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Serum Biochemical Changes in Response to Affection with *Pseudomonas aeruginosa* Mastitis in Holstein Dairy Cows

Dina R.S. Gad El-Karim[1*], Gamal A. El-Amrawi[2], Alyaa R. Salama[1]

[1]Department of Pathology and Clinical Pathology, Faculty of Veterinary Medicine, Alexandria University, Egypt.

[2]Department of Theriogenology, Faculty of Veterinary Medicine, Alexandria University, Egypt.

***Correspondence**
Corresponding author: Dina R.S. Gad El-Karim
E-mail address: dina.shabaan@alexu.edu.eg

Abstract

Despite of the great efforts to develop effective control programs for mastitis, it is still one of the most economically important diseases in dairy cattle herds. *Pseudomonas aeruginosa* is a member of coliform Gram-negative bacteria causing treatment-resistant clinical or sub-clinical mastitis in dairy cows. This study aimed to evaluate the influence of affection with clinical *P. aeruginosa* mastitis on some oxidative stress biomarkers, inflammatory cytokines and proteins, in addition to some complement factors in Holstein dairy cows. Affection with *P. aeruginosa* mastitis evoked a state of oxidative stress which accompanied with depletion of cellular enzymatic and non-enzymatic anti-oxidants and elevation of lipid peroxide and advanced oxidation protein product (AOPP) level. Additionally, this affection stimulated the release of some inflammatory cytokines and proteins, enhanced activity of caspase-1. In contrary, the level of complement factor 2 (C2), complement fragments C3b and complement fragment C5a has been decreased upon affection with mastitis. In conclusion, marked oxidative stress state and enhanced release of inflammatory cytokines and proteins with complement system defective activation may share in pathogenesis and virulence of *P. aeruginosa*-induced clinical mastitis in dairy cattle.

KEYWORDS
Pseudomonas aeruginosa, Mastitis, Oxidative stress, Complement, Cows.

INTRODUCTION

Mastitis could be considered as one of the most costly and prevalent disease in dairy herds all over the world, as it causes severe decrement in profitability of the herd (Seegers *et al*., 2003; Hogeveen *et al*., 2011; Adriaens *et al*., 2021). Generally, clinical signs of mastitis range from inflammation of mammary gland with pyrexia, depression and decrease food intake in clinical mastitis to only elevation in milk somatic cell count, watery milk secretion, and presence of milk clots in case of non-clinical chronic infection (sub-clinical mastitis) (Watt, 1990). Nearly about half of the cases suffering from clinical mastitis are infected with Gram-negative bacteria, including *Pseudomonas aeruginosa* (Bannerman *et al*., 2005). *P. aeruginosa* is a Gram negative, rod-shaped motile bacteria, which is associated with a wide range of acute and chronic affections in different species (Chen *et al*., 2018). *P. aeruginosa* could induce both clinical and sub-clinical mastitis in ruminant (Bergonier *et al*., 2003; Sela *et al*., 2007). *P. aeruginosa* induced mastitis may occur as an outbreak in dairy herds or in sporadic manner as it is an environmental pathogen with a high distribution in humid areas. Manure, bedding, contaminated water and milk parlors washing and spraying devices are the main sources of infection in dairy farms (Kirk and Bartlett, 1984; Daly *et al*., 1999). In addition, poor hygienic measures and formation of bio-film within milking parlors may facilitate intra-mammary invasion of the bacteria (Erskine *et al*., 1987; Kawai *et al*., 2017). In most cases, *P. aeruginosa* mastitis is resistible to treatment with anti-bacterial agents, and this would cause severe economic losses in dairy herds (Schauer *et al*., 2021). This resistance could be attributed to low permeability of its cell wall and ability to upgrade its resistance through continuous mutation in resistance genes (Lambert, 2002). Virulence factors of this bacteria include presence of flagella and pili which enhance its motility and tissue adherence (Lyczak *et al*., 2000), presence of bacterial lipopolysaccharide which inhibit lytic effect of complement system (Lerouge and Vanderleyden, 2002), besides secretion of exotoxin A (*toxA*) and exo-enzyme S (*exoS*) (Narayanan, 2013). Also, secretion of pyocyanin by bacteria is a major virulence factor, as it can disturb cellular redox balance resulting in cell death (Hall *et al*., 2016). In this context, the present study aimed to spot the light and investigate the presumptive changes in serum levels of some oxidant/ antioxidant, inflammatory and immunological bio-markers during early affection with clinical mastitis caused by *Pseudomonas aeruginosa* and their role in disease pathogenesis in Holstein dairy cows.

MATERIALS AND METHODS

Cows

A total of twenty Holstein lactating cows, age about 4-6 years old, belonging to private extensive dairy farm in Alexandria Province, Egypt were selected to accomplish this study. The cows were kept in free-stall barns with same nutritional and management

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conditions. Cows were milked twice daily in a tandem style milk parlor. The usage of the animals in this study was approved by Intuitional Animals Care and Use, Alexandria University (IACUC).

Group-1: Consisted of ten apparently healthy lactating Holstein cows with normal udder and milk secretion, California mastitis test (CMT) (DeLavalTM, Australia) was performed to the cows for three consecutive days to exclude presence of sub-clinical mastitis.

Group-2: Consisted of ten cows, suffering from mastitis, based on presence of classical clinical signs of such case (inflamed one or more of udder quarters). Mid-stream milk samples were obtained directly after appearance of clinical signs before administration of any medication for treatment of mastitis. Milk samples were collected in sterile containers and streaked on nutrient agar media plates which were incubated for 24 hours at 37°C. The characteristic *Pseudomonas aeruginosa* bacterial colonies appeared on plates (yellowish green flat disc-shaped mucoid colonies with irregular edges). For morphological characterization, Gram stained films were prepared and Gram negative bacilli were detected. For more confirmation, the colonies were picked and further isolation was done on cetrimide agar media which was incubated at 37°C for 48 hours, the produced fluorescent pigments of the bacterial colonies were visualized under ultraviolent light (245 nm) (Samanta, 2013). Also, isolated bacterial colonies were further tested by strip oxidation test (Oxidase Test Kit, Bioanalyse®, Turkey), the detected bacteria appeared positive for oxidase test. All the tests were carried out twice for more accuracy confirmation.

Blood sampling

Blood samples were collected from tail vein of the animals in plain vacutainers for serum separation; samples were collected from mastitis-affected cows at the same time of milk sampling. Blood aliquots were left at room temperature to coagulate, and then the tubes were centrifuged at 3000 r.p.m for 10 minutes. Separated serum aliquots were kept at -20°C for further biochemical evaluation.

Biochemical analysis of serum

Evaluation of oxidant/antioxidant representative parameters

The level of malondialdehyde (MDA), reduced glutathione (GSH), activities of superoxide dismutase (SOD) and catalase (CAT) enzymes were evaluated in serum using commercially available kits (Biodiagnostic®, Egypt). Serum advanced oxidized protein product (AOPP) level was determined according to the method described by Witko-Sarsat *et al.* (1998).

Evaluation of some inflammatory biomolecules

Highly specific enzyme-linked immune-sorbent assay (ELISA) kits were used to determine serum concentration of concentration of interleukin-6 (IL-6), interleukin-1 beta (IL-1β) (Abcam, USA) and caspase-1 (Mybiosource, USA). Also, serum amyloid-A protein (SAA) was evaluated using ELISA-based kits (Abbexa, UK) and haptoglobin (HP) level was determined via immuno-turbidimetry method (Randox, UK).

Evaluation of some complement factors

Serum level of complement C2 (AFG Bioscience®, USA), and complement fragment 3b (C3b) (MyBiosource®, USA), in addition to complement fragment 5a (C5a) and were evaluated using species specific ELISA kits.

Statistical analysis

The statistical change in serum concentration of the evaluated parameters in both of experimental groups was detected using independent t-test by the aid of statistical software package SPSS 16.0. The comparison between mean values was done at 5% significance level. The results were expressed as mean ±SE.

RESULTS

As shown in Table 1, serum concentration of lipid peroxide (MDA) recorded a significant elevation in cows affected with mastitis; this increase was associated with a significant decrease in serum activity of CAT and SOD antioxidant enzymes, in addition to a significant decrement in serum GSH concentration as compared to control unaffected group. Additionally, the increment in serum level of AOPP was detected significantly in the affected cows when compared to control group. Also, serum concentration of inflammatory cytokines (IL-6 and IL-1β) and proteins (SAA and HP) in addition to caspase-1 level were significantly increased in mastitis affected cows when compared to control group (Table 2). In comparison with control group, serum concentration of complement factors C2, C3b and C5a was significantly decreased in *Pseudomonas aeruginosa* infected cows as present in Table 2.

Table 1. Mean values (Mean± SE) of serum concentration of oxidant/antioxidant biomarkers in healthy and infected cows.

Parameter	Group-1 (Healthy)	Group-2 (Infected)	t-value
MDA (µmol/L)	3.67±0.33	8.46±0.53***	2.03
CAT (U/ml)	4.04±0.36	2.31±0.17***	4.49
SOD (U/ml)	60.80±4.26	37.10±2.70***	3.58
GSH (µmol/L)	6.77±0.39	3.08±0.27***	0.73
AOPP (µmol/L)	62.30±4.27	90.20±5.04***	0.21

* Significant at (P<0.05); ** Highly significant at (P< 0.01); *** Very highly significant at (P< 0.001)

Table 2. Mean values (Mean± SE) of serum concentration of some inflammatory cytokines, inflammatory proteins and complement factors in healthy and infected cows.

Parameter	Group-1 (Healthy)	Group-2 (Infected)	t-value
IL-1β (pg/ml)	4.79±0.40	9.03±0.41***	0.09
IL-6 (pg/ml)	152.80±6.71	224.60±10.65***	3.98
Caspase-1 (pmol/L)	115.70±8.99	172.80±15.89**	1.90
SAA (µg/ml)	21.90±1.90	40.70±5.45**	4.48
HP (µg/ml)	185.40±10.54	250.50±13.37***	0.99
C2 (µg/ml)	52.70±3.50	35.40±2.10**	4.12
C3b (µg/ml)	642.10±37.98	420.90±26.68***	2.7
C5a (ng/ml)	29.50±3.06	17.50±1.57*	6.03

* Significant at (P<0.05); ** Highly significant at (P< 0.01); *** Very highly significant at (P< 0.001)

DISCUSSION

Mastitis continues to threat dairy production due to poor milk quality or decreased milk yield and increased veterinary costs (Rollin *et al.*, 2015), in addition to the increase in mortality and culling rates of affected animals (Erskine *et al.*, 2002; Erskine

et al., 2003). *Pseudomonas aeruginosa* is believed to be one of the most virulent bacteria causing mastitis in dairy cows (Schauer *et al*., 2021) and secretion of pyocyanin by this bacterium has gained more attention recently as a corner stone in virulence of this bacteria (Hall *et al*., 2016). Concerning redox state in association with affection with coliform mastitis (as in case of mastitis caused by *P. aeruginosa*), it was proved recently that production of oxylipids which contributes to oxidative stress and reactive oxygen species production (ROS) is induced due to increased rate of polyunsaturated fatty acids (PUFA) oxygenation (Mavangira *et al*., 2015; Mavangira and Sordillo, 2017). PUFA oxygenation is mediated through different enzymatic systems which includes cytochrome P450 enzyme which is activated during inflammation (Spector and Kim, 2015) or non-enzymatically in presence of ROS (O'Donnell *et al*., 2009). Additionally, pyocyanin secretion may induce a state of oxidative stress as a part of its cytotoxic effect (Muller, 2002) through production of ROS (O'Malley *et al*. 2004) and decrease production of cellular antioxidant capacity via its direct ability to down-regulate CAT mRNA as an example (Rada *et al*., 2011). The previous scenarios may offer an acceptable explanation for the detected increase in serum level of MDA (major lipid peroxide) and AOPP which is derived mainly from oxidative modification of plasma albumin in presence of ROS (Piwowar, 2010). Redox imbalance state in this study was affirmed, as the increase in oxidative bio-molecules (MDA and AOPP) was accompanied with diminished serum concentration of cellular antioxidants (GSH, SOD and CAT enzymes), and this state of imbalance may be implicated in cellular death through different mechanisms including caspase system activation and mitochondrial dysfunctions (Ryter *et al*., 2007). Declined capacity of enzymatic and non-enzymatic anti-oxidant could be attributed to increased Referring consumption to neutralize ROS generated from the inflamed udder, indicating a compromised antioxidant defense mechanism (Jhambh *et al*., 2013; Abdel-Hamid and Mahmoud, 2020). Referring to serum level of pro-inflammatory cytokines, production of bacterial endotoxins is a strong inducer of inflammatory reaction which induces release of inflammatory cytokines (Baumann and Gauldie, 1994), also pyocyanin may induce lipopolysaccharide (LPS)-enhanced production of IL-1β and TNF-α (Ulmer *et al*., 1990) and this may explain the elevated level of IL-1β in cows suffering from mastitis. In the same manner, pyocyanin could increase production of IL-6 to aggravate inflammation (McDermott *et al*., 2013). Flagellin of *P. aeruginosa* is thought to induce production of caspase-1 (Miao *et al*., 2008), which is responsible for triggering special type of programmed cell death (pyroptosis) (Miao *et al*., 2011), also elevated caspase-1 level could share in enhancement of inflammatory state through activation and maturation of IL-1β to exert it effect and share in inflammatory process (Yu and Finlay, 2008). Both of IL-1β and TNF-α are implicated in induction of acute phase response, including pyrexia and production of acute phase proteins (APPs) by liver (Koj, 1996; Suffredini *et al*., 1999), APPs share fundamentally microorganism elimination due to their powerful role in opsono-phagocytosis and activation of complement pathway (Eisen and Minchinton, 2003), as a result, cows affected with mastitis in this study recorded a significant increase in serum level of SAA and haptoglobin concentration as previously detected in several studies (Eckersall *et al*., 2001; Bannerman *et al*,. 2005; Kováč *et al*., 2011). Complement system is the main defensive weapon of the body against *P. aeruginosa*, as it is responsible for preparing of bacteria for phagocytosis, beside its direct lytic effect upon bacteria, but *Pseudomonas aeruginosa* has several strategies to overcome complement attack (González-Alsina *et al*., 2023). In this consistence, previous studies elucidated the ability of *P. aeruginosa* to secrete several proteases enzymes including alkaline protease A (AprA), which could degrade C2, which in turn inhibit formation of C3b and subsequently C5a formation (Laarman *et al*., 2012), and this may demonstrate the decrease in these complement factor in this study. In the same line, Bannerman *et al*. (2005) detected that, level of C5a was increased in milk in response to experimentally induced mastitis by *P. aeruginosa* for only 48 hours after infection and declined again.

CONCLUSION

Finally, it could be concluded that, clinical mastitis caused by *Pseudomonas aeruginosa* in dairy cow was associated with several changes in redox state, inflammatory cytokines and inflammatory proteins release, in addition to complement system activation defect, these changes may play a pivotal role in pathogenesis and severity of mastitis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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