بسم الله الرحمن الرحيم

اقرأ باسم ربك الذي خلق خلق الانسان من علق اقرأ

وربك الاكرم الذي علم بالقلم علم الانسان ما لم يعلم

صدق الله العظيم

Ministry of higher education and scientific Research

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College of veterinary Medicine

Detection of bovine mastitis in Diyala province

By

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Dedication

Have done my best to completed this research to dedicate his success if the God will help me and my mother who missed always because she taught me the determination to achieve my ambitions ,despite of the difficulties of life and the surprise of fate , and I dedicate To my brother And two sisters , I ask God that sustains for me their vision and safety from all evil and accept my supplication that include us the intercession of our prophet Muhammad by the best payer .

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To Become my research a first step in the success and discretion I hope you turn the pages with interest and attention I'm admitting with modest in science and I do not dream more than acceptation ..

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My lecturers you have a favor in my learning as a favor of mother for born

I can only pray and ask the God for you to reward you great boon

The Researcher

Ragad Kasem Hameed

Contents **pages**

Summary

This study was conducted to investigate the bacterial causes of bovine mastitis. The samples were subjected to physical tests, as well as Somatic cell count (SCC) and bacteriological examination,

For these purposes, 25 lactating cow , 2.5 – 5 years old of local and Frisian breeds were applied. 100 milk sample were collected a aseptically .

Total isolated were 40 isolate caused by gram positive &19 caused by gram negative .The results of the bacterial culturing confirmed the constant fact that mastitis is a multifactorial entity , different bacterial isolates were obtained from infected halves as follows, *Staphylococcus aureus* 57.14% Coagulase negative Staphylococci 14.28%, *Pseudomonas Spp.* 8.92% , *Pasteurella Spp.* 5.3% , *Escherichia coli* 5.30 %, *Proteus Spp.* 1.78% , *Klebsiella Spp.* 5.3% and *Salmonella Spp* 5.3%.

The result showed that A SCCs of ≥ 500000 cell/ml milk were considered to be positive for mastitis.

Introduction

Mastitis is inflammation of parenchyma of mammary gland, regardless of the causative agents , but it may also be caused by injury and less commonly by allergy and neoplasm (Menzies and Ramanoom, 2001 ; Radostits *et al*., 2007).

Most of the research works conducted to study the impact of mastitis in the livestock industry have focused on dairy cattle. However, many researchers have begun to evaluate the extensiveness and significance of this problem.(Gonzalo et al .,2002) It is well documented that there is a high relationship between amount of milk produced and consumed and pre weaning calve growth performance. Subclinical and clinical cases of mastitis may reduce milk production, and therefore reduce calve performance, as well as cow longevity., a reduction in mastitis problems would increase cow longevity and decrease culling losses. Curtailing mastitis problems would also reduce the need for cross fostering or artificial rearing of lambs which increases costs, labor, and management inputs (Leitner *et al*., 2004).

Mastitis is the outcome of a complex interaction between host, causative agents (micro-organisms) and environment and remains a major cause of both an extreme zoonotic and economic importance throughout the world *Arcanobacterium pyogenes, Staphylococcus aureus, Streptococcus agalactiae, Strepococcus uberis, Escherichia coli, Klebsiella pneumoniae, Micrococcus, Pasterulla haemolytica* and *Pseudomonas aeruginosa* were isolated from mastitic mammary glands either in the pure or mixed form from infected cases (Menzies and Ramanoom, 2001)

This study aims at:

1- Isolation and identification of different bacterial causes of mastitis in cow ,diagnosis and classification of clinical and subclinical mastitis .

2- Relationship between Somatic cell count and bacterial isolation.

Review of literature

2-1- **Definition of mastitis**

Mastitis is major production limiting disease of dairy animal all over the world, the term is derived from the Greek words mastos meaning (breast) and itis meaning (inflammation of) (Petersson *et al*., 2011).

Mastitis is inflammation of the mammary gland parenchyma and characterized by rang of physical and chemical changes in milk and pathological changes in the glandular tissue (Radostitis *et al*., 2007).

2-2- **Economic importance of mastitis**:

Clinical and subclinical mastitis directly affect mammary gland function and have a great economic impact on the dairy industries The forms of loss in milk cow are the same as those for dairy animal. In meat cow the losses take the form of deaths, due usually to gangrenous mastitis, and to decreased growth in the neonates, calve have access to supplemental feed the effect of subclinical mastitis on calve negligible, in cows are milked commercially for the production of cheese (Guerreiro *et al*., 2013). Any factor that adversely affects the quantity and quality of bovine milk is of great financial interest (Radostitis *et al*., (2007).

2-3- **Etiology**

As previously reported, Coagulase positive Staphylococci are the predominant bacteria causing subclinical mastitis in cow (Bergonier *et al.,* 2003).

The primary cause of subclinical mastitis in cow is *Staphylococcus spp*. ,*Streptococcus* spp. and *Micrococcus* spp. *Mannheimia glucosida, M. haemolytica,* and *M. ruminalis* were isolated from cases of acute mastitis in cow (Ndegwa *et al.,* 2000). In study of Green, (1984) reported that Coagulase negative *Staphylococci* (CNS), are the most prevalent isolations, ranging from 25 to 93 %, before *S. aureus* (3 to 37%) mainly isolated from infections then a chronic (less severe ones). Among the *CNS*, *S. epidermidis* then *S. xylosus*, *S. chromogenes* and *S. simulans* are the more frequently isolated in cows.

The identification of *Nocardia spp*., is also considered important due to their potential for causing disease in humans, and because *Nocardia farcinica* is known to cause mastitis in cow (Berriatua *et al*., 2001; Maldonado *et al*., 2004). Indeed, *N. farcinica* is a significant public health concern owing to its aggressiveness, tendency to disseminate, resistance to antibiotics and laborious biochemical identification (De La Iglesia *et al*., 2002).

Nevertheless, despite the accepted role of these bacteria as major IMI-causing pathogens in small ruminants, the pathogenicity of the different *CNS* species varies widely. The most commonly isolated CNS species in persistent subclinical IMI in cow are *Staphylococcus epidermidis*, *S. simulans*, *S. chromogenes* and *S. xylosus* (Contreras *et al*., 2003).

Although a wide range of microorganisms may cause bovine mastitis, most cases are reported to be due to staphylococci. while both *CNS* and *Staphylococcus aureus* are frequent causes in cow (Hariharan *et al*., 2002) *Mannheimia haemolytica* (Bocklisch *et al*.,1994), *Escherichia coli*, various *Streptococci* , Salmonella Spp., Proteus Spp., were also concerned However, *S. aureus* can also be cultured from milk and other body sites, transfer during milking is considered an important mechanism for the spread of this organism (Vautor *et al.,* 2003), transmission of *S. aureus* between ewes could be a result of the herdsman transmitting where *S. aureus* could be transmitted between cow during manual udder control, or the udder being exposed to bedding material contaminated from infected cows (Kirk and Glenn*.,* 2004).

Staphylococcus aureus was the most prevalent pathogens in the clinical mastitis (44.44%) while *Staphylococcus epidermidis* was the most prevalent pathogens in the subclinical mastitits (53.84%) in Baghdad (Al-obaidy ., 2010).

2.4- Forms and clinical signs of mastitis

According to clinical classification mastitis can be divided into clinical and subclinical categories. Clinical mastitis can be determined by a change in milk secretion, a changes in the udder, and or a systemic changes in the diseased animal. Whereas Subclinical mastitis cannot be diagnosed by gross examination of the animal, its udder, or milk and therefore requires a determination of the somatic cell count of the milk and/or a milk culture to determine the presence or absence of an intramammary pathogen. Moreover according to the stage of inflammation clinical mastitis can be further divided into three categories: peracute, acute, and chronic (Menzies and Ramanoon, 2001).

Peracute or gangrenous mastitis is characterized by depression, a fever which may progress to subnormal temperature, when the animal going to die dehydration, anorexia, and a swollen mammary gland (Bor *et al.,* 1989). The gland may be hyperemic and warm initially and progress to cyanotic and cold, milk secreted from the gland may be serum-like, blood-tinged, and mixed with gas, affected animals frequently become recumbent and case fatality may reach 30-40% if animals are left untreated, if the animal survives the initial infection, the affected half may slough off over a period of weeks (Ameh *et al.,*1994).

Acute mastitis is characterized by a warm, swollen, hyperemic, painful gland, and/or secretion of abnormal milk, may be serum-like or contain clots and flakes. Affected animals may show signs of anorexia, depression, fever, tachycardia, and recumbency but are not usually as severely affected as those with per acute mastitis The organisms listed as potential causes of peracute mastitis are also associated with acute mastitis (Anderson, 2009).

Chronic clinical mastitis may lead to palpable changes in the consistency of the udder, leading to an udder which is fibrotic and may have palpable abscesses or nodules. Long term, the udder may become shrunken and produce less milk. (Menzies and Ramanoon, 2001).

Materials and Methods

3.1- Materials:

3.1.1- Animals used in clinical study:

3.1.1.1- lactating cow:

Twenty five lactating cow located diyala province, were clinically examined, to confirm infection with mastitis or apparently normal, The study was carried out over a six month starting from November 2013 to April 2014.

3.1.2-Equipments:

Table (3-1) Apparatuses:

No.	**Apparatus name**	Company
1.	Light microscope	Olympus (Japan)
2.	Incubator	Heracus (England)
3.	Autoclave	Daikyo (Japan)
4.	Centrifuge	MSEE(England)
5.	Sensitive balance	Sartorius(Germany)
6.	Microtiterpipete	Karl Kalb(Germany)
7.	Refrigerator	Concord (Lebanon)
8.	Water bath	Gallen Kamp(England)
9.	Oven	Gallenkamp(England)
10.	pH meter	Pye-Unicam (Germany)
11.	Hood	Memmert (Germany)

3.1.3-Chemicals :-

Table (3-2) Chemicals :

No.	Chemicals	Company
1-	Gram Stain Set	Syrbio- Syria
2-	Catalase reagent H_2O_2 3%	Oxoid
3-	Oxidase Reagent 1%	MAST ID (USA)
4-	Gimsa Stain powder	Syrbio- Syria
5-	Tincture Iodine 5%	Local
6-	Ethanol 70%	Merck(Germany)
7-	Glycerol	Difco (USA)
8-	Heparin	BDH(England)
9-	Bromocresol purple	Sigma(USA)
10-	Crystal Violet	Sigma(USA)
11-	Phenol red	Sigma(USA)
12-	PBS	BDH(England)

3.1.4-Media:

Table (3-3) Media :

1-	Blood Agar	Oxoid
2-	Nutrient Agar	Oxoid
3-	Brain heart infusion Agar	Hi Media
4-	Brain heart infusion broth	Oxoid
5-	Mannitol salt agar	Hi Media
6-	MaCconkey agar	Hi Media
7-	Agar Agar	Hi Media
8-	Triple Sugar Iron (TSI)	Hi Media

3-2- Methods :

3.2.1-Media Preparation, according to manufacture :

3.2.1.1 -Blood Agar:

This media used for bacterial isolation from milk samples were Prepared by dissolving 40 g. of blood agar base in 1 litter of distal water, pH was adjusted to 7.4, autoclaved 121 C° /15 pound pressure for 15 minutes, then media were cooled for 45 C. in water bath then adding sheep blood 5%, mixed gently and poured into petri dishes.

3.2.1.2 -Nutrient Agar:

This media were Prepared by dissolving 23 g. in 1 litter of distal water, pH was adjusted to 7.4, autoclaved 121 C° /15 pound pressure for 15 minutes, then media was cooled for 45 C. and poured into petri dishes.

3.2.1.3- Brain heart infusion Agar:

This media Prepared was done by dissolving 52 g / 1 litter distal water, autoclaved then poured in petri dishes .

3.2.1.4-Brain heart infusion broth:

This media Prepared by dissolving 34.5 g. / 1 litter distal water then autoclaved

3.2.1.5-Mannitol salt agar:

This media prepared by dissolving 111 g/1 litter distal water, autoclaved then poured in Petri dishes .

3.2.1.6- Triple Sugar Iron (TSI):

This media Prepared by dissolving 63.6 gm/1000 ml distal water and after autoclaving, the test tubes were put in slant position yet solidifying the medium.

3.2.2-Biochemical tests :

All Biochemical tests were done according to (Quinn *et al.,* 2004).

A-Catalase test :

It was made by spreading single colony of bacteria from nutrient agar on a clean slide, then some drops of (H_2O_2) was added, the production of O_2 bubbles means a positive reaction .

B- Oxidase test :

This test was done by spreading single colony of bacteria from nutrient agar on a filter paper which soaked previously with oxidase reagent, and conversion the color of colony to dark purple during 2-10 seconds indicated a positive reaction.

C- Urease test :

It done by culturing the isolate on slant of urea agar and incubated 37 C° for 24 hours, and if color of media converted from yellow to pink color which indicated as a positive reaction .

D -Gelatin liquefaction test :

Bacterial colonies were cultured by stripping on gelatin media and incubated at 37 C°. for 24 hours. Non solidifying of gelatin after stored at 4 C° for 10 minutes was indicated as positive reaction .

E- Oxidation / Fermentation glucose test.

This test was prepared by dissolving the following substance in (1) liter of distal water,

- Peptone 2 g
- NaCl 5 g
- K_2HpO_4 1.5 g
- agar 15 g

pH Adjusted to : 7.1

After sterilization 15 ml of 0.2% of Sterile Bromothymol blue was add which sterilized by filtration (Millipore filter 0.22 μm), after that 10 ml of glucose sugar 10% was add (sterilize by filtration), after cooling, it was poured in sterile tubes .

F-Coagulase test.

The test was done according to Charles and Margi, (2002).

1-Preparation of the plasma:

The plasma was prepared by aspirating (4-5) ml of blood (with EDTA) directly from the heart of the rabbit after general anesthesia, and the plasma was separated by centrifugation and kept by freezing until use .

2-Test procedures :

Two versions of the test were used: Slide coagulase test and tube coagulase test.

the tube coagulase was done by using 0.5 ml of plasma placed in a test tube and inoculated with a loopful of the organism cultured on non-inhibitory medium, such as blood agar. The tube was incubated at 37 C°. and read hourly for 4 hours, a negative reaction was indicated by no clot formation, whereas a positive reaction is indicated by clot formation. if the test result remains negative ,the sample was incubated again for 24 hours and then read.

The slide coagulase test was done by A loopful of *Staphyllococci* from a colony is first emulsified on a drop of water or saline solution to yield a thick suspension. A drop of fresh rabbit plasma is then added and stirred with a sterile loop. A positive reaction is indicated by clumping within 5 to 20 seconds.

3.2.3-Examination of milk samples:

Milk samples were conducted in the Laboratory for physical, chemical and bacteriological tests, as follows:

3.2.3.1- Physical Examination: Which include:

Color, odor and consistency of milk.

3.2.3.2-Chemical Examination of Milk Samples:

-Somatic cell count (SCC) :

This test was done according to, Schalm *et al*., (1971) as follows:

0.01 mL of milk was smeared over a surface of 1 cm^2 on a microscope glass slide. To achieve this piece of thin white cardboard marked with a 1 cm x1 cm square was placed under the glass slide. After drying-up the sample at room temperature for 24hours, fats from the smear were removed with xylol, dried up and fixed in ethanol for 5 minutes. Then after the samples were dried and staining with Newman lambert stain, A SCCs of $\geq$ 500000 cell/ml milk were considered to be positive. The number of somatic cells was measured under

microscopic with magnification of 10 × 40 in 50 fields and were multiplied by the microscopic factor to get the cells per ml of milk.

3.2.3.3-Bacteriological examination:

A- Culturing

Isolation and identification of bacteria from milk samples were performed according to Quinn *et al.,* (2004) All milk samples from clinical mastitis and apparent normal milk samples were centrifuged at 3000 rpm/15 minute, and the precipitate was cultured on blood agar, macConkey agar and nutrient agar, incubated at 37 C° for 24 hour, Diagnosis depends on morphological character (shape, color and size of colony).

B-Microscopic examination :

A single colony from nutrient agar was spreaded on a clean slide and fixed with heat and staining with gram stain according to Jawetz *et al*., (2001) and then the bacterial cell was examined under oil immersion .

C-Biochemical tests :

The bacterial isolates were identified by catalase, oxidase, Gelatin liquefaction, urease, O/F test , tube coagulase test and indol test.

3.2.4-Preservation of bacterial isolates:

All isolates after definitive isolation were cultured on sterile brain heart infusion broth and glycerol 20 % and incubated at 37 C° for 24 hours, then after turbidity occurred ,stored in a freezing .

Result and discussion

4.1. Bacterial isolates

Results of culturing and isolation of the causative agents revealed that *S.aureus* were the most predominant bacteria as they were isolated from 32 (57.14%) in mammary glands infected with subclinical mastitis while , *Staphylococcus coagulase negative* (CNS) was isolated from 8 (14.28%) samples (table 1).

This result was agreed with the most studies on bovine mastitis where many investigators demonstrated that *S.aureus* were the most common pathogens isolated from bovine milk and the common bacteria isolated from the teat skin , the teat – end .(Al – kubaysi ,(2008) ; Contreas ,A; Sierra .D., (2007) and (;Al-obaidy., 2010).

The predominance of *Staphylococcus* spp. is also agreed with results of Ebrahimi ,A.&Lotfalian ,S.H (2007) who found that (36.8%) of bovine milk samples infected with *S.aureus* , while 10.5% were infected with *CNS.*

The presence of *S.aureus* on the teat skin , and teat – ends, and their ability to resist the penicillins , and other antibiotics , as well as the arbitrary use of the antibiotics in treatment of mastitis may explain the high rate of *S.aureus* mastitis in this study .

In respect to gram negative bacteria, *Pasteurella Spp.* was isolated from 3 (5.3%) , *pseudomonas Spp.* 5 (8.92%), The relatively high rate of mastitis due to *Pasteurella Spp.* in this study is in compliance with results of Ebrahimi ,A.&Lotfalian ,S.H (2007) who indicated that approximately 50% , 21% and 5.26% , respectively of bovine mastitis caused by *M. haemolytica* , in addition to results of other studies performed in Iraq by Al - kubaysi ,(2008) who demonstrated that *Pasteurella Spp.* is one of the important causes of bovine mastitis.

Enterobacteriaceae were also isolated from several cases of bovine mastitis in this study like *E.coli* 3 (5.30%) , *Klebsiella Spp.* 3(5.3%) , *Salmonella Spp.* 3 (5.3%) and *Proteus Spp.* 1(1.78%), this result is agreed with results obtained

from other studies in Iraq and in other countries (Yousif, 1982 ; Tormod ,M.&Steinar ,W. 2007 ; Raham .,B.& Jalal .S.H.(2010).

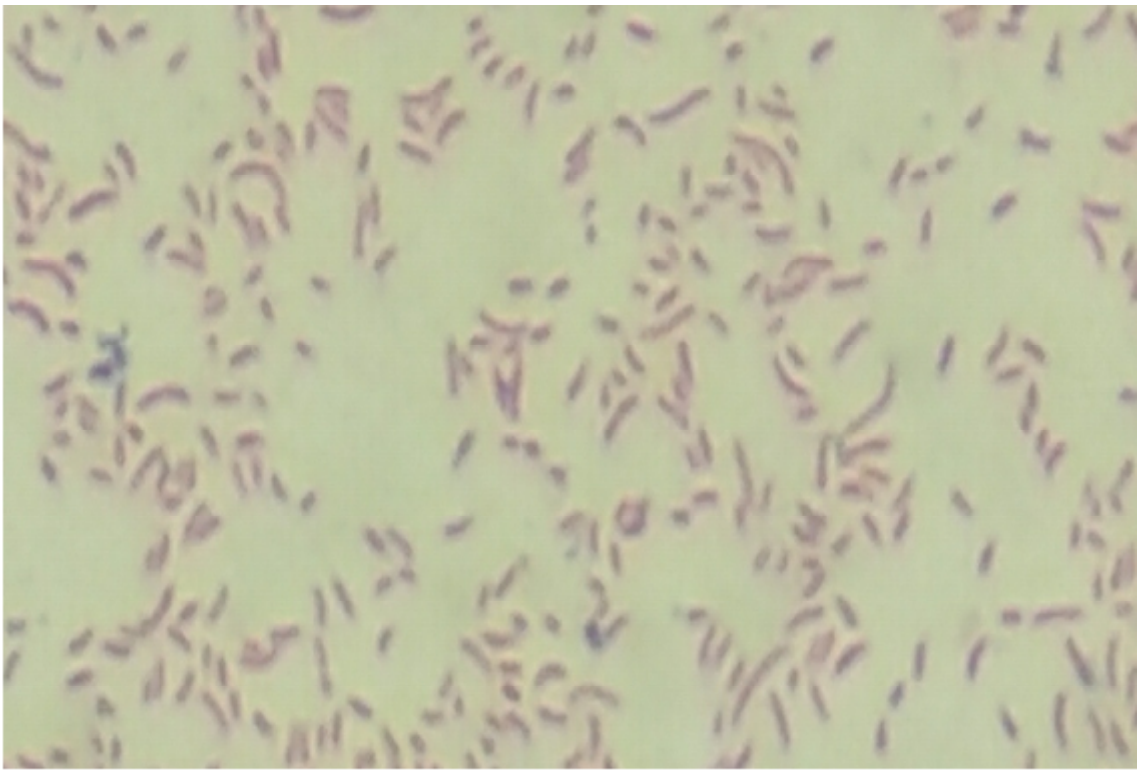
The prevalence of cases caused by these bacteria may be ascribed to their abundant presence in the environment of the animals as a cause of environmental mastitis (Radostits *et al.*, 2007; especially when cow, become recumbent for somewhat long period during parturition which may expose the udder to the environmental pathogens .

It is worthy to mention that the frequency of bacterial species isolated from clinically normal sheep milk was ascribed to many factors such as breed differences , different hygiene and management practices followed in each farm ,age and parity of the animal and type of milking (Radostits et al 2007).

Table (1) Show relation between age, breed, type of bacteria and No. of isolates.

Case number		Age	Breed	Type of bacteria	No. of isolates
2	FL FR	3.5 years	Friesian	*Pasteurella Spp.*	2
4	HL FL FR HR	3 year	cross	*Staph.aureus*	4
5	HL FR	2.5 year	Cross	*Pasteurella Spp* *Pseudomonas Spp.*	1 1
6	HL FL	2.5 year	Friesian	*Salmonella Spp.*	2
7	FL FR HL HR	3 year	local	*Staph.aureus*	4
8	FR HL	2 year	local	*Klepsiella Spp.*	2
9	HL FL FR HR	3.5 year	local	*Staph.aureus*	4
10	HR FL	2 year	local	*Staph.aureus*	4

	HL FR				
11	HL	2.5 year	local	Klepsiella Spp.	1
12	HL FR HR FL	2 year	local	*Staph.aureus*	4
13	HR	2.5 year	Friesian	*Staph. aureus*	1
14	FL FR HL	2.5 year	Friesian	E.coli	3
15	HL HR FR FL	2.5 year	Friesian	*Pseudomonas Spp*.	4
16	HL	4 year	cross	*Staph. aureus*	1
17	HR	3 year	cross	*Salmonella Spp.*	1
18	HL HR FL FR	2.5 year	local	*Staph. coagulase negative*	4
19	FR FL HL HR	2.5 year	local	*Staph. aureus*	4
20	FL HR FR HL	4 year	local	*Staph. aureus*	4
21	FL HR FR HL	3 years	local	*Staph. coagulase negative*	4
22	HR, HL FL, FR.	2.5 years	local	*Staph. aureus* E.coli	2 2
23	HL	3.2 years	cross	proteus	1



Figure(1):Microscopical examination of *E.coli* (Gram stain X 1000).

4.6.4-Relation between SCC and Bacterial isolation:

Fifty six samples from 60 samples +ve for SCC test gave +ve result for bacteria, table (2) shows the results of SCC number in relation to bacterial isolation, (No. SCC more than 500000).

The present results showed that somatic cell count (SCC) is good indirect method for detecting subclinical mastitis and the threshold level of which were used for differentiated between infected and non-infected value in our result was (500,000) cells /ml . this results was agreement with Kaskous (2000) who recorded that SCC in milk can be used as reliable methods for detecting subclinical mastitis in bovine. But some authors recorded that SCC threshold values had difficult to established for cow. This value is affected by different factors, such as the type of breed lactation period and organisms producing Intramammary infections (Gonzalo *et al.,* 2002).

Our results showed –ve result for bacteria from milk sample with SCC low than (500,000) cells /ml, this is a disagreement with result of Schukken *et*

al.,(1989) who isolated mastitis pathogens from milk sample with very low SCC and Poutrel and Rainard (1982) who found that the increase in somatic cell count (SCC) was reliable index of on-going mastitis.

Results showed that subclinical mastitis according to SCC and its relation to isolation of bacteria was very important due to the high percentage of occurring. This is incompatible with Al-majali and jawabreh (2003) found that subclinical mastitis gives a percent of (24.8%).

Table (2) Relation between SCC No. and bacterial isolation.

SCC No.	No. of samples	bacterial isolation
100,000 – 500,000(-ve)	40	0
500,000 – 1,000,000	10	8
1,000,000 – 1,500,000	20	18
1,500,000 – 2,000,000	18	18
More than 2,000,000	12	12
Total	100	56

5. Conclusions and recommendations

5.1. conclusions

1. *Staph. aureus* in bovine mastitis were more predominant than other intramammary infecting pathogens .
2. Environmental pathogens play an important role in bovine mastitis.
3. Somatic cell count test was important test for detection of subclinical mastitis in bovine.
4. High relationship between somatic cell count and bacterial isolation.

5.2. Recommendations

1. Periodic clinical examination of pregnant bovine for early detection, treating or culling of mastitis cases .
2. Providing abundant fresh bedding for pregnant cow in confinement to reduce teat contamination &trauma .
3. Conducting more studies to prepare specific effective vaccines against the predominant causative agents of mastitis.
4. Early weaning or hand feeding of calve to decrease the injuries caused by vigorous suckling of the calve.
5. Bulk milk somatic cell counts should be done monthly for early detection of sub clinical mastitis .

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