ISOLATION OF *STAPHYLOCOCUS AUREUS* AND COAGULASE NEGATIVE STAPHYLOCOCCIFORM BOVINE SUBCLINICAL MASTITIS AND THEIR IMPACT ON THE CHEMICAL COMPONENTS OF MILK

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ABSTRACT

This study was conducted to isolate *Staphylococcus aureus* and Coagulase Negative Staphylococci (CNS) from bovine subclinical mastitis cases and study their effect on chemical composition of milk. A total of 152 milk samples were collected from apparently normal cows from Basrah province, subclinical mastitis (SCM) was detected in 51.97% of tested samples by using California mastitis test (CMT). *Staphylococcus aureus* and CNS were isolated from 20.25% and 16.45% cases of tested SCM respectively.

The affected samples with SCM have high concentrations of fat and protein, the difference from normal samples was statistically highly significant (P <0.001) for fat; lactose was lower in affected samples. The pH of affected samples was higher than that of normal, however, pH of samples containing *S. aureus* was the highest (7.8375) and the difference was statistically significant (P< 0.05).

Antimicrobial susceptibility assay revealed that all isolates were susceptible to chloramphenicol, ciprofloxacin, gentamycin and vancomycin. However, the resistance to oxacillin and penicillin exhibited by CNS and *S. aureus* were 76.9%, 84.6%, 62.5% and 68.75% respectively.

It have been concluded that, subclinical mastitis caused by Staphylococci(in particular *S. aureus*) which carried resistance to antibioticsused in human medicine represents a big problem.However, the changes caused by *S. aureus* and other staphylococci in pH and chemical composition of mastitic milk may reduce the shelf life and processing of the products.

INTRODUCTION

The consumption of animal milk is a by-product of animal domestication, which occurred about ten thousand years ago(1).Milk is a prime source of dietary energy, high-quality protein and fat, it can contribute significantly to meet the required nutrition intakes of calcium, magnesium, selenium, vitamin B12 and pantothenic acid(2).The standard cow's milk ingredients include fat comprise approximately 3 to 4 percent of the solid content of cow milk, protein about 3.5 percent and lactose 5 percent (3).

Mastitis is a common disease entity of dairy cows, accompanied by physical, chemical, pathological and bacteriological changes in milk and glandular tissue (4).There are different predisposing factors such as poor management and hygiene, teat injuries and faulty milking machines are known to hasten the entry of infectious agent (5).Mastitis is generally classified as clinical or subclinical depending on the degree of inflammation in the mammary gland.Clinical mastitis is characterized by visible abnormalities of the milk while sub-clinical mastitis, no change in the milk is obvious, however, both cases may reduce milk production (6). One of the responses of the most obvious reaction is to increase the flow of immune cells from the blood to the milk which has led to a significant increase of milk somatic cell count (7). The subclinical form of mastitis (SCM) in dairy cows is important because the following; 15 to 40 times more prevalent than the clinical form (8).It usually precedes the clinical form; long duration; difficult to detect; reduces the milk production more than 3 times than the clinical mastitis as described before (9).In addition, it adversely affects milk quality (10).

Several causative agents have been implicated in mastitis in dairy cows including bacterial, mycoplasmal and algae, however bacteria are the most frequent pathogens of the disease (11).*Staphylococcus aureus* is one of the most frequently isolated pathogens from both subclinical mastitis and chronic infections (12).

The present study was carried out to determine; the percentage of *S. aureus* and Coagulase Negative Staphylococci (CNS) that cause subclinical mastitis in bovine, and to study antimicrobial susceptibility of isolated strains. Furthermore, the role of subclinical mastitis in alteration of milk composition was also highlighted.

MATERIALS AND METHODS

-Samples collection and detection of subclinical mastitis

A total of 152 milk samples were collected from apparently normal cows from different parts of Basrah province. Diagnosis of subclinical mastitis was done by using California mastitis test as described previously (13).

- **Analysis of milk components:-**

The constituents of milk were assayed by using lactoflash system (Germany).The system directly measures (Fat, Protein, and Lactose).The lactoflash were used 12 ml of milk sample and passes it through thermal and optical sensors to get the results. The results were expressed as a percent.

-Measurement of milk pH:-

The pH of milk was measured by using the pH meter (Trans, China).

-Isolation and identification of bacteria

All milk samples that were positive for CMT test were inoculated on mannitol salt agar (Himedia, India) and on blood agar (Himedia, India). The plates were incubated aerobically for 24hrs at 37°C. The suspected colonies on mannitol salt agar were identified by Gram's stain, catalase test; oxidase test by using oxidase discs from (Himedia, India) as described before (14) and hemolysis were detected on blood agar. Confirmation of isolates was done by using tube coagulase test with rabbit plasma, DNase production and voges-proskauer test (15).

- Determination of the antibiotic susceptibility of isolates

All the isolates that were identified as Staphylococci were tested for antimicrobial susceptibility according to Kirby-Bauer method (16). Six antibiotics were chosen for the study according to their common use in research. The antibiotic discs were provided by (Bioanalyse/ Turkey) including Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Gentamycin (10 µg), Oxacillin (1 µg), Penicillin (10 µg) andVancomycin (30 µg).

- Statistical analysis

The results were analyzed statistically by using Minitab program v.14.

RESULTS

The total number of milk samples collected from apparently normal cows was 152 samples; seventy nine samples which represent 51.97% of total number of samples gave positive results with California Mastitis Test (CMT) (table 1). The number of Staphylococci strains which isolated from CMT positive samples was 29 (36.7%), coagulase negative Staphylococci (CNS) represent 13/29 (44.83%), whereas *Staphylococcus aureus* represent 16/29 (55.17%). The ratio of CNS to the total number of subclinical mastitis samples was 13/79 (16.45%), whereas the ratio of*Staphylococcusaureus*was16/79 (20.25%).

Table (1): Number of positive samples to CMT and isolated *Staphylococcus spp.*

Total number of samples	Samples of sub-clinical mastitis	Number of*Staphylococcus spp.*Isolates	Coagulase Negative Staphylococci	*Staphylococcus aureus*
152	79	29	13	16
100%	51.97 %	36.7%*	44.83%**	55.17%***

*= the percent represent divided the number in the upper raw on total number of CMT positive samples.

; *= the percent represent divided the number in the upper raw on total number of isolated Staphylococci.

Table (2) : Comparison between milk constituents of normal samples.Samples positively react to the CMT (induced by other microorganisms)and samples contain *Staphylococcusspp*. isolates.

Milk constituent	Normal milk samples Mean ± S	Subclinical mastitis Samples caused by other organisms Mean ± S	Samples which contain CNS Mean ± S	Samples which contain *Staphylococcus aureus* Mean ± S	Significance
Fat %	3.3600% ± 0.3362	3.9381 % ± 0.1830	3.9385% ± 0.1758	3.9313% ± 0.1852	**
protein%	3.2760 % ± 0.2945	3.5333 % ± 0.2456	3.4615 % ± 0.2694	3.5813% ± 0.2167	NS
lactose %	4.6120 % ± 0.4193	4.3714 % ± 0.4002	4.300% ± 0.410	4.5313 % ± 0.3591	NS
pH	6.7320 ± 0.1994	7.5571 ± 0.2357	7.6308 ± 0.2323	7.8375 ± 0.2754	*

Values were represented as Mean ± standard deviation.

[NS= not significant (P>0.005), * = P< 0.05, ** = P < 0.001]

CNS= Coagulase negative Staphylococci.

Normal milk samples(negatively reacted to CMT).

With regard to milk composition, Table (2) shows the changes in chemical composition of mastitic milk. Milk fat concentration wasincreased in samples that positively reacted with CMT; the changes were statistically highly significant (P < 0.001). The concentrations of protein and lactose were increased and decreased respectively in comparison with normal samples; however, the changes were statistically not significant. The changes in pH were statistically significant.

Table (3): Antibacterial susceptibility assay of CNS and *S. aureus* isolates against six antimicrobials.

Antimicrobials	Antibiogram of CNS isolates						Antibiogram of *S. aureus* isolates					
	Resistant		Intermediate		Susceptible		Resistant		Intermediate		Susceptible	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Chloramphenicol	0	0	0	0	13	100	0	0	0	0	16	100
Ciprofloxacin	0	0	0	0	13	100	0	0	4	25	12	75
Gentamycin	0	0	0	0	13	100	0	0	0	0	16	100
Oxacillin	10	76.9	0	0	3	23.1	10	62.5	0	0	6	37.5
Penicillin	11	84.6	2	15.4	0	0	11	68.75	1	6.25	4	25
Vancomycin	0	0	0	0	13	100	0	0	0	0	16	100

The antibiogram of isolates illustrated in Table (3), all isolates were susceptible to chloramphenicol, ciprofloxacin, gentamycin and vancomycin. The resistance to oxacillin and penicillin exhibited by CNS were76.9%, 84.6%and by*S. aureus* were 62.5% and 68.75% respectively.

DISCUSSION

Subclinical mastitis are of importance since their incidence is about 40-50% more than that ofclinical mastitis and causes 3-26% loss in milk yield (17). However, SCM is one of the most prevalent, important and costly diseases of dairy animals worldwide, with losses of over 1.7 billion dollars a year in the USA alone (18).

The positive samples to CMT constitute 51.97% of total sample number. This result is in agreement with (19 and 20) who diagnosed the SCM in Korea in 54.3% of tested cows; and in Argentina 54% of tested samples respectively. Whereas, (21) found that 36.7% of assayed milk samples are affected with SCM in Ethiopia.

The isolation rate of *Staphylococci* from SCM was 36.7% this ratio represents 16.45% of CNS and 20.25% of *S. aureus*. These results are in agreement with (22), who concluded that, Staphylococci are the bacteria most commonly isolated from subclinical mastitis. Concerning isolation rate of CNS, (23) reported almost similar result 18%. Higher isolation rates of *S. aureus* from SCM were recorded in Algeria40% by (24),in Egypt52.5% by (25) and in Uruguay62.2% by(7). On the other hand, lower rate of Staphylococci isolation 23.75% was recorded by (26).

The onset of udder infection (mastitis) is known to influence the chemical composition of milk constituents(27).Table (2), illustrated that the difference in milk fat between the normal and affected samples with SCM was significant, and theconcentration of fat was increased in affected samples, this result is in agreement with other authors (28,29 and 30). However, (31) reported a decrease in fat concentration in samples of SCM.

The concentration of milk protein was shown in table (2). Protein increase in affected samples and especially in samples of SCM caused by *Staphylococcus aureus,* the changes statistically were not significant. These results are in accordance with majority of authors (32;33;34 and35). The increment in protein concentration is caused by alteration in the permeability of the secretory epithelium and capillary wall, these changes induced by bacterial toxins (36).Hence, increase the concentration of blood albumin and immunoglobulins in whey milk(37). Moreover, (38) noted that, the change in milk protein related to type of microorganisms.

The concentration of milk lactose was decreased in samples taken from SCM cows; however, the changes were not significant. This result is in consistency with (34; 39 and40).Furthermore, (38) noted that mastitis causes a decrease in milk lactose through damaging the secretary cells that produce milk in mammary glands.

The milk pH of positive samples was higher than that of normal samples. This result is in consistent with (41) who noted that the pH of SCM milk was higher than that of normal milk.The somatic cells count (SCC) in milk has a significant effect on pH (42 and 38). The difference in pH was statistically significant;however, the samples taken from SCM caused by *S. aureus* have higher pH than that of mastitis caused by CNS. These results are in agreement with (23) who suggested that SCM induced by*S.aureus*is associated with high SCC; whereas, that caused by CNS is responsible for a discrete increase of SCC as compared to culture negative animals.

The results of antibacterial susceptibility showed that all CNS isolates were highly susceptible to chloramphenicol, ciprofloxacin, gentamycin, vancomycin; however, 76.9% and 84.6% of these isolates were resistant to oxacillin and penicillin, respectively. These results are in accordance with (43; 44 and 45).The antibiogram of *S. aureus* was similar to that of CNS except in ratios of resistance to oxacillin and penicillin which were 62.5% and 68.75%, respectively.With regard to results of penicillin,(46) in South Ethiopia, recorded 67.9% of isolates were resistant; however, other researcher found 100% of *S. aureus* isolates from SCM were resistant to penicillin (47). Concerning oxacillin 62.5% of isolates was resistant, this result is in accordance with (48) who found 60% of *S. aureus* isolates from bovine mastitis was resistant. The isolates were highly susceptible to chloramphenicol, ciprofloxacin, gentamycin, vancomycin.These results are in agreement with (48; 47 and 49). High resistance rate to penicillin could be due to the widespread use of β-lactam antibiotics such as penicillin and ampicillin for the treatment of mastitis.

Mastitis represents the most costly disease of dairy cows and the major economic loss results from reduced milk production (50).In addition, subclinical mastitis leads to a diversity of compositional changes in milk either because of local effects or because of serum components entering the milk (39), leading to changes in nutritional quality of milk. Moreover, the most important species of staphylococci is *S. aureus* because of their pathogenicity and enterotoxin production causing food intoxication. The raise in incidence of staphylococci mastitis between animals is a serious source of *S. aureus* (51). Concerning the massive treatment of mastitis with antibiotics, resistant *S. aureus* strains have a chance to multiply inducing certain problems for public health among consumers of dairy products (52).

In conclusion, the results of this study indicated that, the subclinical mastitis which is caused by Staphylococci (in particular *S. aureus*) which carried resistance to antibiotics represents a big problem. However, the changes caused by *S. aureus* and other staphylococci in pH and chemical composition of mastitic milk may reduce the shelf life and processing of the products.

عزل المكورات العنقودية الذهبية والمكورات العنقودية السالبة لانزيم التجلط من التهاب الضرع البقري تحت السريري وتأثيرهاعلى المكونات الكيميائية للحليب

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الخلاصة

أجريت هذه الدراسة لعزل المكورات العنقودية الذهبية والمكورات العنقودية السالبةلانزيم التجلط (CNS) من حالات التهاب الضرع البقري تحت السريري ودراسة تأثيرها على التركيب الكيميائي للحليب .

جمعت 152 عينة حليب من أبقار سليمة ظاهريا من محافظة البصرة، تم تشخيص التهاب الضرع تحت السريري (SCM) في 51.97٪ من العينات التي تم فحصها باستخدام اختبار التهاب الضرع كاليفورنيا(CMT). عزلت المكورات العنقودية الذهبية والمكورات العنقودية السالبة لانزيم التجلط بنسبة 20.25٪ و 16.45٪ من حالات التهاب الضرع تحت السريري على التوالي.
احتوت العينات المتأثرة بالتهاب الضرع تحت ا لسريري على تراكيزعالية من الدهون والبروتين، وكان الفرق عن العينات الطبيعية ذو دلالة إحصائية عالية (P <0.001) للدهون .تركيزاللاكتوزكان أقل في العينات المتأثرة .الأس الهيدروجيني للعينات المتأثرة كان أعلى من الطبيعية، والأس الهيدروجيني للعينات التي تحتوي على جراثيم المكورة العنقودية البرتقالية هو الأعلى (7.8375) وكان الفرق ذو دلالة إحصائية(P<0.05) .

أظهر فحص الحساسية للمضادات الميكروبية أن جميع العزلات كانت حساسة للكلورامفينيكول، سيبروفلوكساسين، الجنتاميسين وفانكومايسين .فضلا عن ذلك، كانت المقاومة لأوكساسيلين والبنسلين التي أظهرتها والمكورات العنقودية السالبة لانزيم التجلط والعنقودية الذهبية 76.9٪، 84.6٪، 62.5٪ و 68.75٪ على التوالي.
أستنتج من هذه الدراسة ان التهاب الضرع تحت السريري الناجم عن المكورات العنقودية يمثل مشكلة كبيرة و خصوصا جراثيم المكورة العنقودية البرتقالية التي تحمل المقاومة للمضادات الحيوية المستخدمة في الطب البشري .التغييرات التي تسببها المكورات العنقودية وغيرها في الأس الهيدروجيني والتركيب الكيميائي للحليب قد يقلل من مدة صلاحية الحليب ومنتجاته.

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