BACTERIOLOGICAL, CYTOLOGICAL, AND HEMATOLOGICAL CHANGES ASSOCIATED THE OVINE SUBCLINICAL MASTITIS

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ABSTRACT

The problem of subclinical mastitis and the associated bacteriological, cytological, and hematological changes were studied in a sheep flock consists of 28 lactating ewes. 56 milk and 28 duplicate blood samples were collected. The milk samples were tested firstly by California mastitis test (CMT) and the animal prevalence rate was 85.7% (24/28) while the udder halves prevalence rate was 67.9% (38/56). By bacteriological examination, Coagulase Negative Staphylococci (CNS) were isolated from 37 samples (as a single pathogen from 33 samples and mixed with *Staph aureus* from 4 samples) with a prevalence rate 66.1%, while *Staph aureus* was isolated from 5 samples with a prevalence rate 8.9% (as a single pathogen from 1 sample and as mixed infection with CNS from 4 samples). The isolated bacteria were subjected to the antibiotic sensitivity test and it was found that all the CNS isolates were 100% sensitive to both Enrofloxacin and Gentamicin, while the *Staph aureus* isolates were sensitive to Enrofloxacin and Oxytetracycline. Somatic cell count (SCC) were estimated in these milk samples and 7 samples were found to have SCC in the range from $250x10^3$-$500x10^3$ cells/ml and the rest of samples contain SCC level above $500x10^3$ cells/ml. The results of hematological and serum biochemical examinations revealed that there is no significant changes in the examined parameters between infected or control groups.

Keywords: *Subclinical mastitis, Staph aureus, Coagulase-negative Staphylococci, Sheep, SCC, CMT*

INTRODUCTION

Subclinical mastitis is a worldwide problem and its economic importance attributed mainly to its higher prevalence and effect on quality and quantity of the produced milk. Due to its economic, hygienic and legal importance in Europe (EU directives 46/92 and 71/94 defining the bacteriological quality of milk), subclinical mastitis constitute a significant problem in sheep with a prevalence rate ranged from 7.05% to 92% (Ergün *et al.*, 2009).

Subclinical mastitis is the most important factor affecting quality and quantity of sheep milk, and coagulase negative *Staph* (CNS) is the mostly isolated pathogen from sheep mammary gland (Leitner *et al.*, 2004).

Somatic cell count (SCC) considered a very helpful diagnostic tool in diagnosis of subclinical mastitis in dairy ewes (Davasaztabrizi *et al.*, 2013). It correlate very well with the total bacterial count and in case of bacteria free udder the level of SCC is approximately $200x10^3$ cells/ml (Olechnowicz and Jaśkowski, 2012). Level of milk SCC is greatly affected by the type of the invading pathogen (Gonzalo *et al.*, 2002).

Staphylococcus spp are the most frequently isolated pathogens from cases of intramammary infection in sheep (Burriel 1998, Ariznabarreta *et al.;* 2002 and Bergonier and Berthelot 2003).

Staph aureus is mostly isolated from sheep cases with clinical mastitis and the Coagulase-negative Staphylococci are the most frequently isolated pathogen from subclinical intramammary infection (Bergonier *et al.*, 2003).

Due to their opportunistic nature, the prevalence of CNS increased greatly with the bad milking hygiene (Contreras *et al.*, 2007).

Although bovine subclinical mastitis has been widely studied in different regions of Egypt, studying of such problem in sheep to some extent is limited so this study aims to thorough light on the prevalence and the main etiologic agents associated with subclinical mastitis in a sheep flock located in Assiut governorate, Egypt. Study the relation between udder infection and level of milk SCC, determine the effective antibiotics against the isolated pathogens and also study the possible hematological and biochemical changes associated with subclinical mastitis.

MATERIALS and METHODS

Animals:
28 lactating Ewes were used in this study and they were apparently healthy, and free from clinical mastitis and any other visible udder lesions. Milking hygiene is poor and teat dipping procedures were not used.

Samples:

a- Milk samples:
Milk samples were collected under aseptic conditions from each udder halves after washing of the udder with running water, dried with separate paper towels and the teat orifices were disinfected with cotton swabs soaked in 70% ethyl alcohol. Under aseptic precautions 56 milk samples, representing different 56 udder halves of 28 milking ewes, were separately collected in sterile bottles after discarding the fore milk. Each milk sample was examined for abnormal colour and presence of clots. The milk samples were transported refrigerated to the laboratory.

b- Blood samples:
For each ewe two blood samples were collected by puncture of the jugular vein: one with heparin and the other one without anticoagulant.

California mastitis test (CMT):
CMT was performed on all milk samples using the method described by Schalm *et al.* (1971). According to visible reactions the results were classified into 5 scores: (0) = negative, (±) = trace, (+1) = weak positive, (+2) = distinct positive, and (+3) = strong positive.

Somatic Cell Count (SCC) according to Alekish *et al.* (2014):

Milk samples were thoroughly mixed and from each sample 0.01 ml was spread over an area measures 1 cm^2 of a glass slide then left to air dried and stained by Newman-Lampert stain and examined microscopically.

Bacteriological examination according to (Ariznabarreta *et al.*, 2002):

A standard loopful from each milk sample was spread evenly on 5% sheep blood agar (Himedia laboratories Pvt. Ltd. India) the plates were incubated aerobically at 37°C and examined after 24 and 48 h. Subclinical IMI was defined as growth of five or more identical colonies. In the case of *Staph aureus*, isolates from one colony perinoculum were considered positive. Growth of three or more bacterial types was considered as contaminated culture and eliminated from the study. Isolated bacteria were identified according to colony morphology, hemolytic pattern on blood agar, microscopic examination (Gram staining) and standard biochemical techniques (catalase test and coagulase test) according to Quinn *et al.* (1994).

Antibiotic sensitivity testing:
Antimicrobial susceptibility testing by using disc diffusion standard technique according to Andrews (2008) was applied. The isolated micro-organisms were tested against Penicillin G 10 u, Amoxicillin/ Clavulanic acid 20/10 mcgm, Gentamicin 10 mcgm, Enrofloxacin 10 mcgm and Oxytetracycline 30 mcgm.

Hematological and biochemical examination:
Blood gas analysis and a complete blood count including erythrocyte count, hematocrit, hemoglobin, and total leucocyte count were carried out on the first sample. The blood samples were analysed for pH, bicarbonate (HCO_3), partial tension of carbon dioxide (pCO_2), partial tension of oxygen (pO_2), and base excess (BE) using blood gas analyzer (ABL 5, Radiometer, Copenhagen, Denmark). Hematological analyses of erythrocyte count, hematocrit, hemoglobin and total white blood cells were performed using a Medonic Vet. Hematology Analyzer (Medonic CA 620, Sweden). After centrifugation of the second blood sample, serum samples were collected and then frozen at −20°C for one week; subsequently, analysis of biochemical parameters was carried out. With the serum samples, commercial test kits were used to determine the concentrations of total proteins, albumin, blood urea nitrogen, creatinine and total bilirubin. The activities of aspartate aminotransferase (AST) and γ-glutamyltranspeptidase (GGT) were also measured in serum samples. The biochemical analyses of the selected parameters were spectrophotometrically measured according to the standard protocols of the suppliers.

RESULTS

Fifty six milk samples from 28 ewes were tested firstly by CMT and 38 samples were found to be positive with different scores with a prevalence rate 67.9%. These positive samples were subjected to bacteriological and cytological examinations and the results in details illustrated in the following tables.

Table 1: Prevalence of subclinical mastitis by CMT.

	Examined	CMT			
		+ve	%	-ve	%
Ewe	**28**	**24**	**85.7**	**4**	**14.3**
Milk samples	**56**	**38**	**67.9**	**18**	**32.1**

Table 2: Prevalence of CNS and *Staph aureus*.

	Examined	Single CNS		Single *Staph aureus*		Mixed		Total CNS		Total *Staph aureus*	
		No.	%	No.	%	No.	%	No.	%	No.	%
Milk samples	**56**	**33**	**58.9**	**1**	**1.8**	**4**	**7.1**	**37**	**66.1**	**5**	**8.9**

Table 3: Relation between different scores of CMT and isolated bacteria.

	CMT +1	CMT +2	CMT +3	Total
Single CNS	**12**	**13**	**8**	**33**
Single *Staph aureus*	**1**	**0**	**0**	**1**
Mixed	**2**	**1**	**1**	**4**
Total	**15**	**14**	**9**	**38**

Table 4: Relation between SCC and isolated bacteria.

	SCC (10^6)			
	0.25 - 0.5	0.5 - 1	1 - 2	> 2
CNS	**6**	**6**	**8**	**13**
Staph aureus	**-**	**1**	**-**	**-**
Mixed	**1**	**-**	**2**	**1**
Total	**7**	**7**	**10**	**14**
% (n= 38)	**18.4**	**18.4**	**26.3**	**36.8**

Table 5: Results of antibiotic sensitivity test.

	CNS (n=37)				*Staph aureus* (n=5)			
	Susceptible		Resistant		Susceptible		Resistant	
	No.	%	No.	%	No.	%	No.	%
Amoxicillin/Clavulanic acid	24	64.9	13	35.1	4	80	1	20
Enrofloxacin	37	100	0	0.0	5	100	0	0.0
Gentamicin	37	100	0	0.0	4	80	1	20
Oxytetracycline	33	89.2	4	10.8	5	100	0	0.0
Penicillin G	12	32.4	25	67.6	2	40	3	60

Table 6: Hematological and biochemical findings in subclinical lymastitic and healthy sheep.

parameters	Udder groups			
	Control	**CNS**	***Staph. aureus***	**Mixed**
Erythrocytes, T/L	8.5 ± 0.2	7.8 ± 0.6	8.6 ± 1.3	8.1 ± 1.0
Hematocrit, %	31 ± 2	32 ± 1	33 ± 2	31 ± 1
Hemoglobin, g/L	95 ± 2	92 ± 1	90 ± 2	92 ± 3
Leukocyte count, G/L	6.0 ± 0.2	5.6 ± 0.1	5.5 ± 0.2	5.7 ± 0.2
Total proteins, g/L	73 ± 1	75 ± 0.8	74 ± 1.5	75 ± 1.2
Albumin, g/L	37 ± 0.9	37 ± 0.5	37 ± 0.9	37 ± 0.7
Globulins, g/L	36 ± 2	38 ± 1	37 ± 2	38 ± 1.5
A/G	1.1 ± 0.1	0.97 ± 0.04	1.0 ± 0.07	0.97 ± 0.06
γGT, U/L	27 ± 2	26 ± 1	25 ± 4	27 ± 1
AST, U/L	36 ± 3	32 ± 2	37 ± 5	37 ± 4
Total bilirubin, µmol/L	4.5 ± 0.7	4.9 ± 0.4	4.5 ± 0.7	5.0 ± 0.6
BUN, mmol/L	3.7 ± 0.2	3.6 ± 0.1	3.9 ± 0.2	3.7 ± 0.2
Creatinine, µmol/L	109 ± 2	111 ± 1	113 ± 2	112 ± 1
pH	7.35 ± 0.01	7.34 ± 0.01	7.35 ± 0.01	7.36 ± 0.01
HCO_3, mmol/L	24 ± 4	25 ± 5	26 ± 1	25 ± 1
pCO_2, mm Hg	43 ± 1	44 ± 0.6	44 ± 1.2	43 ± 0.9
pO_2, mm Hg	35 ± 1.1	34 ± 0.6	35 ± 1.1	35 ± 1.0
BE, mmol/L	0.3 ± 0.01	0.3 ± 0.04	0.4 ± 0.08	0.4 ± 0.06

DISCUSSION

Subclinical mastitis is the most prevalent type of mastitis characterized by no detectable changes in the udder and no visual abnormalities in milk. Giadinis *et al.* (2012) reporting the absence of clinical signs specific to subclinical mastitis in sheep. Diagnosis of such cases mainly depends upon bacterial culture results and/or indirect methods as CMT and SCC.

Concerning the results of table (1) it is clear that the animal prevalence of subclinical mastitis was 85.7% (24/28), while the udder half prevalence of subclinical mastitis was 67.9% (38/56). Similar results recoded by Alekish *et al.* (2014) in Jordan as they found that the prevalence of subclinical mastitis between Awassi sheep was 66.9%. While Oliveira *et al.* (2013) in Brazil recorded 40.45% prevalence rate of subclinical mastitis in 42 first partum Santa Ines ewes. Beheshti *et al.* (2010) recorded 17% prevalence rate of subclinical mastitis based on the results of CMT. Difference in prevalence rates may be attributed to difference in mangemental system and study area.

Results in table (2) showing that the prevalence of CNS as a subclinical ovine mastitic pathogen was 66.1%, while the prevalence of *Staph aureus* was 8.9%. Similar results were recorded also by Oliveira *et al.* (2013) as they isolated CNS and *Staph aureus* from sheep with subclinical mastitis and the prevalence rates were 66.93% and 6.3% respectively. Also nearly similar results (76% for CNS and 8% for *Staph aureus*) recorded by Beheshti *et al.* (2010), while Vasiu *et al.* (2008) reported a prevalence rate 54.75% for CNS and 9.52% for *Staph aureus*. Gonzalo *et al.* (2002) isolated CNS and *Staph aureus* from cases of ovine subclinical mastitis in a percentage about 62.25% and 4.3% respectively. Also Ergün *et al.* (2009) recorded prevalence rates 76.5% and 3.1% for CNS and *Staph aureus* respectively. Sayed *et al.* (2012) in Assiut recorded a prevalence rate for CNS about 48.15% and for *Staph aureus* 22.22%.

The high prevalence of CNS may be attributed to the fact that CNS are normal flora of healthy teat skin and so has a great ability to colonize the teat end (Kudinha and Simango, 2002). The ability of different pathogens to colonize the teat canal is very important step for the mammary gland to be infected (Forbes, 1969).

Intramammary infections caused by *staph aureus* is very important from public health point of view due

to its ability to produce thermostable enterotoxins and leukotoxins.

The variation in the prevalence rates between the present study and other studies could be related to differences in managemental system, health status of the flock, geographical distribution, weather, nutritional status and finally the size of the study sample.

Table (3) gives information about the relation between the isolated pathogens and different scores of CMT. It is clear that there're a good correlation between the results of CMT and the bacteriological results so it can be used as an easy and simple indirect test for diagnosis of ovine subclinical mastitis and this is completely agree with Ergün *et al.* (2009) as they stated that the CMT is very useful technique in diagnosis of subclinical mastitis in lactating ewes.

Results in table (4) showing that 27 CNS isolates, 1 *Staph aureus* and 3 mixed infections were associated with high SCC (more than $500x10^3$/ml) while 6 CNS and 1 mixed isolates were associated with low SCC (less than $500x10^3$/ml). Ariznabarreta *et al.* (2002) and Gonzalo *et al.* (2002) recorded that *Staph aureus* infection characterized by high log SCC while within CNS some species showing high log SCC and others have less intense inflammatory response and low log SCC. The difference in SCC response may be attributed to immune status of the host at time of infection and the pathogenicity of the causative bacteria (Alekish *et al.*, 2014). Many authors suggest different normal SCC limits for ewe's milk, Bergonier and Berthelot (2003) mentioned that sheep with healthy udder having average SCC below 500000 cells/ml throughout the lactation period. Hammadi and Yousif (2013) concluded that ewes with SCC $500x10^3$ cells/ml considered positive for subclinical mastitis, while Pengov (2001) stated that measurements of SCC can be used effectively to detect subclinical lymastitic ewes and the comparison with the bacteriological results showing that the threshold value regarded as upper limit for normal SCC of ewe's milk should be $250x10^3$ cells/ml.

Results of table (5) showing the results of antimicrobial sensitivity testing, it is clear that the effective antimicrobial agents are Enrofloxacin and Gentamicin (for CNS), and Enrofloxacin and Oxytetracycline (for *Staph aureus*). The least effective one was Penicillin G, followed by Amoxicillin/Clavulanic acid and this may be attributed to the misuse of antibiotics and frequently incomplete antibiotic treatment courses (Tras *et al.*, 2007). Similar results recorded also by (Aydin *et al.*, 2009 and Abdel-Naser *et al.*, 2010).

Table (6) declared the results of hematological and biochemical findings in subclinical lymastitic ewes (24/28) and healthy control (4/28), it was found that there is no significant changes among the different groups, indicating subclinical mastitis in ewes may has no systemic reaction as the infection is localized in the udder (Ozenc *et al.*,2011).

In conclusion CNS was found to be a major cause of subclinical mastitis in sheep. CMT and milk SCC with threshold level $250x10^3$ cells/ml can be used effectively when rapid diagnosis of subclinical mastitis is needed. The application of antibiotic sensitivity test on the isolated bacteria is quite beneficial before start the treatment. As it is a localized inflammation subclinical mastitis has no effect on hematological or biochemical parameters.

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التغيرات البكتريولوجية، الخلوية والدموية المصاحبة لإلتهاب الضرع الخفى فى الأغنام

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اجريت هذه الدراسة للتعرف على التغيرات البكتريولوجية والخلوية والدموية المصاحبة لإلتهاب الضرع الخفى فى قطيع من الأغنام (يبلغ عدده ٢٨ نعجة حلوبة) وقد تم تجميع عدد ٥٦ عينة لبن و٢٨ عينة دم مزدوجة. تم إختبار عينات اللبن أولا بواسطة إختبار الكاليفورنيا ووجد أن ٣٨ عينة كانت إيجابية والتى خضعت للفحص البكتيريولوجى والعد الخلوى. وقد زرعت العينات على مستنبتات آجار الدم وقد تم عزل ٣٧ عترة من ميكروب المكور العنقودى السالب للتجلط (٣٣ كعترة منفردة ، ٤ عترات مختلطة مع الميكروب المكور العنقودى الذهبى) وكذلك تم عزل ٥ عترات من الميكروب المكور العنقودى الذهبى (١ عترة منفردة ، ٤ عترات مختلطة مع ميكروب المكور العنقودى السالب للتجلط). وقد أظهرت نتائج العد الخلوى للخلايا الجسمية باللبن أن ٧ عينات كانت تحتوى على خلايا جسمية بين ٢٥٠ ألف إلى ٥٠٠ ألف خلية/ مل أما باقى العينات فقد وجد أنها تحتوى على عدد أكبر من ٥٠٠ ألف خلية/مل. وعند إخضاع العترات المعزولة لإختبار الحساسية للمضادات الحيوية وجد أن جميع عترات الميكروب المكور العنقودى السالب للتجلط حساسة بنسبة ١٠٠% لكلا من الإنروفلوكساسين والجنتاميسين ، بينما كانت عترات الميكروب المكور العنقودى الذهبى حساسة بنسبة ١٠٠% لكلا من الإنروفلوكساسين والأوكسيتتراسيكلين. وبالنسبة للفحوصات الدموية والبيوكيميائية على مصل الدم فقد أظهرت النتائج عدم وجود أى تغير معنوى بين الحيوانات السليمة والمصابة بإلتهاب الضرع الخفى. وقد تم مناقشة جميع النتائج بالتفصيل بالبحث.