: Some biochemical parameters (Mean values±S.E) of healthy and bloody milk buffaloes (N=5)

Parameters	Healthy control buffaloes Group (I)	Bloody milk buffaloes Group (II)
Total protein (g/dl)	7.68 ±0.09	7.97 ± 0.12
Albumin (g/dl)	3.86 ± 0.17	3.69 ± 0.20
Globulins (g/dl)	3.82 ± 0.15	4.28 ± 0.29
Calcium (mg/dl)	10.26 ±0.13	10.82±0.21
Phosphorous (mg/L)	6.93 ± 0.23	6.44 ±0.30
C-reactive protein (mg/L)	5±0.71	6.75 ±0.63
Haptoglobin (g/L)	0.49 ± 0.04	0.66 ±0.06

***group(II) was contained 4 groups and group (I) was a healthy control. *values did not show any significant differences at p< 0.05.**

The results in Table (4) revealed a non significant reduction in RBCs, Hb, PCV and platelets values in buffaloes secreted blood tinged milk. The values of TLC, Neutrophil, serum total protein, globulins and inflammatory markers (C-reactive protein and Haptoglobin) showed a non significant elevation in the affected buffaloes (Tables 4 and 5). These findings indicated the localization of the infection in the udder that produced minimal systemic reaction. Serum Calcium and Phosphorous were within normal levels.

Complete blood count (CBC) including the leukon and erythron evaluation is commonly used to assess the systemic status of sick animals [39]**.** However, it has been reported that changes in the hematological and biochemical analyses in cases of mastitis were limited to those caused by Gram-negative bacteria but not Gram-positive bacteria [39-42]. Lack of endotoxemia in cases of clinical mastitis caused by Gram-positive bacteria was suggested as an explanation for this difference [40]. Our results were in contrary to Faris and Selim [43] who noticed significant decrease in the values of RBCs, Hb, PCV and Platelets in addition to serum calcium, while TLC, neutrophil, serum total protein, and globulin were significantly increased in buffaloes secreting bloody milk.

Conclusion

It could be concluded that, biochemical and bacteriological examination of bloody milk cases must be conducted before beginning of treatment. Gentamycin 10% in addition to coagulant induced the best results. The annual occurrence of bloody milk cases in the period extended from December to April denotes the need for further epidemiological investigations.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربى

اللبن المدمم في الجاموس: التشخيص ومحاولات العلاج

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أجريت هذه الدراسه على ٨٠ عينه مجمعة من إناث الجاموس الحلاب التى تفرز لبن مدمم من جميع الأرباع دون ظهور أى أعراض التهابات على الضرع أو إنخفاض ملموس فى إنتاج اللبن من الحيوانات المصابة فى محافظة الشرقيه. وقد أظهرت نتائج إختبار العدد الكلي للخلايا الجسدية أن ٤٦ عينه بنسبة ٥٧.٥% ينحصر العدد الكلى للخلايا الجسدية ما بين ٢٠٠٠٠٠ إلى ٢٥٠٠٠٠ في حين كانت ٣٤ عينه بنسبة ٤٢.٥% تحتوى على عدد خلايا جسدية أقل من ٢٠٠٠٠٠ . أظهر إختبار الكاليفورنيا أنه سالب لجميع العينات فيما عدا ١٥ عينة فقط كانت إيجابيه له بنسبة ضعيفة. الفحص البكتريولوجي كان ايجابى فى ٥٦ من ٨٠ عينة تم فحصها بنسبة ٧٠% وكان سالبا فى ٢٤ من ٨٠ عينه بنسبة ٣٠% . تم تصنيف ١٤ عتره فقط على أنها ميكروب المكور العنقودى الذهبى بنسبة ٢٥% بينما كانت ٤٢ عتره بنسبة ٧٥% تتبع ميكروب المكور العنقودى السالب التلزن، عشرون منهم كان العدد الكلي للخلايا الجسدية أقل من ٢٠٠٠٠٠ x ١٠^{٣}، في حين كانت جميع عينات الميكروب المكور العنقودى الذهبى تحتوى على عدد خلايا الجسديه ما بين ٢٠٠٠٠٠ الى ٢٥٠٠٠٠ خليه / مل^{٣} . وقد أظهر اختبار الحساسية للمضادات الحيويه أن الجنتاميسين والأموكسيسيلين + حمض الكلافيولينك والإنروفلوكساسين تمثل أفضل المضادات الحيويه تأثيرا على المعزولات سواء المكور العنقودى الذهبى أو المكور العنقودى سالب التلزن . عولجت المجموعات ١و٢و٣ (إيجابية العزل البكتريولوجى) بالمضادات الحيوية وهي الجنتاميسين والاموكسيسيلين + حمض الكلافيولينك والإنروفلوكساسين مع إعطاء مساعدات التجلط مثل فيتامين ك وكانت نسبة الشفاء ٨٠و٨٠و٦٠% على التوالي. أما المجموعة الرابعة والتي تشمل حيوانات سالبة للعزل البكتريولوجى وعولجت باستخدام مساعدات التجلط فقط مثل فيتامين ك فقط فقد أظهرت نسبة شفاء ٦٠%. في حين كان معدل الإنتكاسة المرضية صفر،٤٠،٤٠،٢٠% علي التوالي وقد اظهرت القياسات الهيماتولوجية والبيوكيميائية عدم وجود إختلاف معنوي بين الحالات المصابة والسليمة. نستخلص من هذه الدراسة أن الميكروب العنقودي سواء الموجب أو السالب التلزن هو الميكروب المتهم في حالات اللبن المدمم في الجاموس الحلاب في مصر. كما أثبتت الدراسة أن الجنتاميسين بالإضافة إلي مضاد التجلط (أمري –ك) هو العلاج الأفضل لها.