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Bacterial Bovine Mastitis and Formation of Specific antibodies in Milk and Sera, Sudan

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Abstract

The aim of the study to isolate and identify the bacteria which can cause mastitis in cows and the antibodies forming during the infection by these bacteria and appear in milk and serum. A total of 50 samples of milk were collected from all quarters of each cow and sera were collected from cows with clinical and subclinical mastitis in two different farms in Khartoum. The bacteria were isolated from 38 (76%) of the milk samples and the total number of isolates was 45. Sixteen (36%) bacterial isolates were obtained from cows with clinical mastitis, whereas 29 (64%) were obtained from cows with sub-clinical mastitis. Mixed infection was detected in 10 (20%) of the milk samples. Both Gram positive and Gram negative bacteria were isolated and identified to the species level, using cultural characteristics and biochemical tests. Most isolates were *Staphylococcus* species, other gram positive bacteria isolates: 1: *Streptococcus agalactiae*, 3 species from *Enterococcus* (*avium*, *mundtii* and *faecium*), 2: *Listeria Ivanovii*, 3: *Bacillus* species (two *Bacillus licheniformis* and one *B. mycoides)* and 3 strains of *Corynebacterium pseudotuberculosis*. The gram negative bacterial isolates: three *Enterobacteria* species (2: *Citrobacter freundii* and 1: *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* consisted two strains. Only *Bacillus mycoides* gave clear precipitation lines with both milk immunoglobulin fraction and serum. Other bacteria gave precipitation lines with serum antibodies alone. The results revealed that the Gram positive bacteria, especially *Staphylococcus* species, were the common causative agent of bovine mastitis; the number of cows with subclinical mastitis was higher than cows with clinical mastitis. The bacteria associated with bovine mastitis can induce local and systemic specific antibodies response as measured by agar gel immuno-diffusion test.

Keywords: Bovine mastitis; Bacteria; Antibodies; Serum; Mill

Introduction

Mastitis is inflammation of the mammary gland due to the injury of any type. However, the udder disease of major concern is that associated with microbial infection. The microbes that are associated with mastitis are: *Staphylococcus* species, *Streptococcus* species and other Gram positive and Gram negative rods [1]. Mastitis is characterized by physical, chemical and usually bacteriological changes in milk and by pathological changes in the glandular tissue [1]. Loss of milk production, cows premature culling, milk discarded or downgraded as well as veterinary expenses and large of some of money is lost to dairy farming each year through poor udder health [2]. Immunoglobulins in mammary secretion are derived from blood serum or are made locally by cells of the lymphocyte-plasma cell series situated close to the glandular epithelium. The major immunoglobulin in colostrum and milk of ruminants, IgG1 is derived from the blood and is transferred into secretion selectively relative to IgG2, probably by a mechanism requiring specific receptor sites on the basal or intercellular membrane of the glandular epithelium. Acute inflammation causes suppression of selective transfer of IgG1, but there is a marked increase in the transfer of proteins, such as IgG2 and serum albumin, which enter secretion non selectively. Infusion of antigen into the mammary gland of ruminants some weeks before parturition induces a persisting local production of antibody, most of which is associated with IgA and IgM. IgA antibodies in the mammary gland probably originate in the intestine, and prior antigenic stimulation of the gut may be required for maximal IgA antibody responses in the gland [3].

Substantial increases in immunoglobulin G subclass 1 (IgG1) and IgG2 antibody titers were detected in serum and lacteal secretions of animals immunized through an intestinal fistula. IgM and IgA antibody responses were low or undetectable. Low numbers of IgA and IgG1 plaque-forming cells were occasionally detected. It is proposed on the basis of these data that migration of antigen-stimulated IgG lymphoblasts and perhaps of antigen, to spleen and peripheral lymph nodes may be dominant events after intestinal immunization of ruminants. This is consistent with the predominance of serum-derive IgG antibodies in colostrums and milk. Intramammary infusion of antigen gave rise to increases in antibody titers in all classes which were greater not only in lacteal secretions but also in blood serum than with their systemic route used. Comparison of IgA titers in secretions from the immunized glands with those in serum also suggest that locally synthesized IgA antibodies might have contributed in some measure to serum titers. Local synthesis in both immunized and non immunized glands was also reflected by the presence of increased numbers of IgA and IgG1 plaque-forming cells. It was hypothesized that antibody forming cells responsible for local synthesis originated in lymphoid tissue within the mammary gland or from peripheral lymph nodes, depending upon the route of immunization.

The aim of the study to isolate and identify the bacteria which can cause mastitis in cows and the antibodies forming during the infection by these bacteria and appear in milk and serum.

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Methods

A total of 50 milk samples were collected from all quarters of each cow and 50 serum samples were collected in Khartoum State from dairy farms at Alsalama (Khartoum) and Shambat (Khartoum North). Only milk samples from cows that reacted positively to California Mastitis Test (CMT) were collected and subjected to bacteriological and immunological investigation in the laboratory. The swabs were cultured on blood agar, chocolate gar and MacConkey's agar (Oxoid). The plates were incubated aerobically at 37°C for 1-2 days. Visual examination for detection of growth, pigmentation and colonial morphology. Isolated bacteria were purified by repeated subculture on blood agar and nutrient agar plates and incubated at 37°C for 24 hours until pure colonies were obtained. The purified bacteria were stored at 4°C. Identification of isolates was carried out according to Barrow and Feltham [4]. Smears were prepared by emulsifying small inoculums of the bacterial culture in a drop of sterile normal saline and spreading them on clean slide. The smears were allowed to dry and fixed by gentle heating. Gram stain was done as described by Barrow and Feltham [4]. It was used to study morphology, shape and gram staining reaction of each isolates. Gram-positive bacteria appeared purple, while Gram-negative bacteria appeared red. Biochemical test were conducted and preformed according to Barrow and Feltham [4].

Took 30 ml in two sterile falcon tubes (15 ml to each one) and centrifuged at 4000 rpm for 30 minutes at 28°C to detect milk. The whey and cell depress were removed and the protein was taken and put in clean petri dish and mixed with 20% sodium sulphate, stirred with magnetic stirrer for 30 minutes and centrifuged at 4000 rpm for 30 minutes at 28°C. The deposit dissolved in PBS then concentrated by poly ethylene glycol, kept at -20°C [5]. Whole cell lysate of bacterial isolates prepared from isolate that were grown on blood agar and the colonies, put in 2 ml normal saline, 1 ml of excess fluid was transferred to other blood agar plate using pipette. The plate was incubated at 37°C for 24-48 h. The colonies were covered with 0.5% formalin saline and left overnight at 4°C to kill the bacteria, then the pellet was washed 2 times with normal saline at 4000 rpm for 30 minutes at 28°C. The deposit was suspended in PBS and sonicated in sonicator (MSE-England) for 30 seconds stroke and in short intervals, the amplitude kept 18 rpm per cycles, then concentrated by poly ethylene glycol and kept at -20°C. Agar gel immune diffusion (AGID) test was done to detect the presence of antibodies in milk and sera using Whole cell lysate of the isolates.

Results

In this study 50 milk samples were collected and bacteria was isolated from 38 (76%) of the milk samples. The bacteria were identified to the species level as shown in Tables 1-8. Bacteria were isolated from 29 (64%) of milk samples collected from 37 cows with subclinical mastitis as a previously detected by California mastitis test. Mixed infection was found in 7 (14%) of sample. The main bacteria isolated were Staphylococcus species. Mixed infection was found only 10 milk samples (20%) of both clinical and subclinical mastitis. Most isolates were Staphylococcus 27 (60%) all of them were coagulase positive 10 (22%), 9 (20%) *Staphylococcus hyicus* and 8 (18%) *Staphylococcus intermedius*) (Table 1), *Streptococcus agalactiae* was 1 (2%) of the isolate (Table 2), Enterococcus species were 4 (9%) (1, (2%) *Enterococcus avium*, 2 (4%) *Enterococcus mundtii* and 1 (2%) *Enterococcus faecium* (Table 3), *Listeria Ivanovii* were 2 (4%) (Table 4), Bacillus Species were 3 (7%) (2 (4%) *Bacillus licheniformis* and 1 (2%) *Bacillus mycoides* that show heamolysis on blood agar (Table 5), three strain of *Corynebacterium pseudotuberculosis* (7%) were found with three strains strain 1 (2%) was differed from the main genus

Biochemical Test	*Staphylococcus aureus*	*Staphylococcus intermedius*	*Staphylococcus hyicus*
Gram reaction	+	+	+
Shape	Sphere (cocci)	Sphere (cocci)	Sphere (cocci)
Spore	-	-	-
Motility	-	-	-
Growth in air	+	+	+
Catalase	+	+	+
Oxidase	-	-	-
Glucose(acid)	+	+	+
Carbohydrate (F\O\ -)	F	F	F
VP	+	-	-
Coagulase	+	+	+
Nitrate	+	+	+
Urease	+	+	+
Acid from Maltose	+	-	-
Acid from Mannitol	+	+	-
Acid from Xylose	-	-	-
Acid from Lactose	+	+	+

O=Oxidative; F=Fermentative; VP=Voges-Proskauer

Table 1: Biochemical reaction of *Staphylococcus* species isolates.

Biochemical Test	Streptococcus agalactiae
Gram reaction	+
Shape	Sphere (cocci)
Spore	-
Motility	-
Growth in air	+
Catalase	-
Oxidase	-
Glucose(acid)	+
Carbohydrate (F\O\ -)	F
CAMP test	+

CAMP=Christie Atkins and Munch Petersen

Table 2: Biochemical reaction of *Streptococcus agalactiae*.

Biochemical Test	Enterococcus		
	Avium	Mundtii	Faecium
Gram reaction	+	+	+
Shape	Sphere (cocci)	Sphere (cocci)	Sphere (cocci)
Spore	-	-	-
Motility	-	-	-
Growth in air	+	+	+
Catalase	-	-	-
Oxidase	-	-	-
Glucose(acid)	+	+	+
Carbohydrate (F\O\ -)	F	F	F
Haemolysis	-	Alpha	Alpha
Growth at 45	+	+	+
Yellow pigment	+	_	+
Bile-Aesculin	+	+	+
VP	+	+	+
Hydrolysis of Aesculin	+	+	+
Hydrolysis of Arginine	+	-	+
Hydrolysis of Starch	-	-	-
Fermentation of Arabinose	+	+	+
Fermentation of Adonitol	+	+	+
Fermentation of Sucrose	+	+	+
Fermentation of Lactose	+	+	+

Table 3: Biochemical reaction of Enterococcus Species.

by acid from xylose and hydrolysis of aesculin the two were found positive in this strain but were negative in the main strain, strain 2 (2%) was differed from the main genus by acid from xylose, Salicin and hydrolysis of aesculin the three were found positive this strain opposite to the main strain and the last strain 3 (2%) was differed from the main strain by xylose, salicin, hydrolysis of Aesculin and VP they all were found positive in this strain but were negative in the main strain (Table 6), Enterobacteria species were 3 (7%) 2 (4%) *Citrobacter freundii* and 1 (2%) *Klebsiella pneumoniae* (Table 7), the two strain of *Pseudomonas aeruginosa* (alkali producers) (4%) but they were differed from the main genus by acid from glucose it was negative in the primary biochemical test in this strain but positive in the main strain however the all (the 2 strain and the main genus) were read on the secondary table of the biochemical test after suppose that the acid from glucose is negative (Table 8). The serums' antibodies of all isolates gave positive result in a form clear precipitation lines on AGID test (27%) exception that of the *Staphylococcus*, Streptococcus and Enterococcus species on the other hand the milk's antibody of all isolates gave negative result on AGID test exception that of the *Bacillus mycoides* species (0.02%) (Table 9).

Biochemical test	Listeria Ivanovii
Gram reaction	+
Shape	Rod
Spore	-
Motility	+
Growth in air	+
Catalase	+
Oxidase	-
Glucose(acid)	+ (gas)
Carbohydrate (F\O\ -)	F
B-haemolysin	+
CAMP test	-

Table 4: Biochemical reaction of *Listeria ivanovii.*

Biochemical Test	Bacillus	
	Mycoides	Llicheniformis
Gram reaction	+	+
Shape	Rod	Rod
Spore	+	+
Motility	-	+
Growth in air	+	+
Catalase	+	+
Oxidase	+	+
Glucose(acid)	+	+
Carbohydrate (F\O\ -)	-	-
In ammonium salt media acid from Glucose	+	+
In ammonium salt media acid from Glactose	+	+
In ammonium salt media acid from Salicin	+	+
In ammonium salt media acid from Xylose	-	+
Urease	+	+
VP	+	+
Indol	-	-
Nitrate	+	+

Table 5: Biochemical reaction of *Bacillus* spp.

Biochemical Test	*Corynebactrium psedotuberculosis*		
	1	2	3
Gram reaction	+	+	+
Shape	Rod	Rod	Rod
Spore	-	-	-
Motility	-	-	-
Growth in air	+	+	+
Catalase	+	+	+
Oxidase	-	-	-
Glucose(acid)	+ (gas)	+ (gas)	+ (gas)
Carbohydrate (F\O\ -)	F	F	F
Acid from Lactose	+	+	+
Acid from Maltose	+	+	+
Acid from Salicin	-	+	+
Acid from Starch	+	+	+
Acid from Xylose	+	+	+
VP	-	-	+
Ausculin hydrolysis	+	+	+
Arginine hydrolysis	+	+	+
Urease	+	+	+
Nitrate reduced	+	+	+

Table 6: Biochemical reaction of *Corynebactrium psedotuberculosis.*

Biochemical test	*Klebsiella pneumoniae*	*Citrobacter freundii*
Gram reaction	-	-
Shape	Rod	Rod
Spore	-	-
Motility	-	+
Growth in air	+	+
Catalase	+	+
Oxidase	-	-
Glucose(acid)	+ (gas)	+ (gas)
Carbohydrate (F\O\ -)	F	F
MacConkey growth	+	+
Urease	+	+
Gluconate	-	-
VP	-	-
Negative	-	+
Acid from Glycerol	+	+
Acid from Lactose	+	+
Acid from Maltose	+	+
Acid from Mannitol	+	+
Acid from Raffinose	+	+
Acid from Rhamanose	+	+
Acid from Salicin	+	-
Acid from Sorbitol	+	+
Acid from Sucrose	+	+
Acid from Xylose	+	+
Acid from Starch	-	-

Table 7: Biochemical reaction of Enterobacteria.

Biochemical Test	*Pseudomonas aeruginosa*
Gram reaction	-
Shape	Rod
Spore	-
Motility	+
Growth in air	+
Catalase	+
Oxidase	+
Glucose(acid)	Negative
Carbohydrate (F\O\ -)	O
MacConkey growth	+
Green Pigment	+
Nitrate	+
Simmon's Citrate	+
Urease	+
Gluconate	+
Malonate	+
Ammonium salt media acid from Glucose	+
Ammonium salt media acid from Maltose	-
Ammonium salt media acid from Mannitol	+
Ammonium salt media acid from Fructose	+
Ammonium salt media acid from Arabinose	+
Ammonium salt media acid from Xylose	+

Table 8: Biochemical reaction of *Pseudomonas aeruginosa*.

Bacteria Species	Serum Antibodies	Milk Antibodies
Staphylococcus aureus	-	-
Staphylococcus intermedius	-	-
Staphylococcus hyicus	-	-
Streptococcus agalactiae	-	-
Enterococcus mundtii	+	-
Enterococcus avium	+	-
Listeria Ivanovii	+	-
Bacillus mycoides	+	+
Bacillus licheniformis	+	-
Corynebactrium	+	-
Klebsiella Pneumoniae	+	-
Citrobacter freundii	+	-
Pseudomonas aeruginosa	+	-

Table 9: Detection of specific antibody to causative agent in sera and milk of infected cows in agar double diffusion test.

Discussion

Fifty milk and serum samples were collected and bacteria were isolated from only 38 (76%) milk samples. The most common organism isolated in this study was Staphylococcus spp. Coagulase positive staphylococci (60%) were the most frequently isolated bacteria in this study and all of them were isolated from clinical cases these findings were in agreement with the findings other authors [3,6,7]. In this study *Staphylococcus intermedius* isolated and this in agreement with the finding by Chaffer [8] who was isolate *Staphylococcus intermedius* from mastitis. *Bacillus licheniformis* isolated and this is in agreement with Logan [9]. Also an isolated bacterium was *Listeria ivanovii* from mastitic cow and this is in agreement with Rawool [10]. In this study *Bacillus mycoides* was isolated from clinical mastitis and *Bacillus licheniformis* were isolated from sub-clinical mastitis these findings were in agreement with the findings [11] who reported that Bacillus spp. were isolated in both clinical and subclinical cases. *Klebsiella* spp. isolated and this is in agreement with Cullor [12], who found that 20% of bovine mastitic case, in Nordic countries caused by coliform of which about 85% were *E. coli* and *Klebsiella* spp., and other Enterobacteria. In this study we isolated coliform bacteria like: *Klebsiella pneumoniae* and *Citrobacter freundii* and this is in agreement with findings by Jackson and Bramle [13], mentioned that the Coliform such as *E. coli, Klebsiella Pneumoniae, Klebsiella oxytoca, Enterobacter cloatae, Enterobacter arogenes*an and *Citrobacter freundii* are all associated with bovine mastitis. Other isolated bacteria were *Enterococcus faecium, Enterococcus mundtii* and *Enterococcus avium* and this is in agreement with Jayarao [14]. In this study whole cell lysate of bacterial isolates was tested against sera and milk, in order to detect specific antibodies against the isolates. Some bacterial isolates gave frank precipitation lines with their respecting serum antibodies but not with milk. In contrast, *Bacillus mycoides* gave clear precipitation lines with sera and milk although it is not common within the bacteria that cause bovine mastitis and this is in agreement with the finding by Carneiro [15]. The significance of these antibodies in protection against mastitis [16-21] and the immunology of the udder were reviewed by Carneiro [15].

Conclusion

This study has shown that Gram positive bacteria, especially Staphylococcus species were the common causative agent of bovine mastitis. The number of cows with subclinical mastitis was higher than cows than cows with clinical mastitis. The bacteria associated with mastitis can induce local and systemic specific antibodies response as measured by agar gel immune diffusion test. The research need further study to detect the other antibodies for other bacterial agents to simplify diagnosis of bovine mastitis without culturing milk to detect the specific bacteria caused mastitis and apply the method to detect the antibodies to other microorganism causing mastitis like virus and parasites.

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