




## Concentrations of acute-phase proteins and immunoglobulins in serum and synovial fluid in clinically healthy heifers and steers<sup>1</sup>

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**ABSTRACT.**- Di Filippo P.A., Lannes S.T., Meireles M.A.D., Nogueira A.F.S. & Quirino C.R. 2019. **Concentrations of acute-phase proteins and immunoglobulins in serum and synovial fluid in clinically healthy heifers and steers.** *Pesquisa Veterinária Brasileira* 39(6):388-392. Laboratório de Clínicas e Cirurgia Veterinária, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego 2000, Campos dos Goytacazes, RJ 28013-602, Brazil. E-mail: [pdf@uenf.br](mailto:pdf@uenf.br)

The aim of the study was to determine the concentration pattern of intra-articular acute phase proteins (APPs) and immunoglobulins in healthy crossbred cattle. Synovial fluid (SF) samples were collected from the radiocarpal joint of 25 heifers and 25 steers. Concentrations of APPs were measured by SDS-PAGE. The results were submitted to analysis of variance using the SAS statistical program, and means were compared by the Student-Newman-Keuls test ( $P < 0.05$ ). Thirty-seven proteins with molecular weights ranging from 7 to 37kDa were identified in SF of all animals. Eight were nominally identified with immunoglobulin A (IgA) and G (IgG), ceruloplasmin (Cp), transferrin (Tf), albumin (Ab),  $\alpha_1$ -antitrypsin (AAT),  $\alpha_1$ -acid glycoprotein (AGP), and haptoglobin (Hp). The  $\alpha_1$ -antitrypsin was only identified in the Sf of the heifers. The SF values of Cp, Hp, AGP and IgA were significantly higher in heifers than in steers. In sera, 34 proteins with molecular weights between 7 and 244kDa were identified in heifers and steers. Similar proteins were nominally identified in the sera, however the  $\alpha_1$ -antitrypsin was identified only in SF. The serum values Tf, AGP and IgG were significantly higher in heifers compared with steers. In conclusion, the physiological acute-phase proteins concentrations in synovial fluid of healthy ruminants can be useful in the interpretation of samples from animals with joint diseases. The SF electrophoretic profile of healthy ruminants differs depending on gender. Similar proteins were nominally identified in the sera, but only the SF of  $\alpha_1$ -antitrypsin.

**INDEX TERMS:** Acute-phase protein, immunoglobulin, serum, synovial fluid, healthy animals, heifers, steers, arthritis, biomarkers, disease diagnosis, electrophoresis, lameness, cattle.

**RESUMO.**- [Concentração de proteínas de fase aguda e de imunoglobulinas no soro e no líquido sinovial de bovinos clinicamente saudáveis.] O objetivo do estudo foi determinar o padrão de concentração de proteínas de fase

aguda e de imunoglobulinas intra-articulares (APPs) em bovinos mestiços saudáveis. As amostras de fluido sinovial (SF) foram coletadas da articulação radiocárpica de 25 novilhas e 25 novilhos. As concentrações de APPs foram mensuradas por SDS-PAGE. Os resultados foram submetidos à análise de variância usando o programa estatístico SAS, e os meios foram comparados pelo teste Student-Newman-Keuls ( $P < 0,05$ ). Trinta e sete proteínas com pesos moleculares variando de 7 a 37kDa foram identificadas no SF de todos os animais. Oito foram nominalmente identificadas como imunoglobulina A (IgA) e G (IgG), ceruloplasmina (Cp), transferrina (Tf), albumina (Ab),  $\alpha_1$ -antitripsina (AAT), glicoproteína  $\alpha_1$ -ácido (AGP) e haptoglobina (Hp). A  $\alpha_1$ -antitripsina foi identificada apenas no SF das novilhas. Os valores de Cp, Hp, AGP e IgA

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no SF foram significativamente maiores em novilhas do que em novilhos. No soro, 34 proteínas com pesos moleculares entre 7 e 244kDa foram identificadas nas novilhas e novilhos. Proteínas similares foram identificadas nos soros, mas apenas o SF das novilhas apresentou a  $\alpha$ 1-antitripsina. Os valores séricos de Tf, AGP e IgG foram significativamente maiores em novilhas em relação aos novilhos. Conclui-se que a mensuração das concentrações das proteínas da fase aguda no líquido sinovial de animais saudáveis pode ser útil na avaliação de amostras oriundas de bovinos com afecções articulares. O perfil eletroforético do SF de ruminantes saudáveis difere em função do gênero e as diferenças devem ser levadas em consideração na interpretação dos achados.

TERMOS DE INDEXAÇÃO: Proteínas de fase aguda, imunoglobulina, soro, líquido sinovial, animais saudáveis, artrite, biomarcadores, diagnóstico de doenças, eletroforese, claudicação, bovinos.

## INTRODUCTION

Diagnosis of joint damage is based upon clinical orthopedic examination and radiographic assessment, both of which can be non-specific and insensitive in early joint pathologies (Hurter et al. 2005). Recent studies have investigated other methods to overcome these obstacles. Magnetic resonance imaging, computed tomography and evaluation of biochemical markers in synovial fluid have been used (Hegemann et al. 2002, Jacobsen et al. 2006a). Synovial fluid (SF) is a dialysate of plasma that contains proteins, electrolytes and hyaluronic acid, capable of reflecting infections, immunological, or inflammatory joint conditions by altering its composition and appearance (Basile et al. 2013).

Joint damage induces the production of nitric oxide, interleukin-1, interleukin-6, tumor necrosis factor, metalloproteinases, inhibitors of metalloproteinases, antibodies and others, which trigger the acute-phase response and production of acute-phase proteins (Gruys et al. 2005). The acute phase proteins (APPs) can be produced by both hepatocytes and peripheral tissues, and can be classified according to their concentration in positive APP, if they increase, or negative APP, if they decrease. The APPs are believed to play major roles in several aspects of the systemic reaction to inflammation, including the opsonization of several pathogens, the scavenging of potentially toxic substances and the overall regulation of different stages of inflammation (Petersen et al. 2004). The APPs are sensitive factors that allow the early and precise detection of inflammation in ruminants (Kent 1992).

Intra-articular injection of LPS induced systemic and local acute-phase response in horses (MacDonald & Benton 1996, Jacobsen et al. 2006b). Increases in interleukin-1, interleukin-6, and tumor necrosis factor- $\alpha$  have also been measured in cases of naturally occurring acute and severe chronic synovitis in horses (Bertone et al. 1993). By demonstrating SF-specific intraarticular serum amyloid (SAA) isoforms, the results obtained by Jacobsen et al. (2006b) suggested that acute-phase protein is synthesized locally in the inflamed equine joint, similar to what has been demonstrated in humans previously. Measuring local APP levels improves precision of diagnosis, because it provides information on inflammatory/infectious status of the particular organ of interest. However, this potential has been explored only to a very limited degree, and much more research is needed in

this field (Jacobsen 2007). To our knowledge, SF-APPs have not been investigated specifically in cattle.

Limb pathology is also a serious problem in cattle, since lameness negatively affects welfare and economic production. However, the diagnosis of inflammatory processes in these animals is difficult because the clinical symptomatology is quite poor. Also, alterations in the classic parameters of the inflammatory response are relatively slight and not very specific. Serum amyloid A was useful for monitoring and early detection of arthritis in dairy cows (Jawor et al. 2008). In addition, severe sole ulcers and white line abscesses increased serum APPs in cows (Kujala et al. 2010). Based on the dynamic of haptoglobin, Smith et al. (2010) found which treatment was more effective in dairy cattle diagnosed with claw disorders.

Although these studies have demonstrated the importance of APPs in the diagnosis, monitoring and treatment of limb pathology in cattle, so far the local levels of APPs have not been detected. Therefore, the aim of this study was to determine the physiological concentrations of acute-phase proteins and immunoglobulins in synovial fluid (SF) and compare them to isoforms detected in serum of healthy heifers and steers.

## MATERIALS AND METHODS

All procedures applied in this study were approved by the animal research ethics committee of "Universidade Estadual do Norte Fluminense Darcy Ribeiro" (protocol number 291). Fifty crossbred cattle with average age of  $18 \pm 1.2$  months and weighing  $350 \pm 70$ kg, free from evidence of joint disease (as determined by thorough clinical examination, flexion tests, complete blood count and synovial fluid analysis) were used in the study (25 heifers and 25 steers). The animals were kept in pastures (*Brachiaria decumbens*) on the same farm. They had ad libitum access to water and mineral salt and received commercial feed containing 16% crude protein (min). The animals were immunized against clostridiosis (Poli-Star®, Vallé), rabies (Raivacel Multi®, Vallé) and foot and mouth disease (Bovicel®, Vallé).

Synovial fluid was collected at the radiocarpal joint site using a 25 x 0.7mm needle with the animals standing positioned in a head gate. Before arthrocentesis, the joint was shaved, washed using neutral detergent (three times), and rinsed with 70% alcohol. From each animal, 5 mL of jugular venous blood was drawn for serum samples. These were immediately centrifuged (8°C, 2,500g, 10 min at room temperature), and the supernatant was aliquoted and frozen within two hours of collection. The SF and serum samples were kept frozen in sterile Eppendorf tubes at -20°C until analysis.

Total proteins were determined by the Biuret method, and the serum and synovial proteinogram were obtained by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Laemmli 1970). Molecular weights and concentrations of protein fractions were determined by computed videodensitometry (CS 9000, Shimadzu Corp., Kyoto, Japan). Reference markers (Sigma Chemical Co., St Louis) were used to characterize proteins, with molecular weights of 29, 45, 66, 97.4, 116, and 205kDa. Also, electrophoretic migration of proteins was compared with that of pure proteins, including albumin, transferrin, haptoglobin, ceruloplasmin, IgA, IgG,  $\alpha$ 1-antitripsin and acidic glycoprotein.

Analysis of variance (Proc GLM) was performed with a model including the fixed effect of time, sex and simple interactions. Data were expressed as the mean  $\pm$  standard deviation (SD). The means were compared by the Student-Newman-Keuls test (SNK) of SAS (SAS Institute Inc., Cary/NC, 2012). Differences were considered significant with  $P < 0.05$ .

## RESULTS

The concentrations of the serum and SF proteins are summarized in Table 1 and 2. Thirty-seven proteins with molecular weights ranging from 7 to 37kDa were identified in SF of all animals. Eight were nominally identified with immunoglobulin A (IgA) and G (IgG), ceruloplasmin (Cp), transferrin (Tf), albumin (Al),  $\alpha_1$ -antitrypsin (AAT),  $\alpha_1$ -acid glycoprotein (AGP), and haptoglobin (Hp). Cp, Hp, AGP and IgA levels in the synovial fluid were significantly higher in the heifers than in the steers. Only heifers SF presented  $\alpha_1$ -antitrypsin.

In sera, 34 proteins with molecular weights from 7 to 244kDa were identified in heifers and steers. Similar proteins were nominally identified in the sera, but only the SF contained  $\alpha_1$ -antitrypsin. The serum values of Tf, AGP and IgG were significantly higher in heifers than steers. APP levels in the synovial fluid were significantly lower than the levels in the serum of all animals.

## DISCUSSION

The diseases that affect the locomotor system of cattle are one of the main health problems of the dairy industry and generate important questions about economic and animal welfare aspects (Warnick et al. 2001, Green et al. 2002). The economic losses result from lower milk production, ill

temper of animals, fertility problems, increased need to cull animals and costs for diagnosis and treatment. Many studies have shown to the presence of an acute-phase response associated with locomotor infections or inflammation (Jawor et al. 2008, Smith et al. 2010, Tóthová et al. 2011). In horses, has been shown that APPs levels in synovial fluid can help distinguish infectious from non-infectious joint disease, and when synovial fluid APPs levels were measured sequentially in the same patient, levels reflected effect of treatment (Jacobsen et al. 2006a). This is the first study to determine the APPs levels in synovial fluid of healthy ruminants.

Ruminants are significantly different to other species in their acute phase response in that Hp is a major APP. In healthy cattle the serum Hp concentration is <20mg/L but can increase to >2g/L within 2 days of infection (Petersen et al. 2004). Elevations in this protein have also been reported in dairy cows with pododermatitis septica, pododermatitis circumscripta, interdigital necrobacillosis and papillomatous digital dermatitis. In the animals suffering from pododermatitis septica and interdigital necrobacillosis, the Hp concentrations decreased after treatment lasting one to five days, indicating the effectiveness of treatment for these diseases. In contrast, the treatment did not affect the concentrations of Hp in animals with pododermatitis circumscripta (Smith et al. 2010). Hp binds with free hemoglobin (Hb), which is toxic and pro-inflammatory, thus reducing the oxidative damages associated with hemolysis (Yang et al. 2003). Hp can inhibit the proliferation of mastocytes, prevent the spontaneous maturation of Langerhans cells and suppress the proliferation of T cells (Xie et al. 2000, Arredouani et al. 2003). It also has an inhibitory effect on granulocyte chemotaxis, phagocytosis and bactericidal activity (Rosbacher et al. 1999). In this study, regardless of sex, Hp concentrations were detected in serum and synovial fluid (Table 1 and 2). Hp was also detected in the synovial fluid of healthy horses (Basile et al. 2013).

Jawor et al. (2008) evaluated the concentrations of APPs at distinct times during the treatment of cows with limb diseases. The highest concentrations of Hp, serum amyloid A (SAA) and fibrinogen (Fbg) were observed at the start of treatment. The authors noted a gradual reduction in the concentration of APPs in the cows whose treatment occurred without complications. On the other hand, in cows showing additional complications (e.g., viral infections, bronchitis, occurrence of other inflammatory states of the limbs), they observed increases in one or two of the APPs measured. They concluded that monitoring APP concentrations can be a valuable complement for the clinical assessment of treatment and early detection of possible disease complication. Fibrinogen (Fbg) is involved in homeostasis, supplying a substrate for the formation of fibrin and tissue repair, in turn providing a matrix for migration of inflammatory cells (Thomas 2000). Serum amyloid A is involved in the recruitment of inflammatory cells to the infection site (Xu et al. 1995), promotes the neutralization of endotoxins, inhibits the proliferation of lymphocytes and endothelial cells, and deters the aggregation of platelets and the adhesion of T lymphocytes to the extracellular protein matrix (Urieli-Shoval et al. 2000). In horses, SAA is synthesized locally in the mammary gland and in joints, and the protein has been shown in normal colostrum and synovial fluid from horses with experimentally induced arthritis (MacDonald et al. 1996, Jacobsen et al. 2006b).

**Table 1. Serum protein concentrations determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, in crossbred heifers and steers (mean  $\pm$  SD)**

Protein (mg/dl)	Bovines		P-value
	Heifers	Steers	
Total serum protein	8982.5 $\pm$ 419.1A	8488.7 $\pm$ 166.3A	0.28
Albumin	5142.1 $\pm$ 157.4A	5021.7 $\pm$ 98.8A	0.52
Ceruloplasmin	11.00 $\pm$ 0.81A	13.96 $\pm$ 1.46A	0.08
Transferrin	335.60 $\pm$ 22.01A	267.54 $\pm$ 22.01B	0.03
Haptoglobin	9.44 $\pm$ 1.44A	7.65 $\pm$ 0.92A	0.30
$\alpha_1$ -Acid glycoprotein	13.42 $\pm$ 0.73A	7.98 $\pm$ 0.73B	0.00
Immunoglobulin A	174.90 $\pm$ 17.81A	138.60 $\pm$ 17.81A	0.15
Immunoglobulin G	2799.36 $\pm$ 95.162A	2070.74 $\pm$ 95.162B	0.00

P-value = significance of the differences in means; different letters in the same row indicate differences between groups (P<0.05).

**Table 2. Synovial fluid protein concentrations determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, in crossbred heifers and steers (mean  $\pm$  SD)**

Protein (mg/dl)	Bovines		P-value
	Heifers	Steers	
Total serum protein	1195.00 $\pm$ 394.33A	1116.50 $\pm$ 510.38A	0.60
Albumin	514.92 $\pm$ 389.47A	571.98 $\pm$ 323.06A	0.62
Ceruloplasmin	1.56 $\pm$ 0.92A	0.43 $\pm$ 0.45B	0.0001
Transferrin	44.38 $\pm$ 27.94A	44.51 $\pm$ 24.26A	0.98
Haptoglobin	3.01 $\pm$ 1.59A	1.64 $\pm$ 1.26B	0.005
$\alpha_1$ -Acid glycoprotein	3.25 $\pm$ 1.20A	2.20 $\pm$ 1.23B	0.01
Immunoglobulin A	17.35 $\pm$ 12.48A	10.09 $\pm$ 7.81B	0.03
Immunoglobulin G	224.88 $\pm$ 100.23A	204.71 $\pm$ 130.35A	0.59
$\alpha_1$ -antitrypsina	26.802 $\pm$ 17.8	-	-

P-value = significance of the differences in means; different letters in the same row indicate differences between groups (P<0.05).

Significant increases in the serum concentrations of Hp, SAA and Fbg were found by Tóthová et al. (2011) in heifers suffering from hoof diseases (pododermatitis, laminitis, sole ulcer, and digital dermatitis). In turn, Laven et al. (2004) did not find a correlation between the presence of an acute-phase response and the development of sole hemorrhages in postpartum first-lactation heifers. According to them, only more severe pathologies, such as sole ulcers and interdigital necrobacillosis would be able to induce a systemic APR. The APPs measured by Laven et al. (2004) (albumin, Fbg, Hp, seromuroid and Cp) are considered to have high sensitivity to the species, despite the SAS being considered the most sensitive APP in bovines, with rapid increase in the blood after inflammatory stimulus (Werling et al. 1996, Kujala et al. 2010). The SAA is not measured by polyacrylamide gel electrophoresis and its determination has high costs, which often makes it difficult to determine it routinely. Therefore, Kujala et al. (2010) reported that cattle diagnosed with sole ulcers and/or e/or white line abscesses showed higher serum concentrations of SAA, although the concentrations of Hp remained unchanged. According to Young et al. (1996) and Kujala et al. (2010), Hp requires a stronger stimulus to induce an increase in the blood. Furthermore, the acute-phase response can vary between animals faced with the same challenge (Lomborg et al. 2008). Smith et al. (2010) and Jawor et al. (2008) reported that animals with the same hoof injuries presented high or undetectable serum concentrations of Hp. According to Jacobsen et al. (2004), the ability to produce determined APPs is an innate trait of the individual.

The analysis of the acute-phase proteins present at the site of interest (e.g., synovial fluid) increases the precision of diagnosis and helps to distinguish between inflammatory and infectious processes affecting the joints. It also allows evaluating the effectiveness of treatment (Jacobsen et al. 2006b). APPs have already been measured in the synovial fluid of equines (Jacobsen et al. 2006a, Basile et al. 2013, Di Filippo et al. 2014) and humans (Catterall et al. 2010). Nevertheless, little is known about the APPs present in the synovial fluid of cattle. In this study, we identified eight proteins in the SF - ceruloplasmin, transferrin, albumin,  $\alpha_1$ -antitripsin,  $\alpha_1$ -glycoprotein acid, haptoglobin and immunoglobulin A and G. The identification and quantification of the APPs in the SF of healthy cattle can allow comparison with samples suffering from inflammatory and/or infections processes of the joints, as already performed in other species.

In this study, the male and female cattle studied were the same age and were raised on a single farm with the same management practices. Therefore, the differences between the levels of APPs present in the serum and SF between males and females can be attributed to gender. Differences between genders have already been described for competitive horses (Escribano et al. 2008, Cywinska et al. 2011, Di Filippo et al. 2016, Martins et al., 2017). Nothing has been demonstrated previously in ruminants, so further investigation is necessary. Thus, age, gender, and physiological condition should be considered when interpreting a measured APP concentration.

## CONCLUSIONS

The establishment of the physiological acute-phase proteins concentrations in synovial fluid of healthy ruminants can be useful in the interpretation of samples from animals with joint diseases.

Further investigations are needed to establish the utility of synovial protein electrophoresis in bovine clinical practice in relation to inflammatory or infectious diseases.

The synovial fluid electrophoretic profile of young ruminants changes based on sex.

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