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Isolation and Identification of Staphylococci Isolated From Bovine Mastitic Milk in River Nile State, Sudan

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Abstract

A total of 133 staphylococci were isolated from 100 bovine mastitic samples in different localities of River Nile State. In some cases more than one isolate was recovered from the same sample. These isolates were subjected for further confirmation by using API staph. 35 (26.3%) of the staphylococci were coagulase-positive Staphylococcus aureus and 98 (73.7%) were coagulase-negative. These findings show a high prevalence of staphylococci in dairy herds in River Nile area. A high prevalence rate of coagulase-negative staphylococci was observed in cows with a high SCC i.e. more than 660,000 cell/ml. Coagulase-negative staphylococci are suspected to be significant as a cause of mastitis especially in quarters with high SCC. The coagulase-negative staphylococci isolated in order of frequency were: S. epidermidis (10.3%), S. chromogenes (9.0%), S. capitis subsp. Ureolyticus (6.8%), S. haemolyticus (6.0%), S. hyicus (6.0%), S. caseolyticus (5.3%), S. simulans (5.3%), S. xylosus (5.3%), S. saprophyticus (4.5%), S. carnosus (3.8%), S. lugdunensis (3.8%), S. capitis (3.0%), S. saccharolyticus (2.3%) and S. sciuri (2.3%).

Introduction

Mastitis is defined as inflammation of the udder irrespective of the cause. Two forms of mastitis are known viz clinical and subclinical mastitis [1]. Despite extensive research and control efforts, mastitis remains a major problem for the dairy industry. The disease is complex and may be caused by a large number of organisms [2]. Over 130 different microorganisms have been isolated from bovine mastitic milk samples, but *Staphylococcus aureus*, *Streptococcus spp* and members of *Enterobacteriaceae* are among the most common aetiological agents in cows and in other animal species. Invasion of the mammary gland by microorganisms is characterized by an increased leukocyte count in the milk, the majority of cells being neutrophils [3].

In Sudan clinical and subclinical mastitis leads to substantial (20%) drop in milk [4]. Over 95% of subclinical and more than 60% of clinical cases of mastitis in the Nordic countries are caused by Gram–positive cocci. Of these, the most common pathogen is

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Staphylococcus aureus, which was found to be responsible for 30–40 % of subclinical cases and 20–30% of acute cases. The importance of coagulase – negative staphylococci (CNS) has increased during recent years. Over 30% of subclinical cases and nearly 20% of acute cases were found to be caused by CNS [5]. In Sudan several agents were isolated from cases of subclinical mastitis, these include: *S. aureus, S. epidermidis, Corynebacterium spp, Pseudomonas spp, Str. agalactiae, Str. dysagalactiae*, and *Micrococcus spp* [6]. High incidence of subclinical mastitis was reported in Khartoum and commonest species of bacteria isolated were: *Enterococcus faecalis, Enterococcus faecium, Str. bovis, Str. equi subsp equi, Lactococcus lactis* and *Str. pyogene*s [7]. *S. aureus* was considered as the major bacterium isolated from bovine clinical mastitis [8] followed by *Streptococcus agalactiae* [9]. Other organisms isolated include: *Bacillus cereus* [9], *Escherichia coli* [10], *Klebsiella pnumoniae* [11] and *Staphylococcus epidermidis* [9]. [12] isolated *S. aureus* from 20.48% of mastitic bovine milk samples and *S. epidermidis from* 28.7% samples. [13] reported that staphylococci were responsible for 24.5% of mastitis in cows. [14] isolated *S. aureus, S. capitis subsp. ureolyticus, S. lugdunensis, S. simulans, S. caseolyticus, S. chromogenes, S. hyicus, S. epidermidis, S. hominis* and *S. capitis,* from mastitic milk of cows. Staphylococcal mastitis has a great economic importance. [15] reported that milk yield from quarters shedding staphylococci was found to be less than that from quarters free from staphylococci.

The objective of this research work was to investigate the characteristics of staphylococci obtained from bovine mastitic milk samples using conventional and rapid systems.

Materials and Methods

Area of the Study

A total of 100 bovine milk samples positive for California Mastitis Test (CMT) were collected from the three localities (Atbara, El Damar and Barber) in River Nile State in Sudan. In the three localities samples were collected from special dairy cattle farms markets and veterinary hospitals.

Sampling Procedure

Before collection of milk samples from the tested cows, the udder was thoroughly cleaned with soap and water, rubbed dry, and the teat area was rubbed thereafter with a piece of cotton soaked in 70% alcohol . The first stream of milk was discarded. The California Mastitis Test was directly applied for quarter's milk and samples were collected from positively reacted milk into sterile bottles. The collected samples were put in ice box containing ice and transported to the laboratory. In most cases the time between collection and arrival to the laboratory was 1-2 hrs. In the laboratory mastitic milk samples were kept in a deep-freezer and swabs were soaked in tubes containing nutrient broth and incubated at 37C. All samples were examined on the next day. On the next day mastitic milk samples were removed from the deep–freezer and left on the bench to thaw.

Isolation, Identification and Characterization of the Staphylococcal Isolates

All media (Oxoid media) were prepared and sterilized according to the manufacturer instructions. For the primary isolation of staphylococci, a loop full milk sample was streaked onto blood agar, McConkey's agar, and nutrient agar using sterile wire loop. The cultures were incubated aerobically at 37oC for 18-24

hours. Cultures on semi-solid media were examined grossly for colonial morphology and haemolysis on blood agar. Whereas, broth media were checked for turbidity, change in colour, accumulation of gases in CHO media and for sediment formation. One half colony from each plate was used for performing gram staining. Colonies which showed Gram positive cocci were sub cultured on nutrient agar. Purification was based on the characteristics of colonial morphology and smear. This was obtained by sub culturing of a typical discrete colony on blood agar plate. Pure cultures were preserved on slants of blood agar and egg media at 4^{o}C.

Biological and Biochemical Identification

The purified isolates were identified as previously described [16, 17]. The identification include: Gram's reaction, presence or absence of spores, shape of organism, motility, colonial characteristics on different media, aerobic and anaerobic growth, sugars fermentation ability and biochemical tests (staining of smear, catalase test, oxidase test, coaggulase test, oxidation fermentation test, motility test, glucose breakdown test, fermentation of carbohydrates, urease activity, citrate utilization, gelatin hydrolysis test, nitrate reduction test).

Identification of Staphylococcal Isolates Using API staph (BIOMERIEUX, France) Identification System

According to [18] API staph (Analytical Profile Index for identification of the Genus *Staphylococcus*) is a standardized system for the identification of the Genera: *Staphylococcus*, *Micrococcus* and *Kocuria*, which uses miniaturized biochemical tests and specially adapted database.

Pure staphylococci isolates were sub cultured on blood agar and incubated at 36oC ± 2 for 18–24 hours. The identification test of staphylococci isolates was conducted according to the manufacturer BIOMERIEUX protocol. Homogeneous bacterial suspension was obtained by using API staph medium. Both tubes and cubules of API staph were filled with the inoculated API staph media. Anaerobiosis was ensured in the ADH, LDC, ODC, URE and H2S tests by filling the cubules with sterile mineral oil to form a convex meniscus. The incubation boxes were closed and incubated at 36oC ± 2 for 18–24 hours. Identification was obtained according to the numerical profile of API staph.

Statistical Analysis

Statistical analysis was done through Microsoft office Excel 2007.

Results and Discussion

A total of 100 CMT positive mastitic milk samples were collected from different localities of River Nile State.

California Mastitis Test (Cmt)

This test was performed according to [17]. The test was done in white plastic paddle with four receptacles. In this test the milk was drown into the cups of the paddle and promocresol purple solution was added in an estimated equal quantity to milk in each cup by squirting reagent from polyethylene wash bottle. A gentle circular motion was done to the paddle. Occurrence of any degree of precipitation or gel formation is considered as a positive result. Table (1) illustrates the approximate ranges in somatic cell concentrations associated with each of the CMT scores.

Somatic Cell Count (SCC) and Isolation of Staphylococci

As shown in table (2), 56 of 100 mastitic milk samples had a SCC score ranging between 2,400,000 and 10,000,000 cells/ml, 38 of 100 mastitic milk samples had a SCC score ranging between 660,000 and 2,400,000 cells/ml and six out of 100 mastitic milk samples had a SCC score more than 10 million cells/ml.

According to the cultural characteristics, bacterial morphology and biochemical reactions results, and API staph results, a total of 133 staphylococci were isolated. Thirty five of staphylococcal isolates (26.3%) were coagulase-positive and 98 (73.7%) were coagulase-negative (Table 3).

Identification of Staphylococci Using Api Staph System

According to API staph identification system results (Table 4), the identified staphylococci were:

1. Coagulase – positive *Staphylococcus aureus* (26.3%).

2. Coagulase – negative Novobiocin sensitive staphylococci were:

S. epidermidis (10.5%), *S. chromogenes* (9.0%), *S. capitis subsp. ureolyticus* (6.8%), *S. haemolyticus* (6.0%), *S. hyicus* (6.0%), *S. caseolyticus* (5.3%), *S. simulans* (5.3%), *S. carnosus* (3.8%), *S. lugdunensis* (3.8%), *S. capitis* (3.0%) and *S. saccharolyticus* (2.3%).

3. Coagulase–negative Novobiocin resistant staphylococci were:

S. xylosus (5.3%), *S. saprophyticus* (4.5%), and *S. sciuri* (2.3%) (Figure 1).

Cultural characteristics and morphology of *Staphylococcus species* are shown in figures from (1 to 8).

Table 1 Approximate Ranges in Somatic Cell Counts for California Mastitis Test Scores

C.M.T Score	Average SCC (cell/milliliter)	Description of reaction
N negative	0-480,000	No thickening and homogenous.
T (Trace)	Up to 640,000	Slight thickening and reaction Disappears in 10 sec.
1	660,000	Distinct thickening and no gel formation.
2	2,400,0000	Thickens immediately, begins to gel and levels in the bottom of cup.
3	> 10,000,000	Gel is formed, surface elevates with a central peak above the mass.

Table 2 Results of the Somatic Cell Count (SCC) of Mastitic Milk Samples as Estimated by CMT

Farms	No. of Samples	CMT score 1	CMT score 2	CMT score 3
Omer Amir	11	9	2	0
Barbar	12	6	6	0
El Damer	20	0	7	2
El Damar market	10	5	8	0
Abdelrahman	10	1	8	1
Alaa Eldin	10	3	6	1
Atbara hospital	10	8	10	0
Research center	7	4	6	1
Akram	10	2	3	1
Total	100	38	56	6

- C.M.T. score 1 = 660,000 – 2,400,000 cells / ml
- C.M.T. score 2 = 2,400,000 – 10,000,000 cells / ml
- C.M.T. score 3 >10,000,000 cells / ml

Table 3 Number and Percentage of Staphylococci Isolated From Mastitic Milk

Staphylococcus species	No.of isolates	Percentage
S. aureus	35	26.3%
S. capitis	4	3.0%
S. capitis subsp. ureolyticus	9	6.8%
S. carnosus	5	3.8%
S. caseolyticus	7	5.3%
S. chromogenes	12	9.0%
S. epidermidis	14	10.5%
S. haemolyticus	8	6.0%
S. hyicus	8	6.0%
S. lugdunensis	5	3.8%
S. saccharolyticus	3	2.3%
S. saprophyticus	6	4.5%
S. sciuri	3	2.3%
S. simulans	7	5.3%
S. xylosus	7	5.3%
Total	133	100%

Table 4 Api Staph Identification System Results.

Tests	*S. Aureus*	*S. capitis*	*S.capitis Subsp. Ureolyticus*	*S. carnosus*	*S. chromogenes*	*S. epidermidis*	*S. haemolyticus*	*S.xylosus*
(0)	-	-	-	-	-	-	-	-
GLU	+	+	+	+	+	+	+	+
FRU	+	+	+	+	+	+	+	+
MNE	+	-	-	+	+	-	-	-
MAL	+	-	-	-	+	+	+	+
LAC		-	-	+	+	+	+	+
TRE	-	-	-	+	+	-	+	+
MAN	+	-	-	+	-	-	-	+
XLT	-	-	-	-	-	-	-	-
MEL	-	-	-	-	-	+	-	-
NIT	+	-	-	+	+	+	-	+
PAL	+	-	-	+	+	+	-	+
VP	+	+	+	+	-	+	-	-
RAF	-	-	-	-	-	-	-	-
XYL	-	-	-	-	-	-	-	+
SAC	+	+	+	-	+	+	+	+
MDG	+	-	-	-	-	-	-	-
NAG	+	-	-	+	-	-	+	-
ADH	-	+	+	+	+	+	+	-
URE	+	-	+	-	+	+	-	+
LSTR	-	-	-	-	-	-	-	_

Table 4 (continued)

Tests	*S. hyicus*	*S. lugdunensis*	*S.lentus*	*S. simulans*	*S. scuri*	*S. saccharolyticus*	*S. saprophyticus*
(0)	-	-	-	-	-	-	-
GLU	+	+	+	+	+	+	+
FRU	-	+	+	+	+	+	+
MNE	+	+	+	-	+	-	-
MAL	-	+	+	-	+	+	+
LAC	-	+	+	-	-	+	+
TRE	+	+	+	+	+	+	+
MAN	-	-	+	+	+	-	-
XLT	-	-	-	-	-	+	-
MEL	-	-	+	-	-	-	-
NIT	+	+	-	+	+	-	-
PAL	+	-	-	+	+	-	-
VP	-	+	-	-	-	+	-
RAF	-	-	+	-	-	-	-
XYL	-	-	+	-	-	-	-
SAC	+	+	+	+	+	+	+
MDG	-	-	-	-	-	-	-
NAG	+	+	+	+	+	+	+
ADH	+	+	-	+	-	-	+
URE	+	+	-	+	-	+	-
LSTR	-	-	-	-	-	-	-

Fig. 1: Black colonies of *Staphylococcus aureus* on Baird-Parker medium.

Fig. 2: Mannitol fermenting colonies of *Staphylococcus aureus* on M.S.A. medium.

Fig. 3: Mannitol non-fermenting colonies of *Staphylococcus saprophyticus* on M.S.A. medium.

Fig. 4: Mannitol fermenting colonies of *Staphylococcus caseolyticus* on

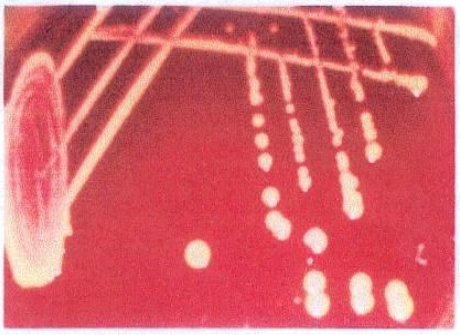

Fig. 5: Non-haemolytic colonies of *Staphylococcus chromogenes* on blood agar.

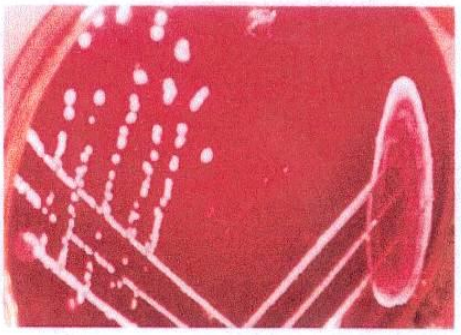

Fig. 6: Non-haemolytic colonies of *Staphylococcus hyicus* on blood agar.

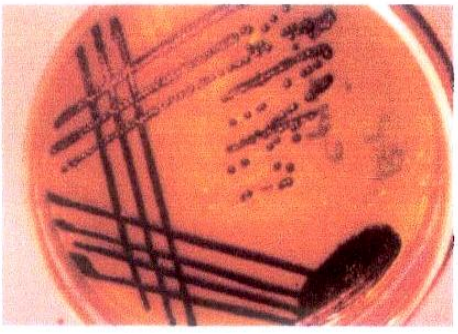

Fig. 7: Black colonies of *Staphylococcus epidermidis* on Baird-Parker medium.

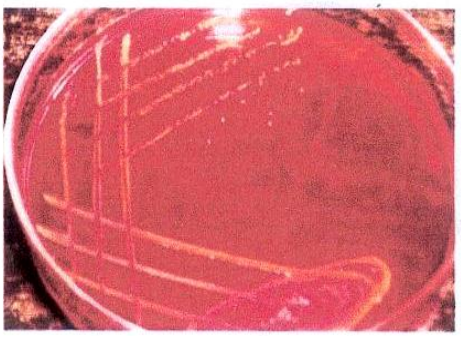

Fig. 8: Mannitol non-fermenting colonies of *Staphylococcus epidermidis* on M.S.A. medium.

Infection or injury of the udder results in an increase in somatic cells. Somatic cells are composed of approximately 75 percent leucocytes and 25 percent epithelial cells. Epithelial cells are in fact dead cells which have been sloughed from alveoli and canals within the udder. Somatic cells are useful in detecting subclinical mastitis which has been shown to be the most costly form of mastitis. Rising levels of somatic cells act as an early indicator of future clinical mastitis. As a cow-side test, the California Mastitis Test (CMT) can be useful in detecting and controlling mastitis since it focuses attention on the individual quarters that are secreting milk with high number of leucocytes. It is practically useful in detecting subclinical and chronic mastitis. Most clinical cases begin as subclinical mastitis while the chronic cases serve as constant reservoir of mastitis causing organisms. Problem cows can be identified by the Somatic Cell Count (SCC) score [19].

Hundred bovine mastitic milk samples were collected from different quarters of the 46 producing dairy cows positive for CMT and were further investigated for SCC and bacteriological cultures.

Elevated somatic cell count was by no means a sure sign of an udder infection. Somatic cells counts only indicate the udder has responded to an irritant. In this study there was high correlation between CMT and SCC, but there was no correlation between cell count and other inflammation parameters such as swelling, pain, heat and abnormal secretion of mammary glands. However, a high prevalence (38.2%) of coagulase-negative staphylococci was obtained in this study from cows with high SCC (more than 660,000 cells/ml). [20] reported that *S. aureus* increased the SCC over 600,000 cells/ml, whereas coagulase-negative staphylococci caused an infection which increased SCC to lesser extent in most cases up to 500,000 cells/ml.

One hundred thirty three staphylococci were recovered from 82 out of the 100 samples. In some cases more than one isolate was recovered from the same sample. Of the staphylococci 35 (26.3%) were coagulase-positive *S. aureus* and 98 (73.7%) coagulase-negative. These findings show a high prevalence of staphylococci in dairy herds in Khartoum area. [15] found that, *S. aureus* was responsible for 30-40% of subclinical cases of bovine mastitis and over 30% subclinical cases of bovine mastitis were caused by coagulase-negative staphylococci. [1] mentioned that *S. aureus* is the first microorganism incriminated in bovine mastitis.

Coagulase-negative staphylococci: *S. epidermidis* (10.5%), *S. chromogenes* (9.0%), *S. haemolyticus* (6.0%), *S. hyicus* (6.0%), *S. simulans* (5.3%) and *S. xylosus* (5.3%), were implicated in bovine mastitis elsewhere [21].

Other coagulase-negative staphylococci: *S. capitis subsp ureolyticus* (6.8%), *S. caseolyticus* (5.3%), *S. carnosus* (3.8%), *S. lugdunensis* (3.8%), *S. saprophyticus* (4.5%), *S. capitis* (3.0%), *S. saccharolyticus* (2.3%) and *S. sciuri* (2.3%), have not been proven to cause mastitis in dairy cattle. However, *S. capitis subsp. ureolyticus*, *S. caseolyticus* and *S. capitis* were recovered from bovine mastitic milk, whereas *S. carnosus* and *S. lugdunensis* were isolated from normal milk of cows [14] and *S. sciuri* was isolated from normal milk of cows [22].

In this study coagulase-negative staphylococci were the most prevalent in bovine mastitic milk samples as they constituted 32.8% out of 299 microorganisms isolated. [12] found coagulase-negative staphylococci in 70.7% of the total Gram-positive bacteria isolated from bovine mastitic milk.

Conclusion

This study clearly revealed that CMT and SCC are valuable in detecting subclinical cases of bovine mastitis and staphylococci are the predominant bacterial spp. isolated from bovine mastitic milk samples in River Nile State. Moreover, Coagulase-positive and coagulase-negative staphylococci are involved in bovine mastitis. Coagulase-negative staphylococci should be suspected as a cause of bovine mastitis when accompanied with a high SCC. The significance of staphylococci in bovine mastitis is becoming well known and further studies should be carried out to investigate the predisposing factors related to the incidence of bovine mastitis and to identify different causes of bovine mastitis. Further studies should include a survey of more animals in different farms and an extensive study of the significance of coagulase-negative staphylococci in bovine mastitis. Moreover the serotyping of staphylococcal isolates obtained from different areas should be given more attention.

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