The impact comparison of Staphylococcus aureus mastitis on the level of milk nitric oxide, immunoglobulin A and complement 3 between cows and buffaloes

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Abstract: Mammary gland secretions derived from secretory cows and buffaloes infected with *Staphylococcus aureus (S. aureus)* was examined for the nitric oxide (NO), immunoglobulin A (IgA) and complement 3 (C3). The examined cows reflected 25 cases of subclinical mastitis, 20 cases of clinically mastitis animals and 10 cases of recurrent (chronic) mastitis and the same number from buffaloes. The level of NO in the normal cows was found to be 9.54 µM/ml to increase to 18.10 µM/ml in the subclinical mastitis animals and to significantly increase further to 23.23 µM/ml in the clinically affected cases and 25.01 µM/ml in recurrent (chronic) mastitis cows, while the level of NO in buffaloes were 6.31, 8.30, 13.12 and17.55 µM/ml for normal, subclinical, clinical and recurrent (chronic) cases, respectively. The level of IgA in cows were 125, 530, 619 and 804 mg/dL for normal, subclinical, clinical and recurrent (chronic) cases, respectively, while the level of IgA in buffaloes were 232, 620, 719 and 934 mg/dL for normal, subclinical, clinical and recurrent (chronic) cases, respectively. The level of C3 in cows were 19, 57, 60 and 92 mg/dL for normal, subclinical, clinical and recurrent (chronic) cases, respectively, while the level of C3 in buffaloes were 20, 58, 62 and 91 mg/dL for normal, subclinical, clinical and recurrent (chronic) cases, respectively. These results suggest the promising use of milk whey NO, IgA and C3 concentration variabilities as prognostic parameters on the degree of the commencement of mastitis differences between cows and buffaloes.
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1. Introduction

Bovine mastitis is an inflammatory disease of cows and buffaloes mammary gland caused by various infectious or non infectious etiological agents. Mastitis was one of the first observed diseases of farm animals when cattle were domesticated over 5000 years ago. Since then it will have been an ever present problem for all those who kept and milked dairy cattle and buffaloes ***(Sharma et al., 2012).*** Mastitis is the most costly disease for dairy farmers and industry ***(Hogeveen et al., 2011),*** also a highly prevalent and costly disease in dairy industry due to antibiotherapy and loss in milk production ***(Rato et al., 2013).***

The major causes of bovine mastitis are *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae*, *Str. ubris* ***(Phuektes et al., 2001)***. *S. aureus* is recognized worldwide as a major pathogen causing subclinical intramammary infections in dairy cows ***(Salasia et al., 2004)***. *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis* are relevant mastitis pathogens ***(Rato et al., 2013).***

This study is mainly focused on mastitis caused by *S. aureus* infections. One reason for this is that the bacterium is one of the commonest udder pathogen in Egypt. However, the main explanation is the fascinating development of persistent cases of mastitis associated with the contagious pathogen *S. aureus*. *S. aureus* infection often starts with an acute phase and generally becomes chronic and subclinical ***(Sutra and Poutrel, 1984).***

Nitric oxide operates in a variety of tissues to regulate a diverse range of physiological processes including the inflammatory response ***(Dawson and Dawson, 1995).*** Macrophage and epithelial cells of the mammary gland produce significant amount of NO, this inducible NO mediates inflammation during mastitis and an increase in milk NO levels from cows with experimentally induced mastitis has been reported, and it has been proposed that the elevated NO concentration in milk resulted from the inflammatory response of the mammary gland ***(Bouchard et al., 1999)***.

Immunoglobulin A is the main Ig of the seromucous tissue, defending the exposed external surface by blocking the adherence of microorganism and their toxins to epithelial cells ***(Avery and Gorden, 1991).***

Phagocytosis is facilitated by opsonins of which IgG and complement component 3 (C3) are the most important. This facilitation is due to the existence of receptors for the Fc part of IgG and for C3b or iC3b on the surface of the Poly Morphnucleur (PMN) ***(Howard et al., 1980)***.

2. Material and methods:

2.1. Animals:

A. Lactating cows:

A total number of 220 lactating Holstein-Friesian cows from 4 dairy herds located in El Bohera Governorate were studied in this investigation.

B. Lactating Buffaloes:

A total number of 110 lactating buffaloes from 2 dairy herds located in El- Bohera Governorate were also studied in this investigation.

2.2. Milk samples:

A. Lactating Cows:

A total number of 880 quarter milk samples were collected from lactating Holstein-Friesian cows and tested for subclinical mastitis, clinical mastitis, recurrent (chronic) mastitis and apparently normal quarter by California Mastitis Test (CMT) and physical examination. All milk samples were collected according to ***Quinn et al. (2002)***. All milk samples were taken during the period between winter and spring of 2011 and 2012.

B. Lactating Buffaloes:

A total number of 440 quarter milk samples were collected from lactating buffaloes and tested for subclinical mastitis, clinical mastitis, recurrent (chronic) mastitis and apparently normal quarter by California Mastitis Test (CMT) and physical examination. All milk samples were collected according to ***Quinn et al. (2002)***. All milk samples were taken during the period between winter and spring of 2011 and 2012.

Milk samples were collected aseptically according to the known routine procedure. Each udder was examined before sampling for presence of clinical signs of mastitis such as symmetry, hotness, hardness, swelling or any physical changes. The examined udders were then thoroughly washed, dried with a clean towel and the teats were sprayed with 70% ethanol. After that the first few jets of milk were discarded and 10 ml of milk samples from each quarter were collected in a sterile McCartney bottle ***(Blood and Henderson, 1986)***. All samples were kept at 4 ^{o}C and transported immediately to the laboratory. Each sample was divided into two parts, each in a sterile McCartney bottle. One was incubated for 24 hrs for bacteriological examination, the second was used to separate milk serum and the extracted milk was stored at -80 ^{o}C until assayed for nitric oxid, immunoglobuline A and complement 3.

2.3. Bacteriological examination:

The sediment was streaked on to the surface of nutrient agar, Baird Paker agar, Mannitol salt agar and blood agar. The isolates were culturally and biochemically screened and identified according to ***(Quinn et al., 2002).***

2.4. Detection of the effect of *S. aureus* mastitis nitric oxide, immunoglobulin A and complement 3 concentration in milk whey of cows and buffaloes:

To estimate the effect of *S. aureus* mastitis on some immunological parameters in milk of cows and buffaloes, a total of 60 milk samples were selected from the total examined cows (n = 220) and 60 milk samples were selected from the total examined buffaloes (n = 110) and classified into 4 groups in both cows and buffaloes:

First group: Control normal *S. aureus* negative milk samples (n = 5).

Second group: Subclinical *S. aureus* positive milk samples (n = 25).

Third group: Clinically mastitis milk samples *S. aureus* positive (n = 20).

Forth group: Recurrent (Chronic) mastitis milk samples *S. aureus* positive (n = 10).

2.4.1. Nitric oxide assay:

The measurement of nitric oxide was assessed according to the assay described by ***Ramadan and Attia (2003).***

2.4.2. Immunoglobulin A assay:

2.4.2. Complement 3 assay:

The measurement of nitric oxide was assessed according to the assay described by ***Young (2000).***

2.5. Statistical analysis:

The statistical procedures used were according to ***Snedecor (1985).*** The Student's t-test was used in addition to the Analysis of Variance Fisher (F test).

3. Results:

The results recorded in Table 1 reveals that the incidence of *S. aureus* being the causative agent in clinically mastitis cases reached 31.5%, subclinical cases in a percentage of 32.8% and in recurrent (chronic) cases in a percentage of 11.1%. At the same time the *S. aureus* was isolated in the normal milk from apparently healthy udders that did not reveal any clinical signs and was negative to the CMT in a percentage of 30.4% in cows.

The results recorded in Table 1 reveals also that the incidence of *S. aureus* being the causative agent in buffaloes in clinically mastitis cases reached 33.3%, subclinical cases in a percentage of 34.6% and in recurrent (chronic) cases in a percentage of 28.6%. At the same time the *S. aureus* was isolated in the normal milk from apparently healthy udders that did not reveal any clinical signs and was negative to the CMT in a percentage of 30.7% in cows.

Table (1): The bacteriological positive samples for *S. aureus* isolated from the normal and mastitis quarters milk samples in both cows and buffaloes.

Item	Subclinical mastitis		Clinical mastitis		Recurrent (Chronic) mastitis		Apparently normal		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Cows	151	32.8	56	31.5	2	11.1	68	30.4	277	31.5
Buffaloes	84	34.6	21	33.3	2	28.6	39	30.7	146	33.2

No.: Positive number. %: Was calculated according to the number of examined samples (880 in cows and 440 in buffaloes).

Table (2): Nitric oxide (NO) level in milk whey of cows and buffaloes at different degrees of *S. aureus* mastitis.

Mastitis degrees	Cows	Buffaloes	t-test
	Mean±Std. Error	Mean±Std. Error	
Control	9.54±0.61D_a	6.31±0.55D_b	5.58***
Subclinical	18.10±0.81C_a	8.30±0.37C_b	4.22***
Clinical	23.23±0.65B_a	13.12±0.43B_b	6.68***
Recurrent (Chronic)	25.01±0.95A_a	17.55±0.71A_b	6.02***

Capital letters: Indicated that, means within the same column of different letters are significantly different at ($P < 0.01$).
Small letters: Indicated that, means within the same row of different letters are significantly different at ($P < 0.01$).
* = Significant at ($P = 0.05$) **= Significant at ($P < 0.01$) ***= Significant at ($P < 0.001$) NS = Non-significant at ($P > 0.05$)

The level of NO was higher in cow's milk whey than buffaloes milk whey in all stages of mastitis with significant at ($P < 0.001$) while the NO level was higher in recurrent (chronic) mastitis then clinical then subclinical and control cases with significant at ($P < 0.01$).

Table (3): IgA concentration (mg/dL) in milk whey in *S. aureus* cows and buffaloes mastitis.

Mastitis degrees	Cows	Buffaloes	t-test
	Mean±Std. Error	Mean±Std. Error	
Control	125.00±4.01 D_b	232.00±3.89 Ca	19.14***
Subclinical	530.00±18.20B_b	620.00±12.82Ba	4.04***
Clinical	619.00±15.09C_b	719.00±19.58 B_a	4.04***
Recurrent (Chronic)	804.00±19.62A_b	934.00±12.13 A_a	5.63***

Capital letters: Indicated that, means within the same column of different letters are significantly different at ($P < 0.01$).
Small letters: Indicated that, means within the same row of different letters are significantly different at ($P < 0.01$).
* = Significant at ($P = 0.05$) **= Significant at ($P < 0.01$) ***=Significant at ($P < 0.001$) NS = Non-significant at ($P > 0.05$)

The levels of IgA were higher in buffaloes than in cows in all stages of mastitis at significant of ($P < 0.001$). Moreover, the IgA level was higher in recurrent (chronic) mastitis followed by clinical mastitis then subclinical mastitis at significant of ($P < 0.01$).

Table (4): Complement 3 (C3) concentrations (mg/dL) in milk whey in *S. aureus* cows and buffaloes mastitis.

Mastitis degrees	Cows	Buffaloes	t-test
	Mean±Std. Error	Mean±Std. Error	
Control	19.00±2.77 C_a	20.00±2.11 C_a	0.28NS
Subclinical	57.00±3.35 B_a	58.00±3.27 B_a	0.21NS
Clinical	60.00±2.58 B_a	62.00±2.49 B_a	0.55 NS
Recurrent (Chronic)	92.00±2.49 A_a	91.00±2.33 A_a	0.29 NS

Capital letters: Indicated that, means within the same column of different letters are significantly different at ($P < 0.01$).
Small letters: Indicated that, means within the same row of different letters are significantly different at ($P < 0.01$).
* = Significant at ($P = 0.05$) **= Significant at ($P < 0.01$) ***=Significant at ($P < 0.001$) NS = Non-significant at ($P > 0.05$)

There were non-significant differences between cows and buffaloes in the level of C3 in different stages of mastitis. Moreover, the C3 level was higher in recurrent (chronic) mastitis followed by clinical mastitis then subclinical mastitis at significant of ($P < 0.01$).

4. Discussion:

Mastitis, an inflammatory reaction of the mammary gland that is usually caused by a microbial infection, is recognized as the most costly disease in dairy cattle. Decreased milk production accounts for approximately 70% of the total cost of mastitis. Mammary tissue

damage reduces the number and activity of epithelial cells and consequently contributes to decreased milk production ***(Zhao and Lacasse, 2008).*** Mastitis, although an animal welfare problem, is a food safety problem and is the biggest economic problem ***(Sharma et al., 2011)***.

Clinical examination of the udder of dairy animals under investigation, especially in the subclinical form of mastitis proved to be of little value in diagnosing the disease, as all examined cases which were found to be mastitis through bacteriological examination revealed no apparent signs or symptoms of udder inflammation by palpation. Hence this method is inadequate and cannot be relied upon for subclinical mastitis unless accompanied with other reliable tests ***(Abdel-Karim and El- Ashmawy, 1979, Alsaied, 2006*** and ***Khalil, 2007).***

Staphylococcus aureus is an important human and animal pathogen that it represents a major agent of contagious bovine mastitis ***(Barkema et al., 1999, Gianneechina et al., 2002*** and ***Ghaleb et al., 2005).*** *S. aureus* is a well-armed pathogen that is a leading cause of bovine mastitis ***(Klein et al., 2012).***

Staphylococcus aureus was the most important and prevalent contagious mammary pathogen, it causes clinical and subclinical intramammary infection with serious economic loss and herd management problems in dairy cows ***(Dego et al., 2002).***

In the current study, results of bacteriological isolation among cows shown in tables (1) revealed that *S. aureus* was isolated with a percent of 31.5% and 33.2% in cows and buffaloes, respectively. Moreover, ***Dego et al. (2002)*** reported that *S. aureus* was the most important and prevalent contagious mammary pathogen, it causes clinical and subclinical intramammary infection with serious economic loss and herd management problems in dairy cows.

Many soluble factors present in milk act in collaboration with innate and immune defense mechanisms of these are nitric oxide (NO) ***(Lacasse et al., 1997)***. NO plays an important role in macrophage mediated cytotoxic activity against a variety of pathogenes including bacteria ***(Kandemir et al., 2002)***.

Nitric oxide (NO) in our study increased and elevated in both milk whey due to *S. aureus* infections to the mammary gland will activate the macrophages to engulf bacteria leading to production of NO due to phagocytosis ***(Stueher and Marletta, 1987).***

The higher levels of IgA in mammary secretions could serve as a first line of protection against a pathogen. Several pathogenic Gram-positive bacteria encode surface proteins capable of binding to immunoglobulins, and it is postulated that this binding might help the bacterium to evade the immunological surveillance of the host ***(Hammerschmidt et al., 1997).***

The IgA was the predominant immunoglobulin defence against microbial infection of mucosal surfaces, sequestering of IgA by the bacterium may be a mechanism for avoiding the first line of host defence. So the binding to IgA might also block the interactions between the IgA Fc part and the host receptors ***(Johnsson et al., 1999).***

The association between *S. aureus* chronic mammary gland infection and the resulting immune response expressed by the production of specific IgA antibodies in blood and milk of Israile Holsten cows ***(Leitner et al., 2000).***

We revealed in this study that the levels of IgA were higher in buffaloes than in cows in all stages of mastitis at significant of ($P < 0.001$). Moreover, the IgA level was higher in recurrent (chronic) mastitis followed by clinical mastitis then subclinical mastitis at significant of ($P < 0.01$).

The system of complement plays an important role in inflammation and defense against invading pathogens. This should apply to the bovine mammary gland with regard to mastitis, although the udder secretes large quantities of milk, in which bacteria find almost inexhaustible nutrients and immunological defenses are impaired. Bovine milk is known to possess poor hemolytic and bactericidal complement-mediated activities. These activities are sometimes found, usually near the lower limit of detection of the tests, towards the end of the lactation period and in colostrum ***(Rainard et al., 1984)***.

Phagocytosis by neutrophilic granulocytes is the main defense of the bovine mammary gland, and the complement fragments of C3 (C3b and iC3b) play a prominent role in opsonization through their interaction with the corresponding membrane receptors of phagocytes ***(Frank and Fries, 1991)***.

Complement-mediated opsonisation by complement component 3 (C3) binding is an important component of the innate immune system. They investigated the role of milk complement as an opsonin and its involvement in the phagocytosis and killing of *S. aureus* isolates

from cases of bovine mastitis by bovine blood PMN. Milk complement enhanced the chemiluminescence response of Poly Morphnucleur (PMN) induced by *S. aureus*. Nevertheless, the association of *S. aureus* to cells and the overall killing of bacteria by bovine PMN were not affected by the presence of milk complement. Therefore, as all milk samples contained antibodies to capsular polysaccharide type 5 and to other surface antigens, it is likely that milk antibodies were responsible for these two phagocytic events. They suggested that the deposition of milk complement components on the surface of *S. aureus* does not contribute to the defence of the mammary gland against *S. aureus* ***(Barrio et al., 2003)***.

There were non-significant differences between cows and buffaloes in the level of C3 in different stages of mastitis. Moreover, the C3 level was higher in recurrent (chronic) mastitis followed by clinical mastitis then subclinical mastitis at significant of ($P < 0.01$).

5. Conclusion:

Worldwide, a lot of effort has been focused on how to minimize the effects of subclinical, clinical and recurrent (chronic) mastitis, for example by preventing intramammary infection using vaccines, or to eliminate ongoing infections by treatment with antibiotics, or to stimulate the immune system of an animal with non-specific immunomodulators. Despite all these efforts the bacteria are ahead of us. To win the battle against *S. aureus* and decrease the number of cows and buffaloes suffering from subclinical, clinical and recurrent (chronic) mastitis, it is essential that all infected cows and buffaloes are rapidly detected and isolated from other animals and that proper milking management and other preventive measures are used to minimize the spread of bacteria.

This study provided an insight into the inflammatory response during *S. aureus* mastitis. Intramammary infection with *S. aureus* elicited local production of cytokines and peak concentrations of IgA reached once clinical signs had been established. A significant increase in the concentration of C3 and NO were also observed. These findings suggest that other factors may be involved in the initial leukocyte recruitment intomammary glands after *S. aureus* infection. Based on the study on healthy cows and cows with naturally occurring cases of subclinical, clinical and recurrent (chronic) *S. aureus* mastitis, milk IgA, C3 and NO were considered to be good indicators on the health of the udder quarters, and also to have a potential as indicators of subclinical, clinical and recurrent (chronic) mastitis. The large variation in IgA, C3 and NO within quarter samples from cows and buffaloes with subclinical, clinical and recurrent (chronic) mastitis suggests that different mechanisms regulate migration of leukocytes into the udder compared to the influx and/or secretion of IgA, C3 and NO into milk.

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