The effect of staphylococcal mastitis including resistant strains on serum procalcitonin, neopterin, acute phase response and stress biomarkers in Holstein dairy cows

Wael El-Deeb[1,2], Mahmoud Fayez[3,4], Naser Alhumam[5], Ibrahim Elsohaby[6,7], Sayed A. Quadri[8] and Hermine Mkrtchyan[9]

[1] Department of Clinical Sciences, College of Veterinary Medicine, King Faisal University, Al-Hofuf, Saudi Arabia

[2] Department of Internal Medicine, Infectious Diseases and Fish Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt

[3] Al Ahsa Veterinary Diagnostic Laboratory, Ministry of Environment, Water and Agriculture, Al-Hofuf, Al-Ahsa, Saudi Arabia

[4] Veterinary Serum and Vaccine Research Institute, Ministry of Agriculture, Cairo, Egypt

[5] Department of Microbiology and parasitology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Al-Hofuf, Saudi Arabia

[6] Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt

[7] Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada

[8] Department of Biomedical Sciences, College of Medicine, King Faisal University, Al-Hofuf, Al-Ahsa, Saudi Arabia

[9] School of Biomedical Sciences, University of West London, London, United Kingdom

Corresponding author
Wael El-Deeb,
drwaeleldeeb@yahoo.com

ABSTRACT

Staphylococcal mastitis (SM) is a frequent disease in the dairy cattle that is costly to treat. This study aimed to investigate the alterations in the levels of procalcitonin (PCT), neopterin (NPT), haptoglobin (HP), serum amyloid A (SAA), proinflammatory cytokines (IL-1β, IL-8, TNF-α, IF-γ) and oxidative stress (OS) biomarkers in Holstein dairy cows with SM under field conditions. In addition, we also evaluated the role of examined biomarkers in disease pathogenesis and their use as diagnostic biomarkers for the disease in dairy cows. Fifty-three dairy cows with SM, including those with infections caused by *Staphylococcus aureus* ($n = 42$) and methicillin resistant *S. aureus* (MRSA) ($n = 11$) were selected for this study. In addition, 20 healthy dairy cows were enrolled as a control group. Higher serum levels of PCT, NP, IL-1β, IL-8, TNF-α, IF-γ, HP and SAA and a state of OS was detected in SM group in comparison with the controls. Moreover, the levels of all examined biomarkers in mastitic cows with *S. aureus* when compared with those infected with MRSA was not significantly different. All examined biomarkers demonstrated a significant degree of discrimination between SM cows and healthy controls (the area under the curve (AUC) ranged from 83.6 for *SAA* to 100 for PCT). Our study showed that SM in dairy cows was associated with substantial changes in serum PCT, NPT, Acute phase proteins (APPs), proinflammatory cytokines, and OS levels. This study demonstrates that clinical examination in tandem with quantification

of PCT, NPT, APPs and cytokines, OS biomarkers could be a useful assessment tool for SM in dairy cows.

INTRODUCTION

Staphylococcus aureus is one of the most common causes of mastitis in cattle internationally, which has become a serious issue with ensuing financial burden for dairy farming due to culling of affected cows (*Roberson et al., 1994*). The infection, once established, is very difficult to treat and infected cattle are a potential source for transmission (*Petersson-Wolfe, Mullarky & Jones, 2010*).

S. aureus is responsible for a common type of chronic mastitis. However, cows, in particular after calving, may develop clinical mastitis causing elevated somatic cell counts (SCC) as highly contagious *S. aureus* remains in teat canals, mammary glands tissues, and teat lesions of infected cattle (*Petersson-Wolfe, Mullarky & Jones, 2010*). Mammary epithelial cells are vital in early immune responses via cytokines secretion (e.g., IL-8) and elements that possess antimicrobial properties (*Strandberg et al., 2005*; *Brenaut et al., 2014*).

The rapidly induced innate immune response at the onset of the infection is the predominant defense strategy. This ubiquitous response that targets a broad range of bacteria is short acting (*Sordillo, Shafer-Weaver & DeRosa, 1997*; *Rainard & Riollet, 2000*). The attacking bacteria is destroyed by neutrophils and macrophages that secrete cytokines (CYT), chemokines but also are involved in further cellular defense strategies (*Paape et al., 2002*; *Leitner et al., 2003*). On the contrary, there is little information available about the factors employed at the infection site (*Gray et al., 2005*).

Pro-inflammatory cytokines (PICs) stimulate quick inflammation in response to bacterial infection or other pathogens, whereas the anti-inflammatory cytokines (AICs) restrict PICs' activity. IL-1, TNF-α and IL-6 are the main PICs (*Dinarello, 2000*; *Heinrich et al., 2003*). The PICs are secreted by variety of cells and are accountable for early immune responses. The activation of an inflammatory cascade by TNF-α and IL-1 causes inflammation, fever, tissue damage, resulting in toxic shock and death in some affected cases (*Dinarello, 2000*). Chemokines stimulate immune response elements to the infection site to facilitate the passage of WBCs from the blood into the affected tissues. Their accumulation at the inflammation site is crucial for an effective acute phase response (APR), culminating in clearance of pathogen and wound healing (*Coussens & Werb, 2002*; *Lawrence & Gilroy, 2007*). Consequently, any disturbances occurring in the balanced order and degree of the APR could potentially lead to chronic inflammatory condition or even infection (*Lawrence & Gilroy, 2007*).

Mastitis leads to over production of reactive oxygen species (ROS) with oxidative stress (OS) in the mammary gland tissue. Consequently, to neutralize these adverse effects, the udder uses its vital antioxidant systems in keeping good milk standards. Bacterial invasion

into the mammary gland has adverse impact on the antioxidant activity; hence, some of these antioxidants (catalase, lactoperoxidase or glutathione-peroxidase) can be used as biological markers of mastitis (*Andrei et al., 2016*). According to the best of authors' knowledge, little is known about the APR and OS state of Holstein dairy cows with staphylococcal mastitis (SM) including resistant strains and the role of these biological markers in disease diagnosis and pathogenesis. In this study, we investigated the levels of APPs, CYT, PCT, NPT and OS biomarkers in Holstein dairy cows with SM including those caused by the resistant strains. It also aimed to determine the role of these biomarkers in the pathogenesis of the disease and to identify the utility of these parameters as a supplementary tool for disease screening in dairy herds.

MATERIALS & METHODS

Schematic flow of the experimental program

Figure 1 depicts the flowchart showing the study design, number of Holstein dairy cows with clinical staphylococcal mastitis (with resistant and non-resistant strains) and number of dairy cows with other causes of mastitis. Five hundred and thirteen lactating Holstein cows with clinical mastitis from three different farms ($n = 5320$) in KSA central region were enrolled in this study. Moreover, 20 healthy dairy cows with normal somatic cell count (SCC), negative California mastitis test (CMT) and negative bacterial culture test were selected as a control group (Group 1). Dairy cows with recent clinical mastitis ($n = 513$) were divided into 2 groups based on the causative agents. A total of 53 cows were included in Group 2, which was formed based on *S. aureus* mastitis ($n = 42$) and MRSA ($n = 11$) cases. Group 3 included mastitic cows infected with other bacterial infectious agents ($n = 460$). The last group (Group 3) was excluded from further investigations to overcome misclassification. The research was approved by the deputyship for Research & Innovation, Ministry of Education, (# 23)

'Dairy cows' enrollment and sampling protocols'

Milk, blood, and serum samples were collected from all cows under investigation. Milk samples were collected after dipping of teats into 0.5% iodine solution and surface cleaning with alcohol following the standards described by *Hogan (1999)*. The milk samples were collected in sterile screw caped plastic containers after discarding the first streams of the quarter milk. The samples were promptly placed on ice and transported to King Faisal University (the transportation time was around 2 h) for immediate analysis.

Analysis of selected Biomarkers
Acute phase proteins

Commercial test kits were used to measure serum amyloid A (SAA) and haptoglobin (HP) in the serum samples of the dairy cows (Tridelta Development Ltd., Kildare, Ireland) according to the instructions of the manufacturer. The hemoglobin peroxidase activity was measured by detecting serum HP levels. In addition, the SAA level was determined using a solid sandwich ELISA.
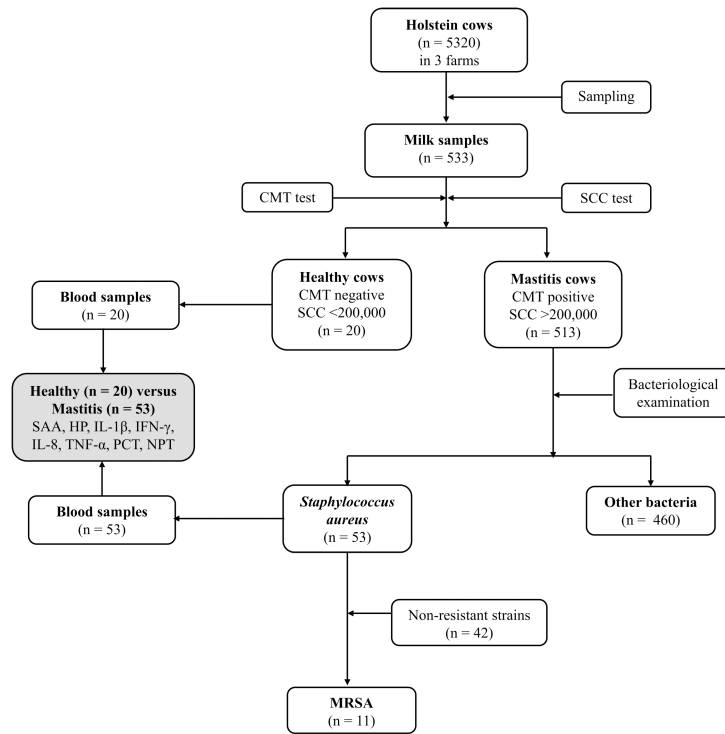
Figure 1 Flowchart showing study design, number of Holstein dairy cows with clinical staphylococcal mastitis (with resistant and non-resistant strains) and number of dairy cows with other causes of mastitis.

Pro-inflammatory cytokines

ELISA (CUSABIO Biotech, Wuhan, China) was used to measure the concentrations of proinflammatory cytokines (IL1-β, IFN-γ, IL-8 and TNF-α) in the serum. Tests were performed according to the manufacturer's instructions for testing the cattle samples.

Procalcitonin (PCT) and neopterin (NPT)

The serum levels of PCT were measured employing a commercial ELISA kits (Cusabio Biotech, Wuhan, China) for cattle (Bovine PCT). the serum NPT concentrations were measured using ELISA kits (Bovine NPT ELISA Kit, FineTest, Wuhan Fine Biotech, Wuhan, China) as instructed by the manufacturer.

Oxidative stress markers

Detection of serum malondialdehyde (MDA), reduced glutathione (GSH) and super oxide dismutase (SOD) levels in both groups of dairy cows was performed by colorimetric method using available test kits (Bio-diagnostic, Egypt). The reaction of MDA with thiobarbituric acid (TBA), forms a TBA-reactive product when it is placed in acidic medium at 95 °C for 30 min. The latter is pink in color and possesses a measurable absorbance at 534 nm. Here, the GSH levels were measured at 405 nm achievable through reduction of 5,5-Dithiobis (2-nitrobenzoic acid) with GSH and measurement of the yellow product produced.

Bacteriological analysis

Aliquots of 0.1 mL from normal and mastitic milk samples were directly cultured onto Trypticase Soy agar (TSA) supplemented with 5% bovine blood and MacConkey agar for isolation of aerobic bacteria (*Hogan, 1999*) and incubated aerobically at 37 °C for 48 h. The plates were inspected for growth and 5 colonies with different characteristics were picked up randomly and sub-cultured onto the Blood agar, Eosin Methylene Blue agar, Mannitol Salt agar and MacConkey agar and incubated for 48 h at 37 °C. Conventional methods, including Gram staining, morphology and macroscopic characteristics were used for initial identification. The isolates were further identified to the species level by Vitek 2 Compact using GP and GN identification kits (bioMérieuxFrance). For differentiation of coagulase positive and coagulase negative staphylococci a coagulase test was performed. To identify possible MRSA, coagulase positive isolates were sub-cultured onto CHROMagar™ MRSA agar (CHROMagar, France).

Somatic cell count (SCC) and California Mastitis test (CMT)

The SCC test was performed for all milk samples using DeLaval cell counter DCC (DeLaval, Ireland). The CMT and the interpretation of the obtained results were performed as described previously (*Schalm & Noorlander (1957)*; *Kivaria, Noordhuizen & Nielen (2007)*).

Statistical analysis

STATA 16.1 (StataCorp, College Station, Texas) was used to conduct statistical analysis. Descriptive data were calculated separately for each parameter in SM and control dairy cows then reported as mean. Normality of the all parameters were assessed using the Shapiro–Wilk test. Data were significantly deviated from normality and the Wilcoxon-Mann–Whitney test was applied to evaluate the differences between each parameter in SM and control healthy cows.

The correlation between parameters was determined using Spearman's rank correlation test. Each assay's diagnostic accuracy was evaluated by creating the ROC (receiver operator characteristics) curve and determining the area under the curve (AUC). An AUC of 0.7 to 0.9 was considered moderately accurate, an AUC of >0.9 highly accurate, and an AUC of 1 perfect (*Gardner & Greiner, 2006*). The Youden index (= maximum [sensitivity + specificity -1]) was used to identify the optimal cut-off values for detection of dairy cow with SM. Furthermore, Cohen's kappa statistic (κ) was used to assess the level of agreement between dairy cows classified as healthy and SM.

RESULTS

The serum levels of PCT, NPT, APPs, CYT and MDA in SM Holstein dairy cows were notably ($P <0.001$) higher than those detected in healthy controls (Fig. 2). However, a significant lower level of antioxidant biomarkers (SOD and GSH) was detected in SM Holstein dairy cows than the control group. In addition, there were non-significant changes in the levels of all examined biomarkers in mastitic cows infected with *S. aureus*
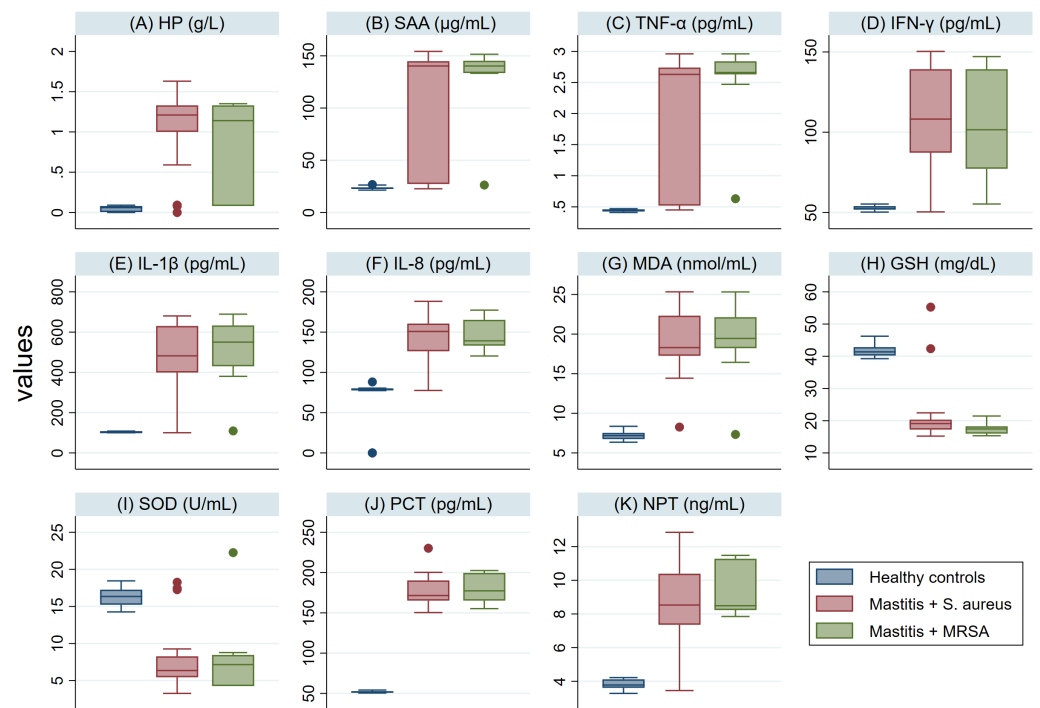
Figure 2 (A-K) Box plots showing variability of haptoglobin (HP), serum amyloid A (SAA), proinflammatory cytokines (IL-1 β, IFN-γ, IL-8, TNF-α), stress biomarkers (MDA, GSH and SOD), procalcitonin (PCT), and neopterin (NPT) in healthy and Holstein dairy cows with clinical staphylococcal mastitis.

when compared with those infected with MRSA (Figs. 2A–2K). The examined biomarkers did not differentiate *S. aureus* mastitis cases from those with MRSA infection. Spearman's correlation coefficient matrix was employed to analyze the study parameters in Holstein dairy cows infected with *S. aureus* and healthy control (Table 1). In addition, the correlation coefficients (r) among biomarkers were investigated. A significant positive correlation ($P < 0.05$) with SM cases for all examined parameters was found except SOD and GSH. SOD and GSH showed a significant negative correlation with SM cases. The highest correlation of SM cases ($r = 0.77$) was associated with PCT. A high correlation was detected between PCT & IL1-β ($r = 0.82$), and among different CYT.

The ROC curves were created (Figs. 3A–3K) and AUC was estimated to assess each parameter's accuracy in order to differentiate between SM Holstein dairy cows and healthy controls. ROCs were generated and the best cut-off values for each biomarker differentiating between SM Holstein dairy cows and healthy controls were determined based on the Youden index. The diagnostic test characteristics (Se, Sp, and accuracy) linked with each parameter's optimal cut-off values are presented in Table 2. PCT, TNF-α, MDA and NPT demonstrated stronger sensitivity and specificity compared to other biomarkers at selected cutoffs (Table 2). The optimal cut-offs when using ROC analysis for discrimination between SM Holstein dairy cows and control dairy cows for PCT, TNF-α

Table 1 Correlation matrix among procalcitonin, neopterin acute phase proteins, proinflammatory cytokines and stress parameters in healthy and Holstein dairy cows with clinical staphylococcal mastitis (20 control and 53 dairy cows with staphylococcal mastitis).

Parameters[a]	Healthy/ Mastitis	HP	SAA	TNF-α	IFN-γ	IL-1β	IL-8	MDA	GSH	SOD	PCT	NPT
HP	0.67	1.00										
SAA	0.70	0.49	1.00									
TNF-α	0.76	0.53	0.58	1.00								
IFN-γ	0.69	0.45	0.48	0.55	1.00							
IL-1 β	0.70	0.51	0.56	0.55	0.76	1.00						
IL-8	0.72	0.43	0.49	0.57	0.27	0.31	1.00					
MDA	0.75	0.51	0.56	0.59	0.82	0.82	0.36	1.00				
GSH	−0.72	−0.42	−0.56	−0.52	−0.45	−0.50	−0.47	−0.54	1.00			
SOD	−0.62	−0.46	−0.44	−0.54	−0.44	−0.59	−0.56	−0.47	0.38	1.00		
PCT	0.77	0.46	0.62	0.59	0.81	0.82	0.34	0.86	−0.56	−0.42	1.00	
NPT	0.71	0.44	0.58	0.57	0.76	0.80	0.34	0.86	−0.58	−0.43	0.89	1.00

Notes.
[a]HP, haptoglobin; SAA, serum amyloid A; TNF-α, tumor necrosis; IFN-, Interferon gamma IL-1 β, interleukin 1-beta; IL-8, interleukin 8; factor-alpha; MDA, malondialdehyde; GSH, reduced glutathione; SOD, super oxide dismutase; PCT, procalcitonin; NPT, neopterin.

Table 2 Diagnostic test characteristics of procalcitonin, neopterin, acute phase proteins, proinflammatory cytokines and stress parameters in healthy and Holstein dairy cows with clinical staphylococcal mastitis.

Parameters[a]	Threshold	Diagnostic characteristics (%)[a]		Accuracy	J^2	κ^3	AUC
		Se (95% CI)	Sp (95% CI)				
HP (g/L)	≥0.09	86.8 (74.7–94.5)	80 (56.3–94.3)	84.9	0.67	0.64	0.93
SAA (µg/mL)	≥26.41	79.2 (65.9–89.2)	95 (75.1–99.9)	83.6	0.74	0.64	0.95
TNF-α (pg/mL)	≥0.47	96.2 (87–99.5)	100 (83.2–100)	97.3	0.96	0.93	0.99
IFN-γ (pg/mL)	≥55.23	86.8 (74.7–94.5)	100 (83.2–100)	90.4	0.87	0.78	0.95
IL1-β (pg/mL)	≥108.36	86.8 (74.7–94.5)	95 (75.1–99.9)	89.0	0.82	0.75	0.95
IL-8 (pg/mL)	≥88.11	94.3 (84.3–98.8)	95 (75.1–99.9)	94.5	0.89	0.87	0.97
MDA (nmol/mL)	≥8.26	98.1 (89.9–100)	95 (75.1–99.9)	97.3	0.93	0.93	0.99
GSH (mg/dL)	≤ 39.26	90 (68.3–98.8)	96.2 (87–99.5)	94.5	0.86	0.86	0.97
SOD (U/ml)	≤14.96	90 (68.3–98.8)	88.7 (77–95.7)	89.0	0.79	0.74	0.90
PCT (pg/mL)	≥54.16	100 (93.3–100)	100 (83.2–100)	100	1.00	1.00	1.00
NPT (ng/mL)	≥4.22	94.3 (84.3–98.8)	100 (83.2–100)	95.9	0.94	0.90	0.96

Notes.
[a]HP, haptoglobin; SAA, serum amyloid A; TNF-α, tumor necrosis; IFN-, Interferon gamma IL-1 β, interleukin 1-beta; IL-8, 4 interleukin 8; factor-alpha; MDA, malondialdehyde; GSH, reduced glutathione; SOD, super oxide dismutase; PCT, procalcitonin; 5 NPT, neopterin.

MDA and NPT were 54.16 pg/ml, 0.47 pg/ml, 8.26 nmol/ml and 4.22 ng/ml respectively (Table 2). APPs, PIC and OS parameters were very similar to each other and showed accurate diagnostic performance (AUC >0.83).

DISCUSSION

S. aureus is a leading cause of mastitis in dairy cows globally. In this study, we assessed the blood changes in APPs, CYT, PCT, NPT and OS biomarkers in mastitic cows infected with *S. aureus* and MRSA to comprehend the role they play in disease pathogenesis and
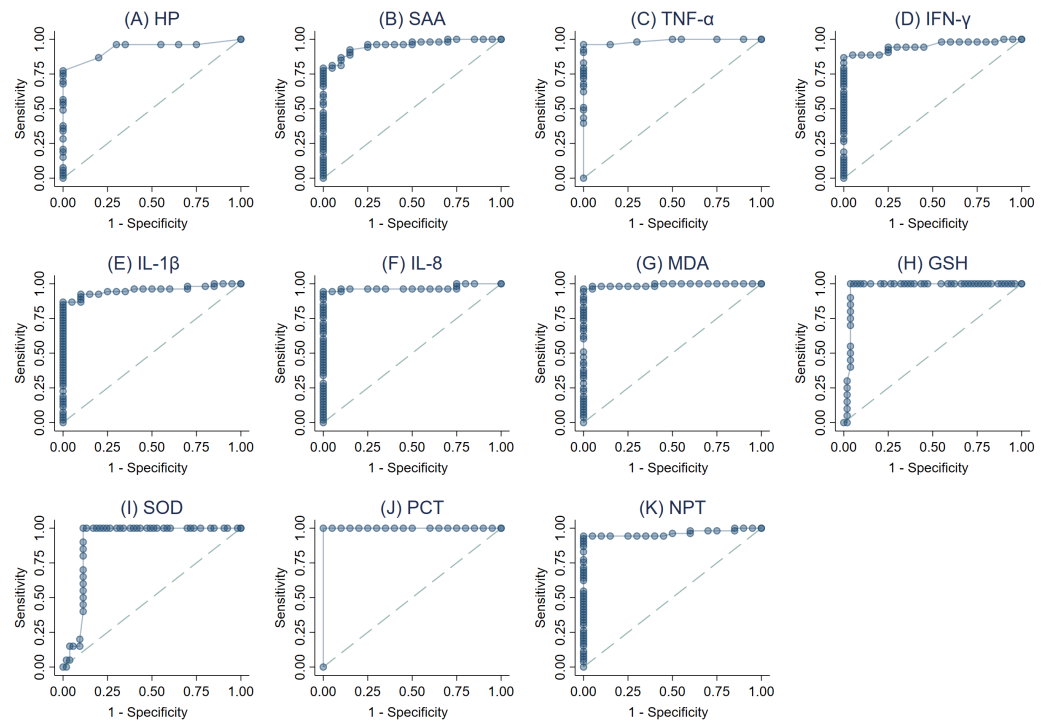
Figure 3 (A-K) Receiver operating characteristic (ROC) curve analysis of acute phase proteins HP, SAA), proinflammatory cytokines (IL-1 β, IFN-γ, IL-8, and TNF-α), stress parameters (MDA, GSH and SOD), procalcitonin (PCT) and neopterin (NPT) in healthy and Holstein dairy cows with clinical staphylococcal mastitis.

immune response and to evaluate their use as a supplementary tool for screening of *S. aureus* mastitis.

In ruminants, HP and SAA are considered dominant APPs that increase throughout infections, inflammatory conditions, surgical trauma, and stresses (*Heegaard et al., 2000*; *Petersson-Wolfe, Mullarky & Jones, 2010*; *El-Deeb, Elmoslemany & Salem, 2017*; *El-Deeb et al., 2020a*). In this study, there were significant elevations in serum HP & SAA levels (Figs. 2A–2B) in mastitic cows with *S. aureus* infection (either *S. aureus* or MRSA), indicating a significant APR to the intramammary *S. aureus* infections. The elevated HP levels could be due to PICs released from damaged udder tissue in mastitic cows following the infection. Pathogens evolve and develop strategies to overcome host defenses (*Mogensen, 2009*). One such sophisticated strategy is to avoid recognition, which is achieved through reduction of the "activation of pattern recognition receptors" (PRR) belonging to the family of Toll-like receptors (TLRs) (*Kawai Kumar & Akira, 2009*), or their downstream signaling. Gram-positive bacteria, such as *S. aureus*, activate the inflammatory response via TLR2 (*Schwandner et al., 1999*). *Yang et al. (2008)* showed that NF-$_k$B activation in bovine mammary epithelial cells (MEC) could be blocked by the heat-inactivated *S. aureus* strain 1,027, even though these particles could activate the bovine TLR2 receptor found in the HEK293 reconstitution system of TLR signaling (*Brightbill et al., 1999*).

Similarly, SAA response in mastitic cows with *S. aureus* infection may be attributed to its significant role in modulating the immune defense of cows during tissue injury. These effects are mainly through induction of leukocyte migration, differentiation of neutrophil, activation of putative receptors of neutrophils and increased secretion of IL-8 (*Urieli-Shoval et al., 2000*; *He, Sang & Ye, 2003*; *Murata, Shimada & Yoshioka, 2004*; *Orro et al., 2011*). Furthermore, it initiates tissue-degrading enzymes' synthesis (*O'Hara et al., 2004*) and PICs (*Furlaneto & Campa, 2000*).

It has been shown that TLR2 is the functional receptor for SAA (*ChengNiRong et al., 2008*) which clearly interpet our results regarding high levels of SAA in blood of mastitic cows with *S. aureus* infection.

Several research groups have reported that the primary bovine MEC cultures (pbMEC) could trigger cytokine-encoding genes such as IL-1 β and IL-8 when challenged with heat-inactivated *S. aureus*, (*Brenaut et al., 2014*; *Gunther et al., 2010*). In compliance with these findings, we have also detected high levels of IL-1 β and IL-8. Similar findings have also been reported for mixed infections of *S. aureus* and *E. coli* (Joshi et al., 2018), in pasteurellosis in sheep (*El-Deeb & Elmoslemany, 2016a*) and dairy cows with urinary tract infection (*El-Deeb & Elmoslemany, 2016b*).

Interestingly, the levels of CYT analyzed in our study were significantly higher among mastitic cows with *S. aureus* and MRSA infections than those in the healthy group. This indicates that *S. aureus* infections are associated with robust APR and several pathological changes in infected dairy cows. A TLR-pathogen association could initiate a downstream signaling cascade, leading to the transcription factors [e.g., Nuclear Factor Kappa B (NF-$_k$B)] activation. Once these factors enter the nucleus, they can bind the target promoters, induce the production of PICs (and other endogenous mediators) and establish cellular resistance to invading pathogens. Ten mammalian TLRs, which generate unique responses via intracellular signaling pathways, can eliminate the pathogen by initiating inflammatory and antimicrobial processes (*Akira, 2003*; *Reuven, Fink & Shai, 2014*).

TNF-α and IL-1 β produce inflammation, fever, further tissue damage, toxic shock and death by triggering inflammatory cascade (*Dinarello, 2000*). Cellular factors of immune defense, which are recruited by chemokines at the infection site (udder tissues), facilitate the entrance of leukocytes from blood stream to tissues. This coordinated induction of the inflammatory mediators at the inflammation site ensues in inflammatory responses such as pathogen elimination and wound healing (*Coussens & Werb, 2002*; *Lawrence & Gilroy, 2007*). Interruptions in the inflammatory response could lead to more chronic conditions (*Lawrence & Gilroy, 2007*) which often occur in the case of *S. aureus* infections among dairy cows.

This study demonstrated that PICs play a significant role in *S. aureus* mastitis pathogenesis and immune response in dairy cows. These results are also consistent with our previously reported data in sheep with bacterial pneumonia (*El-Deeb & Elmoslemany, 2016a*) and *Coxiella burnetii* infected sheep, goats and she-camels (*El-Deeb et al., 2019*).

Interestingly, we detected a major elevation in serum levels of PCT and NPT in mastitic cows with *S. aureus* infection than in healthy controls (Figs. 2J–2K). We believe that this is the first study to record levels of PCT and NPT in SA infected mastitic cows. PCT increases
in blood rapidly as a response to inflammatory conditions, which result from *S. aureus* infection and ensuing production of PICs (*Reinhart, Karzai & Meisner, 2000*). Previous studies have shown that PCT is increased in septicemias (*Assicot et al., 1993*; *Dandona et al., 1994*) and bacterial infections (*Tünger, 2007*; *El-Deeb et al., 2020b*). Recently, comparable results were also reported in goats with contagious caprine pleuropneumonia (*El-Deeb et al., 2020c*).

Remarkably, the higher serum levels of NPT, as noted in our study, could trigger cellular immune response in mastitic cows with *S. aureus* infection. NPT is produced by both macrophages and monocytes once they are stimulated by interferon-γ (which was detected at high levels in mastitic cows infected with *S. aureus* infection in our study). IFN-γ is produced by activated T cells. On the contrary, other research groups have showed that, body cells other than macrophages and monocytes do not produce measurable quantities of NPT following different stimuli. Apparently, the production of NPT is a result of cellular immune response activation (*Werner-Felmayer et al., 1990*). In addition, it was shown that in intracellular pathogenic bacteria NPT acts as an endogenous inhibitor of folate synthesis (Huber et al., 1983). Similar results have been reported in studies involving various bacterial, viral and parasitic diseases (*Shaw, 1991*; *Facer, 1995*) and septicemic colibacillosis in calves (*Ercan, Tuzcu & Başbuğ et al., 2014*).

OS has been detected in several diseased farm animals (*Rodríguez et al., 2011*; *El-Deeb & Buczinski, 2015*; *El-Deeb & Elmoslemany, 2015*). The status of OS can be detected by assessing the change in the balanced antioxidant and oxidant levels in the body cells (*Celi, 2011*). Accumulation of fat is highly susceptible to oxidation process and therefore, the products of lipid peroxidation were determined as parameters for the condition of OS. Peroxidation of fatty acids in the body cells results in the production of MDA. The higher blood levels of free radicals causes MDA overproduction (*Castillo et al., 2005*; *Ayala, Muñoz & Argüelles, 2014*; *El-Deeb, Fouda & El-Bahr, 2014*; *El-Deeb & Tharwat, 2015*). In this study, considerably higher serum MDA levels in mastitic cows with *S. aureus* infection demonstrated a specific lipid peroxidation (OS) (*Ayala, Muñoz & Argüelles, 2014*; *El-Deeb & Younis, 2009*; *El-Deeb & Iacob, 2012*; *El-Deeb, 2013*; *El-Deeb & Tharwat, 2015*). This condition of lipid peroxidation is produced by the releasing numerous oxygen-free radicals, which is due to infection or reduced levels of antioxidants (*Sies, 1997*; *Gupta et al., 2014*). The crucial role of GSH and SOD in the protection of cells against OS may explain their low concentration in mastitic cows with *S. aureus* infection (*Halliwell, 1996*). Nevertheless, if GSH and SOD (antioxidants) ensuing from within the cell do not remove these damaging radicals, the oxidative process rate exceeds the antioxidation rate, resulting in a condition of OS (*Sies, 1997*). Such changes were observed in our study as the serum of mastitic cows revealed increased MDA levels and reduced SOD and GSH levels when compared with the controls. The significant reduction of GSH and SOD in the serum of mastitic cows could be attributed to the reduced ability of antioxidant enzyme system to subdue the OS state.

SOD that plays an important role in superoxide radical dismutation (a by-product of oxygen metabolism), may cause cell injury if not controlled. Previous studies showed the key role played by SOD in the improvement of trinitrobenzene sulfonic acid-induced

colitis by decreasing OS in the intestine (*Quílez et al., 2013*). Likewise, GSH has a significant role in preventing the cellular components damage, which is caused by ROS. Moreover, the peroxidation of cell membrane's lipid layer that occurs due to free radicals is an important feature of cellular damage of infected tissues (*Metwaly et al., 2015*). Here, we have demonstrated correlations between MDA and each of APPs, CYT, PCT and NPT biomarkers, which leads us to hypothesize about successive events that could be occurring in the body of mastitic cows infected with *S. aureus* infection. Therefore, our data reveal that the assessment of OS status in mastitic cows would evaluate tissue injury caused by free radicals. The ROC analysis was employed to assess the ability of APPs, CYT, PCT, NPT and OS biomarkers to differentiate between mastitic cows with *S. aureus* infection and heathy controls. All examined parameters displayed a high level of discrimination between mastitic cows and healthy ones, which is in accordance with the diagnostic accuracy guidelines (*Swets, 1988*).

The Youden index was used to select the optimum threshold representing the highest Se, Sp and accuracy. PCT, TNF-α, NPT and MDA demonstrated the highest Se, Sp and accuracy. Therefore, it could be considered as an additional tool for diagnosing mastitic cows infected with *S. aureus* infections. NPT is considered biochemically dormant due to its half-life in the body. The latter is due to renal discharge (*Hamerlinck, 1999*). Consequently, detection of NPT has a number of advantages than APPs and CYT that have a relatively short half-life and faster degradation. Alterations in the blood levels of PCT, NPT, APPs, CYT, and OS parameters suggest the necessity for a more comprehensive clinical assessment of mastitic cows infected with *S. aureus*.

CONCLUSIONS

In this study, we showed that clinically mastitic cows with infections caused by *S. aureus* and MRSA were associated with a major changes in serum PCT, NPT APPs, CYT and OS levels. Furthermore, we observed higher levels of these biomarkers in mastitic cows with *S. aureus* infections than healthy controls. Moreover, there was no significant difference between the levels of examined biomarkers between *S. aureus* and MRSA infected mastitic cows. Consequently, the tested biomarkers are not able differentiate between *S. aureus* mastitis and those with MRSA cases. Our results propose that measuring the PCT, NPT, APPs, CYT and OS in addition to the clinical examination of mastitic cows may well be a potential diagnostic tool for assessing these cows. Further studies are warranted to evaluate biomarkers in milk samples obtained from mastitic and healthy cows (special focus will be on PCT, NPT and TNF-α) and correlate these biomarker levels with estimated serum levels and the clinical conditions of mastitis in cows before and after treatment. Moreover, we intend to evaluate these biomarkers in subclinical mastitis cases with *S. aureus*, which has a higher prevalence in dairy herds and compare the findings with the results of clinical mastitis cases in this study.

List of abbreviations

SM   Staphylococcal mastitis

PCT	procalcitonin
NPT	neopterin
HP	haptoglobin
SAA	serum amyloid A
PICs	proinflammatory cytokines
OS	oxidative stress
APR	Acute phase response
APPs	Acute phase proteins
MRSA	methicillin resistant *S. aureus*
AUC	area under the curve
CYT	cytokines
MDA	malondialdehyde
GSH	reduced glutathione
SOD	super oxide dismutase

Funding

This work is supported by the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia (No. 23). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia: 23.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Wael El-Deeb conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Mahmoud Fayez, Naser Alhumam and Sayed A. Quadri conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Ibrahim Elsohaby conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Hermine Mkrtchyan conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

Data are available in the Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11511#supplemental-information.

REFERENCES

Akira S. 2003. Mammalian toll-like receptors. *Current Opinion in Immunology* **15**:5–11 DOI 10.1016/S0952-7915(03)00005-0.

Andrei S, Matei S, Rugina D, Bogdan L, Ştefănuţ C. 2016. Interrelationships between the content of oxidative markers, antioxidative status, and somatic cell count in cow's milk. *Czech Journal of Animal Science* **61**:407–413 DOI 10.17221/70/2015-CJAS.

Assicot M, Gendrel D, Carsin H, Raymond J, Guilbaud J, Bohuon C. 1993. High serum procalcitonin concentrations in patients with sepsis and infection. *Lancet* **341(8844)**:15–518.

Ayala A, Muñoz MF, Argüelles S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity* **2014**:360438 DOI 10.1155/2014/360438.

Brenaut P, Lefevre L, Rau A, Laloe D, Pisoni G, Moroni P, Bevilacqua C, Martin P. 2014. Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to *Staphylococcus aureus*. *Veterinary Research* **45**:16 DOI 10.1186/1297-9716-45-16.

Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, Maitland M, Norgard MV, Plevy SE, Smale ST, Brennan PJ, Bloom BR, Godowski PJ, Modlin RL. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* **285**:732–736 DOI 10.1126/science.285.5428.732.

Castillo C, Hernandez J, Bravo A, Lopez-Alonso M, Pereira V, Benedito JL. 2005. Oxidative status during late pregnancy and early lactation in dairy cows. *The Veterinary Journal* **169**:286–292 DOI 10.1016/j.tvjl.2004.02.001.

Celi P. 2011. Biomarkers of oxidative stress in ruminant medicine. *Immunopharmacology and Immunotoxicology* **33**:233–240 DOI 10.3109/08923973.2010.514917.

Cheng Ni Rong H, Tian J, Patrick YP, Richard YD. 2008. Cutting Edge: TLR2 is a functional receptor for acute-phase serum amyloid A. *Journal of Immunology* **181(1)**:22–26.

Coussens LM, Werb Z. 2002. Inflammation and cancer. *Nature* **420**:860–867 DOI 10.1038/nature01322.

Dandona P, Nix D, Wilson MF, Aljada A, Love J, Assicot M, Bohuon C. 1994. Procalcitonin increase after endotoxin injection in normal subjects. *Journal of Clinical Endocrinology and Metabolism* **79**:1605–1608.

Dinarello CA. 2000. Proinflammatory cytokines. *Chest* **118**:503–508 DOI 10.1378/chest.118.2.503.

El-Deeb WM. 2013. Clinicobiochemical investigations of gangrenous mastitis in does: immunological responses and oxidative stress biomarkers. *Journal of Zhejiang University SCIENCE B* **14(1)**:33–39 DOI 10.1631/jzus.B1200123.

El-Deeb WE, Buczinski S. 2015. The diagnostic and prognostic importance of oxidative stress biomarkers and acute phase proteins in urinary tract infection (UTI) in camels. *PeerJ* **3**:e1363 DOI 10.7717/peerj.1363.

El-Deeb WM, Elmoslemany A. 2015. Cardiac and oxidative stress biomarkers in Trypanosoma evansi infected camels: diagnostic and prognostic prominence. *Parasitology* **142(6)**:767–772 DOI 10.1017/S0031182014001899.

El-Deeb WM, Elmoslemany A. 2016a. The diagnostic accuracy of acute phase proteins and proinflammatory cytokines in sheep with pneumonic pasteurellosis. *PeerJ* **4**:e2161 DOI 10.7717/peerj.2161.

El-Deeb WM, Elmoslemany A. 2016b. Acute phase proteins as biomarkers of Urinary Tract Infection in dairy cows: diagnostic and prognostic accuracy. *Japanese Journal of Veterinary Research* **64(1)**:57–66.

El-Deeb WM, Elmoslemany A, Salem MA. 2017. Cardiac troponin I and immuneinflammatory response in horses with strangles. *Equine Veterinary Journal* **51**:18–23 DOI 10.1016/j.jevs.2016.12.003.

El-Deeb WE, Elsohaby I, Fayez M, Mkrtchyan H, El-Etriby D, ElGioushy M. 2020a. Use of procalcitonin, neopterin, haptoglobin, serum amyloid a and proinflammatory cytokines in diagnosis and prognosis of bovine respiratory disease in feedlot calves under field conditions. *Acta Tropica* **204**:105336 DOI 10.1016/j.actatropica.2020.105336.

El-Deeb WE, Fayez M, Elsohaby I, Mkrtchyan H, Alhaider A. 2020b. Changes in blood biomarkers in Arabian Horses with *Clostridium difficile*-induced entero-colitis. *Comparative Immunology, Microbiology and Infectious Diseases* **73**:101525 DOI 10.1016/j.cimid.2020.101525.

El-Deeb WE, Fayez M, Elsohaby I, Salem M, Alhaider A, Kandeel M. 2020c. Investigation of acute-phase proteins and cytokines response in goats with contagious caprine pleuropneumonia with special reference to their diagnostic accuracy. *PeerJ* **8**:e10394 DOI 10.7717/peerj.10394.

El-Deeb WM, Ghoneim I, Fayez M, Elsohaby I, Alhaider A, ElGioushy M. 2019. Acute phase proteins, proinflammatory cytokines and oxidative stress biomarkers in sheep, goats and she-camels with *Coxiella burnetii* infection induced abortion. *Comparative Immunology, Microbiology and Infectious Diseases* **67**:101352 DOI 10.1016/j.cimid.2019.101352.

El-Deeb WM, Iacob O. 2012. Serum acute phase proteins in control and Theileria annulata infected water buffaloes *(Bubalus bubalis)*. *Veterinary Parasitology* **190**:2–18.

El-Deeb WM, Tharwat M. 2015. Lipoproteins profile, acute phase proteins, proinflammatory cytokines and oxidative stress biomarkers in sheep with pneumonic pasteurellosis. *Comparative Clinical Pathology* **24**:581–588 DOI 10.1007/s00580-014-1949-z.

El-Deeb WM, Younis EE. 2009. Clinical and biochemical studies on Theileria annulata in Egyptian buffaloes *(Bubalus bubalis)* with particular orientation to oxidative stress and ketosis relationship. *Veterinary Parasitology* **164**:301–305 DOI 10.1016/j.vetpar.2009.06.002.

El-Deeb WM, Fouda TA, El-Bahr SM. 2014. Clinico-biochemical investigation of paratuberculosis of dromedary camels in Saudi Arabia: proinflammatory cytokines, acute phase proteins and oxidative stress biomarkers. *Pakistan Veterinary Journal* **34(4)**:484–488.

Ercan N, Tuzcu N, Başbuğ O. 2014. The evaluation of important biomarkers in healthy cattle. Kafkas. Ankara Üniversitesi Veteriner Fakültesi Dergisi. **20(5)**:749–755.

Facer CA. 1995. Malaria antigens stimulate neopterin secretion by PBMC and U937 cells. *Microbiology and Immunology* **39(3)**:207–211 DOI 10.1111/j.1348-0421.1995.tb02190.x.

Furlaneto CJ, Campa A. 2000. A novel function of serum amyloid A: a potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1beta, and interleukin-8 by human blood neutrophil. *Biochemical and Biophysical Research Communications* **268**:405–408 DOI 10.1006/bbrc.2000.2143.

Gardner IA, Greiner M. 2006. Receiver-operating characteristic curves and likelihood ratios: improvements over traditional methods for the evaluation and application of veterinary clinical pathology tests. *Veterinary Clinical Pathology* **35(1)**:8–17 DOI 10.1111/j.1939-165X.2006.tb00082.x.

Gray C, Strandberg Y, Donaldson L, Tellam RL. 2005. Bovine mammary epithelial cells, initiators of innate immune responses to mastitis. *Austrialian Journal of Experimental Agriculture* **45**:757–762 DOI 10.1071/EA05046.

Gunther J, Liu S, Esch K, Schuberth HS, Seyfert HM. 2010. Stimulated expression of TNF-alpha and IL-8, but not of lingual antimicrobial peptide reflects the concentration of pathogens contacting bovine mammary epithelial cells. *Veterinary Immunology and Immunopathology* **135**:152–157 DOI 10.1016/j.vetimm.2009.11.004.

Gupta RK, Patel AK, Shah N, Chaudhary A, Jha U, Yadav UC, Gupta PK, Pakuwal U. 2014. Oxidative stress and antioxidants in disease and cancer. *Asian Pacific Journal of Cancer Prevention* **15**:4405–4409 DOI 10.7314/APJCP.2014.15.11.4405.

Halliwell B. 1996. Antioxidants in human health and disease. *Annual Review of Nutrition* **16**:33–50 DOI 10.1146/annurev.nu.16.070196.000341.

Hamerlinck FJ. 1999. Neopterin: a review. *Experimental Dermatology* **8**:167–176.

Hari-Dass R, Shah C, Meyer DJ, Raynes JG. 2005. Serum amyloid A protein binds to outer membrane protein A of gram-negative bacteria. *Journal of Biological Chemistry* **280**:1856.

He R, Sang H, Ye RD. 2003. Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL 1/LXA4R. *Blood* **101**:1572–1581 DOI 10.1182/blood-2002-05-1431.

Heegaard PM, Godson DL, Toussaint MJ, Tjørnehøj K, Larsen LE, Viuff B, Rønsholt L. 2000. The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus. *Veterinary Immunology and Immunopathology* **77**:151–159.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochemical Journal* **374**:1–20 DOI 10.1042/bj20030407.

Hogan JS. 1999. *National Mastitis Council (U.S.).* Madison, WI: National Mastitis CouncilISBN: 0932147038 97809321435.

Kawai Kumar HT, Akira S. 2009. Toll-like receptors and innate immunity. *Biochemical and Biophysical Research Communications* **388**:621–625 DOI 10.1016/j.bbrc.2009.08.062.

Kivaria FM, Noordhuizen JPTM, Nielen M. 2007. Interpretation of California mastitis test scores using Staphylococcus aureus culture results for screening of sucblinical mastitis in low yielding smallholder dairy cows in the Dar es Salaam region of Tanzania. *Preventative Veterinary Medicine* **78**:274–285 DOI 10.1016/j.prevetmed.2006.10.011.

Lawrence T, Gilroy DW. 2007. Chronic inflammation: a failure of resolution? *International Journal of Experimental Pathology* **88**:85–94.

Leitner G, Eligulashvily R, Krifucks O, Perl S, Saran A. 2003. Immune cell differentiation in mammary gland tissues and milk of cows chronically infected with Staphylococcus aureus. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health.* **50**:45–52 DOI 10.1046/j.1439-0450.2003.00602.x.

Metwaly MS, Dkhil MA, Al-Quraishy S, Al Omar SY. 2015. Protective effects of palm pollen aqueous extract against Eimeria papillata induced intestinal damage in mice. *Pakistan Journal of Zoology* **47(4)**:971–979.

Mogensen TH. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews* **22**:240–273 DOI 10.1128/CMR.00046-08.

Murata H, Shimada N, Yoshioka MJ. 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. *Veterinary Journal* **168**:28–40.

O'Hara R, Murphy EP, Whitehead AS, FitzGerald O, Bresnihan B. 2004. Local expression of the serum amyloid A and formyl peptide receptor-like 1 genes in synovial tissue is associated with matrix metalloproteinase production in patients with inflammatory arthritis. *Arthtitis and Rheumatism* **50**:1788–1799 DOI 10.1002/art.20301.

Orro T, Pohjanvirta T, Rikula U, Huovilainen A, Alasuutari S, Sihvonen L, Pelkonen S, Soveri T. 2011. Acute phase protein changes in calves during an outbreak of respiratory disease caused by bovine respiratory syncytial virus. *Comparative Immunology, Microbiology & Infectious Diseases* **34**:23–29.

Paape MJ, Shafer-Weaver K, Capuco AV, Oostveldt K, Burvenich C. 2002. Immune surveillance of mammary tissue by phagocytic cells. *Advances in Experimental Medicine and Biology* **480**:259–277 DOI 10.1007/0-306-46832-8_31.

Petersson-Wolfe CS, Mullarky IK, Jones IK. 2010. *Staphylococcus aureus Mastitis: cause, detection, and control.* Produced by Communications and Marketing, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University, 2010, Publication, 404–229, *Available at* http://www.ext.vt.edu.

Quílez J, Castiblanco CV, Monteagudo L, del Cacho E, Acedo SC. 2013. Host association ofCryptosporidium parvum populations infecting domestic ruminants in Spain. *Applied and Environmental Microbiology* **79(17)**:5363–5371 DOI 10.1128/AEM.01168-13.2-18567.

Rainard P, Riollet C. 2000. Innate immunity of the bovine mammary gland. *Veterinary Research* **37**:369–400.

Reinhart K, Karzai W, Meisner M. 2000. Procalcitonin as a marker of the systemic inflammatory response to infection. *Intensive Care Medicine* **26**:1193–1200 DOI 10.1007/s001340000624.

Reuven EM, Fink A, Shai Y. 2014. Regulation of innate immune responses by transmembrane interactions: lessons from the TLR family. *Biochimica et Biophysica Acta/General Subjects* **1838**:1586–93 DOI 10.1016/j.bbamem.01.020.

Roberson JR, Fox LK, Hancock DD, Gay JM. 1994. Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. *Journal of Dairy Science* **77**:3354–3364 DOI 10.3168/jds.S0022-0302(94)77277-5.

Rodríguez D, de Sotillo AM, Velly Hadley M, Fricton JR. 2011. Evidence of oxidative stress in temporomandibular disorders: a pilot study. *Journal of Oral Rehabilitation* **38**:722–728 DOI 10.1111/j.1365-2842.2011.02216.x.

Schalm OW, Noorlander DO. 1957. Experiments and observations leading to development of the California mastitis test. *Journal of the American Veterinary Medical Association* **130**:199–204.

Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *Journal of Biological Chemistry* **274**:17406–17409 DOI 10.1074/jbc.274.25.17406.

Shaw AC. 1991. Serum C-reactive protein and neopterin concentrations in patients with viral or bacterial infection. *Journal of Clinical Pathology* **44(7)**:596–599 DOI 10.1136/jcp.44.7.596.

Sies H. 1997. Oxidative stress: oxidants and antioxidants. *Experimental Physiology* **82**:291–295 DOI 10.1113/expphysiol.1997.sp004024.

Sordillo LM, Shafer-Weaver K, De Rosa D. 1997. Immunobiology of the mammary gland. *Journal of Dairy Science* **80**:1851–1865 DOI 10.3168/jds.S0022-0302(97)76121-6.

Strandberg Y, Gray C, Vuocolo T, Donaldson L, Broadway M, Tellam R. 2005. Lipopolysaccharide and lipoteichoicacid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine* **31**:72–86 DOI 10.1016/j.cyto.02.010.

Swets JA. 1998. Measuring the accuracy of diagnostic systems. *Science* **240**:1285–1293.

Tünger Ö. 2007. Procalcitonin, CRP and other indicators in the diagnosis and impression of sepsis. Klimik Dergisi, 20, 1, 2007.Stang BV, Koller LD. 1998. Neopterin values in selected groups of normal animals. *Research in Veterinary Science* **65**:87–88.

Urieli-Shoval S, Linke RP, Matzner YJ. 2000. Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states. *Current Opinion in Hematology* **7**:64–69.

Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Wachter HJ. 1990. Neopterin formation and tryptophan degradation by a human myelomonocytic cell line (THP-1) upon cytokine treatment. *Cancer Research* **50**:2863–2867.

Yang W, Zerbe H, Petzl W, Brunner RM, Gnther J, Draing C, Aulock S, Schuberth H, Seyfert H. 2008. Bovine TLR2 and TLR4 properly transduce signals

from Staphylococcus aureus and E. coli, but S. aureus fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNFalpha and interleukin-8 (CXCL8) expression in the udder. *Molecular Immunology* **45**:1385 DOI 10.1016/j.molimm.2007.09.004.