ISOLATION AND IDENTIFICATION of SOME BACTERIAL PATHOGENS FROM SUBCLINICAL MASTITIS of SHEEP in KIRKUK CITY, IRAQ

Almass.M. AL- bayati[1], Hala. M. Majeed[#2] & Ziyad. T. AL- doori[3]

[1]Department of Medicine , College of Veterinary Medicine, University of tikrit, tikrit , Iraq
[#2]Department of Basic Science, College of Medicine/ Ibn sina University of medical and pharmaceutical sciences, Iraq, Baghdad
[3]Department of public health, College of Veterinary Medicine, University of Tikrit, Iraq
[#2]m.hala17@yahoo.com

Abstract:

Background:- Mastitis is the term for a bacterial infection of the udder. It is most common in ewes raising multiple lambs or with high milk production. Most cases occur during the first weeks after lambing or immediately before weaning. Good ewe nutrition and providing a clean lambing environment are important factors in reducing the incidence of mastitis.

Objective: The study was focused on using of rapid & efficient methods for detection of Subclinical Mastitis in 4 small holder dairy flock in central of Kirkuk &its affiliated areas.

Material and Methods:- A total of 94 milk samples sheep were collected from four farms in October to December 2018 to determine pathogens responsible for subclinical mastitis in sheep.

Results:- Screening Subclinical Mastitis(of SCM) were done using California mastitis and White Slide Test (WST). the prevalence percentage of SCM in California mastitis and White Slide Test according the early stage lactation was(74 % and 70%) respectively. While the prevalence percentage of SCM in California mastitis and White Slide test according the according the dry period lactation was(66 % and 64 %) respectively. identification of the isolates was achieved using Gram's staining, hemolytic pattern, colony morphology, Catalase , Coagulase test, IMVIC test and confirmation of bacteria species by using Vitek 2 compact after identification to the primary biochemical tests using Analytical Profile Index. Bacteriological examination of all milk samples found the presence of (82.85%) isolates where Staphylococcus was predominant species (74.28 %) and the coagulase negative Staphylococcus (25.7%) species was identified at the least bacteria .than staph. aureus, while environmental pathogen represented E. coli second common pathogen followed by klebsiella pneumonia (14.8%) , Manhemia haemolytica (12.8 %) proteus spp.(11.4%) And low percentage to the Enterobacter spp. (4.3 %) .

Conclusion:- subclinical mastitis seems to be, as deduced from the high prevalence observed in this study, an important health problem for milking sheeps in the Kirkuk Application of VITEK system could be practiced at selected samples from time to time to confirm identification of causative organisms.

Keywords : Isolation, Identification, Bacterial, pathogens ,sub clinical mastitis.

INTRODUCTION

Mastitis is an important serious economic disease infect widespread dairy sheep's in many countries of the world , causing reduce in milk production , decrease in growth of lamb & cost of treatment [1].

The word mastitis comprises of two words: mastos means breast and itis means inflammation [2].A broad definition of mastitis is inflammation of the parenchyma of the mammary gland regardless of the causative agent can be clinical or subclinical and can be caused by physical or microbial agents therefore characterized by a range of physical and chemical changes   in the milk and pathologic changes in the glandular   tissue[3].

The most important changes in the milk include discoloration, the presence of clots and and the presence of large ، number   of leukocytes as well as swelling, heat, pain and edema in the mammary gland in many clinical cases[4,5].

Sub clinically infected sheep will produces less milk , and the quality of the milk will be changed Subclinical mastitis is very difficult to be diagnosed due to no obvious clinical signs, no visible changes in milk , somatic cell count of the milk is done for its identification[3,6,7]. Both types mastitis in sheep depend on the nature of causative agent ,age ,healthy condition &lactation period [8].

There are more than a reasoned but the bacterial agent are the most common causes, the bacteria causes include contagious pathogen as Staphylococcus spp which include (*Staphylococcus aureus* , *Coagulate negative staphylococcus*) and environmental pathogen such as *Escherichia coli, proteus spp. Klebsiella pneumonia enterobacter spp*.[9,10 , 34]. From the other hand the incidence of mastitis in sheep causes more effect to the animal production, Especially the production of dairy as well as consumption milk production contaminated with residue of pharmacological treatment   that effect to the human healthy [11,12] . For the economical and healthy has been a study in the Kirkuk city in northern of Iraq :

1- Prevalence of subclinical mastitis in sheep according to the stage lactation.
2- Determine by Chemical screen farm test (chalifornia & white sid test).
3- Isolation and identification of some bacterial causes sub clinical mastitis .
4- Confirmation diagnosis using Vitek 2 compact

MATERIAL AND METHOD

samples collection

A total of 94 milk samples were collected from 4 different dairy flocks during the period from October to December 2018 in Kirkuk Governorate (Iraq). After a quarter had been cleaned by removing any possible dirt and washed with tap water, the teat end was dried and swabbed with cotton soaked in ethyl alcohol. Approximately, 100 ml of milk was collected aseptically into sterile bottles after discarding the first three milking streams. Milk samples from each quarter were transported to the research center laboratory in ice cooled box at 4 ºC and analyzed immediately.

Indirect screening test (California mastitis and White Slide Test

The milk samples were subjected to following diagnostic tests: California mastitis test (CMT) and   white side test (WST). The procedure of CMT was followed in this study as per manufacturer's instruction on quarter fore-milk samples. In brief, 2 ml of milk sample was taken in the CMT paddle and equal quantity of CMT Kit (Leucocytest®, Synbiotics

Corporation-2, Lyon, France and marketed by Advance Chemical Co. Bangladesh Ltd.) was added in each cup, rotated for few seconds and the result was recorded within 30 s as 0 (negative), T (trace), 1+, 2++, or 3+++ [13].

The WST was performed as per procedure described by [13], in brief, after thorough mixing avoiding violent shaking, 50 μl (five drops) of milk were placed on a glass slide with a dark background by micropipette. Subsequently 20 μl of WST reagent (4% NaOH) were added to the milk sample and the mixture was stirred rapidly with a toothpick for 20-25 seconds. A breaking up of milk in flakes, shreds and viscid mass was indicative of positive reaction. On the other hand, milky and opaque and entirely free of precipitant was indicative of negative reaction.

Isolation & Identification of bacteria
only positive samples from each milk sample to the CMT & WST Interpretation of the result as follows For bacteriological investigation , 100 microliter Was inoculated in on 5% sheep blood agar (LAB) , Maconkey agar (Oxoid, England) and manitol salt agar (LAB) . Primary identification of bacteria was done based on colony morphology, type of hemolysis , Gram's staining and pure cultures were identified up to genus level as [14,15].Gram staining, Motility test, Catalase activity, coagulase test, sugar fermentation test, Haemolysin test, Indole test, Methyl Red test, Voges – Proskauer test, Citrate utilisation tests were done on a 24-48 hour old pure culture for the identification of the bacteria and confirmation of the species specific characteristic [15, 16].

Identification using Vitek® compact 2 system
Identification has been done by automatic method (Vitek® 2 Compact, Biomérieux, France) . Gram positive and negative bacteria were identify using Gram positive ID card and Gram negative ID card respectively (Biomérieux, France[17- 35].

Significant automated identification was conducted for confirmation diagnosis organism using *staphylococcus spp*. And *enterobacteriace spp.* card contained different substrates for identification different gram-positive organisms and gram negative organism As the results were obtained 8hours (Table 3), slash line identification was done with low discrimination of mixed colonies for two organisms and represented a pathogenic organism of subclinical mastitis in ewes in the current study highly automated identification system the new VITEK gram positive and negative identification cards provide stable and decisive result of bacteria identification[18]. The result from this study will provide a data of specific mastitis-causing bacteria for further rapid detection[19- 20].

RESULTS

Prevalence of subclinical mastitis at different stage of   Lactation
According to the period of the present study, after lambing (early lactation) the prevalence of SCM from 47 milk sample were positive 37 (74%) &35 (70%) according to CMT,WST respectively. On the other hand before lambing (dry period)the prevalence of SCM from 47 milk sample were positive 33 (%66) & 32 (64) according to CMT,WST respectively (table:1and 2)

From milk samples of CMT and WST positive ewes (70) Cultural characterization of bacteria in the Bacterial isolation:

Tab1e- 1:Indirect Screening of subclinical mastitis at different stage of Lactation

TEST Period	Positive	%	Negative	%
CMT(dry period)	33	66	14	28
CMT(early lactation)	37	74	10	20
WST(dry period)	32	64	15	30
WST(early lactation)	35	70	12	24

Table 2 : percentage accuracy of various indirect test used for the diagnosis of mastitis .

Test	Total Number	%
CMT	70	74.46
WST	66	70.2

Table3: Biochemical tests used for identification of *Staphylococcus ssp.*

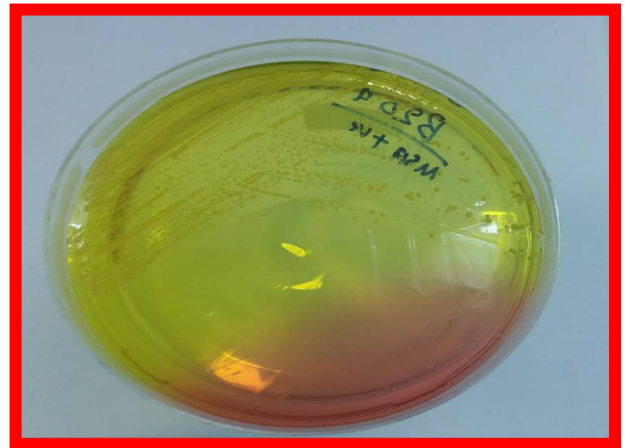| Biochemical test	*Staphylococcus aureus*	*Staphylococcus* Spp. (CNS)
Gram stain	+	+
Haemolysis	+	-
Catalase	+	+
Coagulase	+	-
Oxidase	-	-
Growth on MSA	+	-

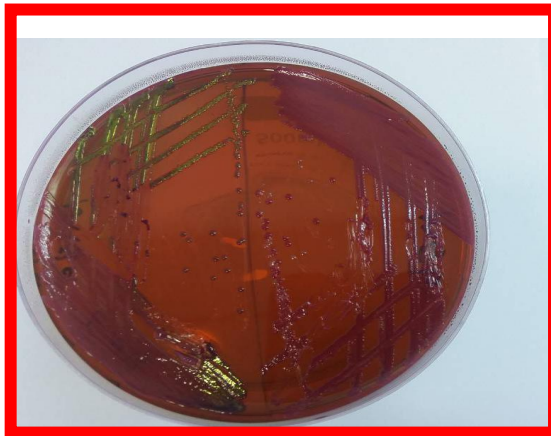Different speciece growth(G+&G-) on blood agar

Fermented& non fermented on the maCconky
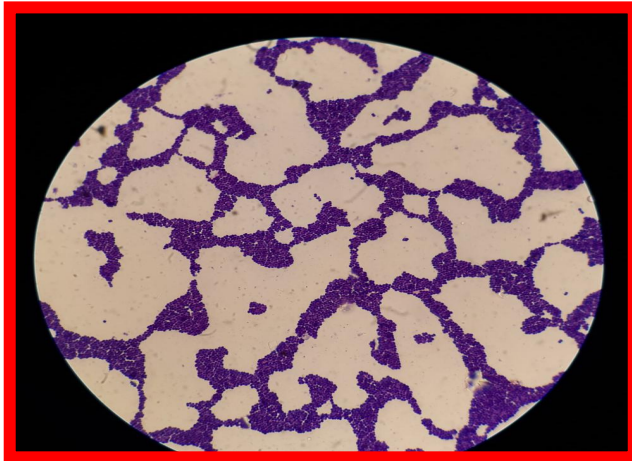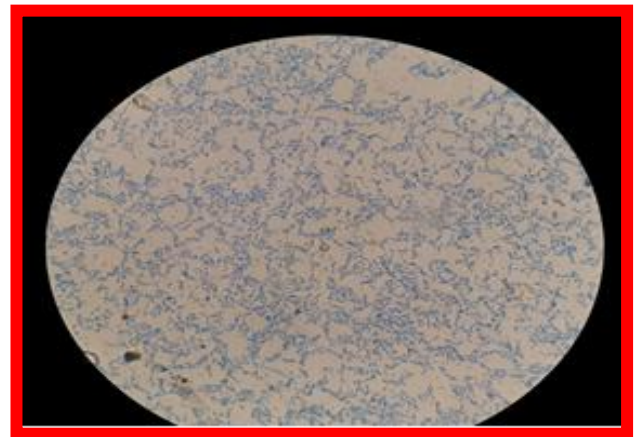
CNS from a colony on nutrient agar Gram's stain

Staphylococcus golden yellow pigment on MSA

E.coli showing metallic sheen on EMB agar   *Pasterella spp* (Bipolar Staining)   .

Figure (1). Bacterial organism isolated from cases of SCM in sheep

Table4: Bacterial organism isolated from cases of SCM in sheep.

Bacteria isolate	Number of isolate before lambing(dry period)	Number of isolate After lambing(early lactation)	Sum	%
Staphylococcus aurues	20	32	52	74.28%
Escherichia coli	8	26	34	37.2%
Staphylococcus spp.(CNS)	13	5	18	25.7%
Klebsiella pneumonia	2	8	10	14.3%
pasterella .Spp	9	-	9	12.8%
Proteus spp	-	8	8	11.4%
Enterobacter spp	1	2	3	4.3%
NO growth	10	2	12	17.14%

Biochemical test
Coil form Bacterial identification was done by biochemical test (Table 5).

Table5: Biochemical properties of bacteria isolated from Subclinical mastitis milk samples

Biochemical tests	E.coli	Klebsiella pnemonia	Pasterella spp.	Enterobacter Spp.
G- rod	-	-	-	-
Motility	+	-	-	-
Oxidase	+	-	+	-
Catalase	+	+	+	-
Urea	-	+	F	-
Methyl red	+	-	-	-
Indole	+	-	-	-
Sugar(Lactose)	-	+	-	+
Maconky grow	+	+	-	+
EMB grow	+	-	-	+
Methylene blue	-	-	+	-

4 Biochemical Profile of Gram-negative Isolates by using Vitek 2 system

The identification of the pathogen was confirmed using the automatic biochemical identifying system Vitek 2 Compact using Gram-Positive and Gram-Negative cards (table 6).

Table 6 : Biochemical Profile of Gram-negative Isolates from Subclinical mastitis milk samples using Vitek 2 system

Code	Reagent	Identification of Gram-negative isolates using GN card		
		E.coli	K. pneumonia	Enterobacter erogenes
9	BGAL	-	+	+
11	BNAG	-	-	-
17	BGLU	-	+	+
18	dMAL	+	+	+
23	ProA	-	+	-
33	SAC	-	+	+
36	CIT	+	+	+
43	NAGA	-	-	-
45	PHOS	-	+	-
48	LDC	+	+	+
57	BGUR	+	-	+
64	ILATa	-	+	-

DISCUSSION

In the present study it was evident that CMT was the most reliable test and closest to the bacteriological results on other hand SCC is found out to increase in the early stage of lactation the SCM recorded in tis study according to the CMT & WST (74%) ,(70%) , where the incidence of SCM in dry period of lactation recorded low percentage as (66%) and (64%) respectively. The result of high level in initial lactation similar to [21].

The reaction of CMT and WST seem to depend on the concentration of somatic cell count in the milk [22]. Present finding are agreement with[23] reported higher reliability of CMT (85.69%) followed by modified WST (79.74%) . For the relationship between indirect screen tests(CMT& WST) positive results and bacteriological isolations, the present study record highly relationship the score of these tests to the bacterial isolation(82.%) similar to the other authors Were reported relationship between the result of CMT and WST with pathogens isolation [24, 25, 35].

Among different bacterial pathogens isolated, *Staphylococcus spp.,* were most prevalent followed by., *Escherichia coli*. Almost similar pattern was noticed by reference.[26] who reported *Staphylococcus spp.* (53.33%) as predominant isolates ,the present study agreement with the result of [27- 29]. [27.]   reported   that reported *Staphylococcus aureus* as predominant pathogen represented (39 %). [28] reported predominant of *Staph.aureus* about 61% in SCM in north of Syria, as well as [29] reported *Staph .aureus* 72.2% as most common bacterial species from SCM .

The main mechanism for prevalence of *Staph. aureus* in sheep was milking procedure lamb can enhance the spread of *Staph.aureus* in flock by suckling ,wash cloths ,bedding ground may increase the prevalence of *Staph.aureus*[30-31]. *Escherichia. coli* constituted 37.3% was the second most prevalent bacterium isolated from the milk samples followed by *Klb. pneumonia*(14.5%),*pasteurlla spp.* (12.8%),*proteus spp.* (11.4 %) and *enterobacter. Spp* at low represented (4.3 %) from total isolate .

The occurance of *E. coli* in sheep milk can be high and the environment main role, the prevalenc of *E .coli* and other environmental pathogen that reported by [32], *E.coli* 14.28% followed by *Kle. pneumonia* 14.28% ,pasterella spp. 10 % as well as, that were *Klebseilla pneumonia* constituted and Proteus spp. constituted 14.6% and *Enterobacter spp* constituted 3% of the total isolates (21.87%) on the other hand, *M. haemoletica* recorded by [33] as the most common causes of mastatic milk in sheep ,the Mavrogianni reported of *m. haemolytica* rate of 11%, the present study nearly to their studies.

CONCLUSION
Staphylococcus is predominant and the coagulase negative Staphylococcus species is identified at the least SCM causing pathogens. The mastitis causing pathogens are *staph. aureus , Stphylococcus coagulate negative , E.coli, Klebseilla pneumonia,Proteus spp. and Enterobacter spp*

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