

Serum concentrations of acute phase proteins in goats and ewes with naturally acquired *Staphylococcus aureus* mastitis

[Concentração de proteínas de fase aguda em cabras e ovelhas com mastite de ocorrência natural causada por *Staphylococcus aureus*]

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ABSTRACT

Serum protein concentrations, including acute phase proteins (APPs), of goats and ewes with naturally acquired *Staphylococcus aureus* mastitis were determined by means of SDS-PAGE electrophoresis to evaluate the relevance of these APPs as biomarkers of the disease in these species. Fifteen healthy goats and 5 goats with naturally acquired staphylococci mastitis, as well as fifteen healthy ewes and 5 ewes with staphylococci mastitis were submitted to daily blood sampling during 7 days. In goats, an increase of 570%, 125%, 621%, and 279% in serum concentrations of ceruloplasmin, fibrinogen, haptoglobin and α_1 -acid glycoprotein, respectively, was observed. In sheep the increase in serum concentrations of ceruloplasmin, fibrinogen, haptoglobin and α_1 -acid glycoprotein was of 337%, 90%, 461%, and 225%, respectively. Our results indicate that these APPs have considerable potencial as early and sensible biomarkers of mastitis caused by *S. aureus* in goats and sheep.

Keywords: ceruloplasmin, fibrinogen, haptoglobin, α_1 -acid glycoprotein

RESUMO

O proteinograma, incluindo proteínas de fase aguda (PFAs), de cabras e ovelhas com mastite de origem natural causada por *Staphylococcus aureus*, foi determinado por meio de eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio (SDS-PAGE) a fim de avaliar a importância destas PFAs como biomarcadores da enfermidade nestas espécies. Amostras de sangue foram colhidas diariamente de cinco cabras e cinco ovelhas com mastite estafilocócica naturalmente adquirida, bem como de quinze cabras e quinze ovelhas saudáveis durante 6 dias consecutivos. Nas fêmeas caprinas, foi verificado aumento dos teores séricos de ceruloplasmina (570%), fibrinogênio (125%), haptoglobina (621%), e α_1 -glicoproteína ácida (279%). Nas fêmeas ovinas as concentrações de ceruloplasmina, fibrinogênio, haptoglobina e α_1 -glicoproteína ácida elevaram-se em 337%, 90,9%, 461% e 225%, respectivamente. Os resultados permitem inferir que estas PFAs são marcadores sensíveis e precoces de mastite causada por *S. aureus* em cabras e ovelhas.

Palavras-chave: Ceruloplasmina, fibrinogênio, haptoglobina, α_1 -glicoproteína ácida

INTRODUCTION

Clinical and subclinical mastitis are serious problems in dairy herds worldwide. Although there are several bacteria causing mastitis in goats and ewes, *Staphylococcus aureus* is by far the most important agent causing udder

infection, especially since it can evolve to persistent infection with recurring clinical episodes (Aires-de-Sousa *et al.*, 2007; Radostitis *et al.*, 2007; Erskine, 2013).

The increasing concern with expeditious identification and solution of sanitary problems in herds has also raised interest in the

identification of early biomarkers. Research along the last few decades showed that quantification of acute-phase proteins (APPs) either in blood plasma or serum is useful for diagnosis and prognosis of disease, as well as to evaluate the severity of inflammation; APPs quantification may also contribute with human food security by early disease detection at slaughterhouse (Eckersall, 2000; González *et al.*, 2007). Proteins whose serum concentrations decrease as a response to inflammation are called negative acute-phase proteins and include albumin and transferrin. Contrarily, proteins whose serum concentrations increase during inflammation are defined as positive acute-phase proteins, e.g., ceruloplasmin, fibrinogen, haptoglobin, α_1 -acid glycoprotein. Concentrations of the latter proteins are mostly low or non-detectable in healthy animals. Thus, increased serum levels of these proteins are used as auxiliary tools for diagnosing and monitoring disease. The type of APPs increasing as a result of inflammation, as well as the time that these APPs remain elevated varies among species and disease (Thomas, 2010; Ceciliani *et al.*, 2012).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) allows the identification of up to 30 protein bands. It is an easy, low-cost technique which requires small samples, allowing detection of extremely low concentrations of protein. By using this technique, it is possible to evaluate the APPs of interest in veterinary medicine (Fagliari *et al.*, 2007).

Despite the importance of determining APPs in ruminant clinic, there are only few reports available on this subject, particularly on goats and sheep. Thus, the aim of the present study was to quantify APPs in blood serum from goats and ewes with naturally acquired *Staphylococcus aureus* mastitis using SDS-PAGE, in order to assess the importance of such proteins as early biomarkers of the disease in these species.

MATERIALS AND METHODS

Fifteen healthy goats and 15 healthy ewes (control groups) as well as five goats and five ewes with naturally acquired Staphylococcal mastitis were used. The experimental protocol was approved by the Committee of Ethics and Animal Welfare of the School of Agrarian

Sciences and Veterinary Medicine, São Paulo State University (Unesp), Brazil, under protocol number 029033-08.

Selection of animals was performed by physical examination and microbiological isolation and identification of *Staphylococcus aureus* in milk samples by assessing the colony characteristics on blood agar, Gram staining and positivity to biochemical tests such as catalase, coagulase, mannitol, Voges-Proskauer and anaerobic fermentation.

Control animals that presented no clinical signs of mastitis and were negative for bacterial detection in milk culture belonged to the School of Agrarian Sciences and Veterinary Medicine (UNESP). Ill animals were selected from among patients with acute mastitis (i.e. fever, swelling and/or local pain in the mammary gland, inappetence, changes in the characteristics of milk, etc) admitted into the School Veterinary Hospital at the same institution. Animals were included in the study after acknowledgment (through anamnesis) that the manifestation of clinical signs begun no longer than 3 days before admittance.

Once included into the experiment, consecutive daily blood samples were taken for six days and moments identified as M1, M2, M3, M4, M5 and M6. During the study period (M1 - M6), animals remained in the Hospital and were treated (M2-M4), once daily for three days with M1 being the first sampling moment when the animal had no therapy influence on its immune response to disease. Treatment consisted of intramammary administration of 200 mg of gentamicin once daily. All females were only discharged from Hospital after microbiological cure of the infection.

Blood samples were collected in tubes without anticoagulant and centrifuged to obtain serum, which was frozen in aliquots at -80°C for ulterior analysis.

Serum total protein was determined by the biuret method using a commercial kit (Labtest, Labtest, Labtest Diagnostica, Lagoa Santa, Minas Gerais, Brazil). Separation of protein fractions and determination of ceruloplasmin, haptoglobin, α_1 -acid glycoprotein concentrations were accomplished by sodium dodecyl sulfate-

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polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Concentrations were obtained through computerized densitometer (Shimadzu CS-9301PC, Tokyo, Japan). A marker solution (Sigma, St. Louis, MO, USA) with molecular weights of 29,000, 45,000, 66,000, 97,400, 116,000 and 205,000 Daltons (Da) was used to identify protein fractions associated with the electrophoretic mobility of patterns of purified immunoglobulin A, immunoglobulin G, ceruloplasmin, transferrin, and haptoglobin (Sigma, St. Louis, MO, USA). Plasma fibrinogen levels were measured by the heat precipitation method, as described by Jain (1993).

Results were analyzed by ANOVA. The means were compared using the Tukey's test set at 5.0% significance value (Petrie and Watson, 2009).

RESULTS AND DISCUSSION

Goats with mastitis presented mild increase in concentrations of total protein in serum when compared with the control group. Total protein levels subsequently decreased five days after admission (M5). There was no difference in serum total protein in ewes between groups and moments (Table 1, Figure 1).

Table 1. Means and standard deviations of serum total protein concentrations (g/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6)

Groups	Moments (days of admission)					
	M1	M2	M3	M4	M5	M6
Healthy goats	6.1±0.66Ba	5.9±0.58Ba	5.8±0.61Ba	6.1±0.55Ba	6.2±0.71Aa	6.0±0.60Aa
Mastitis goats	7.0±0.67Aa	6.9±0.71Aa	6.7±0.75Aa	6.8±0.60Aa	6.7±0.66Aa	6.5±0.59Aa
Healthy ewes	6.6±0.59Aa	6.4±0.70Aa	6.2±0.53Aa	6.5±0.71Aa	6.3±0.80Aa	6.3±0.69Aa
Mastitis ewes	7.3±0.79Aa	7.1±0.70Aa	7.3±0.62Aa	7.0±0.58Aa	6.8±0.65Aa	7.0±0.88Aa

Means followed by different capital letters in the same column and different small letters in the same line indicate significant difference by the Tukey's test (P>0.05).

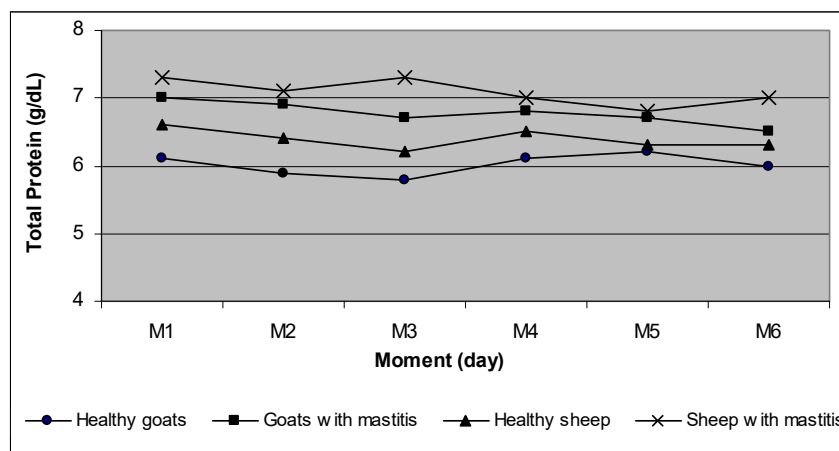


Figure 1. Means of serum protein (g/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experimental period (M6).

Contrary to the present study, Patelli *et al.* (2008) did not report an increase in total protein concentrations in goats experimentally infected with *Trypanosoma evansi* when compared to the control group. Brum *et al.* (2007) reported similar results to the findings of the present study in sheep naturally infected with facial eczema. The mild increase in concentrations of serum total protein detected in the goats of this

experiment may have been caused by the significant increase of APPs, e.g., ceruloplasmin.

Ceruloplasmin levels in goats with mastitis were considerably elevated (i.e. 570%) when compared with control group, with maximum concentration at the second day in hospital (M2), and gradual decrease until the end of the experimental period (Table 2, Figure 2). Also,

ceruloplasmin concentrations in ewes with mastitis were 337% higher than in the control group. Between moments, a gradual decrease in

ceruloplasmin levels it was evident along the experimental period in the mastitis groups.

Table 2. Means and standard deviations of serum ceruloplasmin (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6)

Groups	Moments (days of admission)					
	M1	M2	M3	M4	M5	M6
Healthy goats	9.2±3.53Ba	11.3±3.77Ba	9.8±2.95Ba	12.3±3.11Ba	10.9±3.44Ba	13.0±3.16Ba
Mastitis goats	83.7±7.45Aa	90.7±8.33Aa	84.5±6.65Aa	73.1±7.32Ab	60.9±5.89Ac	52.7±6.12Ad
Healthy ewes	12.4±3.32Ba	10.8±2.92Ba	13.0±2.87Ba	11.7±3.06Ba	14.6±3.21Ba	16.1±2.21Ba
Mastitis ewes	77.1±9.1Aa	65.3±7.21Aab	60.3±7.03Ab	53.4±5.22Ab	46.5±4.66Ac	40.8±4.06Ad

Means followed by different capital letters in the same column and different small letters in the same line indicate significant difference by the Tukey's test ($P>0.05$).

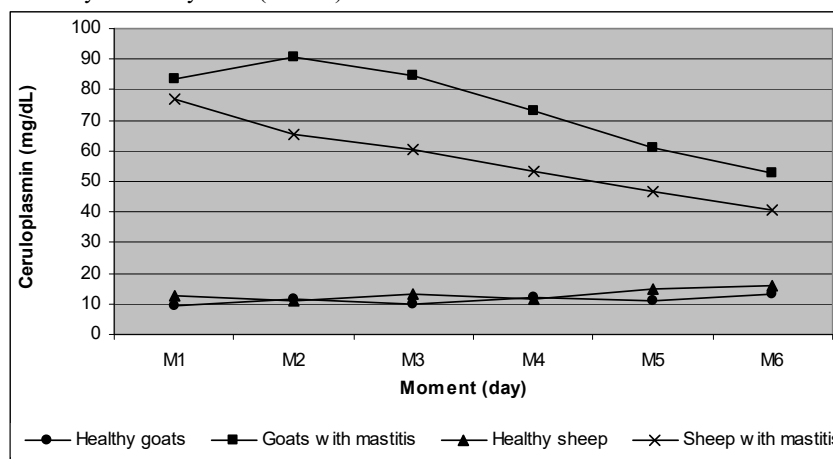


Figure 2. Means of serum ceruloplasmin (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6).

Costa *et al.* (2010) reported an increase of 298% in serum levels of ceruloplasmin in ewes with experimentally-induced mastitis, which agrees with the results of the present study. There are few publications on ceruloplasmin levels in small ruminants presenting mastitis (Eckersall, 2006). Nevertheless, the increase of this protein's concentration reaffirms its anti-inflammatory and biocatalytic effect reported by Patel *et al.* (2002). In cows with clinical or sub-clinical mastitis, serum ceruloplasmin levels drastically increased in both blood plasma and milk (Eckersall *et al.*, 2001; Grönlund *et al.*, 2005). Therefore, although using ceruloplasmin as inflammatory marker is less common than using other APPs, it seems to be a reliable signaler of bovine

infection (Segelmark *et al.*, 1997; Murata *et al.*, 2004). These results emphasize the clinical importance of this biomarker in goats and sheep.

Goats with mastitis presented fibrinogen concentrations 125% higher than the control group, and maximum levels were found at the third day in hospital (M3). Although the lowest serum level of fibrinogen was detected at M6 in goats with mastitis, values were still significantly higher than in the control group (Table 3, Figure 3). Regarding ewes, plasma fibrinogen increased 90.9%, with higher levels in M2 and similar pattern when compared with goats throughout the experimental period.

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Table 3. Means and standard deviations of serum fibrinogen (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6)

Groups	Moments (days of admission)					
	M1	M2	M3	M4	M5	M6
Healthy goats	379±41Ba	382±44Ba	375±36Ba	402±49Ba	391±45Ba	432±51Ba
Mastitis goats	883±92Aa	907±98Aa	923±94Aa	886±91Aa	875±83Aa	845±90Aa
Healthy ewes	442±38.7Ba	439±40.1Ba	455±33.7Ba	471±39.9Ba	463±41.2Ba	456±37.5Ba
Mastitis ewes	912±90.3Aab	930±94.1Aa	919±87.3Aab	865±81.7Aab	821±75.6Ab	757±70.5Ab

Means followed by different capital letters in the same column and different small letters in the same line indicate significant difference by the Tukey's test ($P>0.05$).

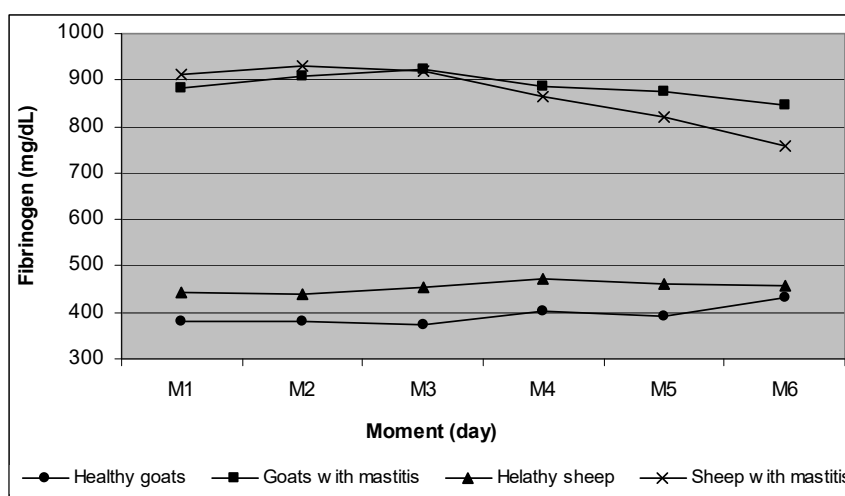


Figure 3. Means of serum fibrinogen (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6).

Costa *et al.* (2010) reported that the peak in plasma fibrinogen occurred 132 hours after the experimental intra-mammary infection with *Staphylococcus aureus* in ewes. This time lapse matches with M5-M6 in our experiment, when, contrary to the report of the previous authors, there was a significant decrease in serum fibrinogen concentrations. However, it is important to consider the more accurate control regarding the beginning of clinical signs in experimental works, as compared to naturally occurring disease, in which it is also difficult to control some variables, as in the present study.

In goats with mastitis there was an increase of 621% in levels of haptoglobin when compared with the control group. The highest concentration was observed at M3, whereupon levels decreased and were significantly lower at M6, although still

higher than in the control group. Serum levels of haptoglobin were also high in ewes with mastitis, with maximum value at the second day in hospital (M2). Despite serum concentrations of haptoglobin decreased throughout the experimental period in mastitis groups, levels of this parameter remained higher than in the control group until the end of the experimental period (M6).

Haptoglobin is a glycoprotein with a two to four days half-life, and its concentration increases quickly, 24-48 hours after tissue damage. Also, it is considered to be a major APP in goats and sheep (Cooray *et al.*, 2007; Ceciliani *et al.*, 2012). Data confirming this statement are shown in Table 4 and Figure 4. Jain *et al.* (2011) reported that haptoglobin is the main APPs in sheep. The present study results also show that

this protein presented the higher responsiveness in ewes, with an increase of 461% in ill animals when compared with the control group. Costa *et*

al. (2010) reported concentrations that rose as high as 607% in experimental infection with *S. aureus* when compared to control group.

Table 4. Means and standard deviations of serum haptoglobin (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6)

Groups	Moments (days of admission)					
	M1	M2	M3	M4	M5	M6
Healthy goats	20.7±3.09Ba	22.5±3.17Ba	19.8±3.01Ba	21.0±2.87Ba	23.1±3.18Ba	20.5±3.03Ba
Mastitis goats	160±25Aa	168±26Aa	175±30Aa	151±26Aa	139±32Aab	127±24Ab
Healthy ewes	27.0±2.21Ba	30.2±2.89Ba	28.2±2.33Ba	31.2±2.17Ba	28.6±2.33Ba	26.8±2.71Ba
Mastitis ewes	167±22.7Aa	174±25.1Aa	163±20.3Aa	166±24.3Aa	153±20.8Aab	142±17.5Ab

Means followed by different capital letters in the same column and different small letters in the same line indicate significant difference by the Tukey's test ($P>0.05$).

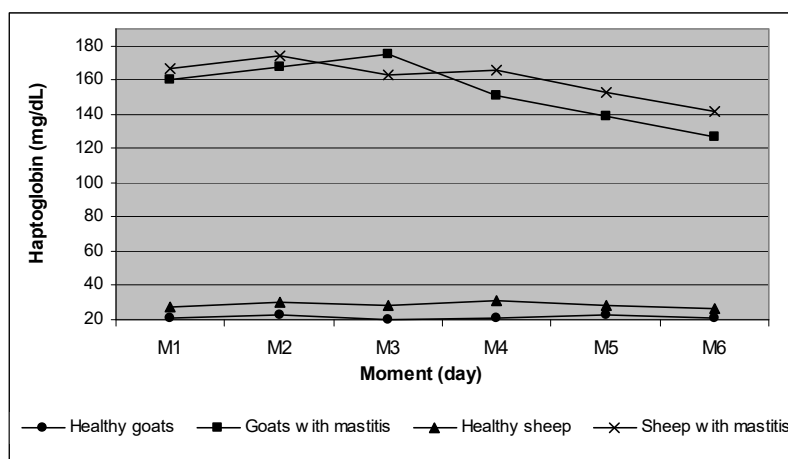


Figure 4. Means of serum haptoglobin (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6).

In general, haptoglobin is considered a sensitive and reliable inflammatory biomarker in goats and ewes suffering staphylococcal mastitis. The present study agrees with Eckersall *et al.* (2001) who stated that haptoglobin is highly sensitive in detecting inflammatory/infectious diseases in ruminants; and with Skinner and Roberts (1994) who reported that this protein may be used as a safe indicator of bacterial infection and inflammation in sheep.

Serum concentrations of α_1 -acid glycoprotein in goats with mastitis were 279% higher when compared with the control group and decreased along the experimental period. Likewise, levels of α_1 -acid glycoprotein in ewes with mastitis were higher than in the control group (maximum increase 225% at M2 - Tab. 5, Fig.5).

Table 5. Means and standard deviations of serum α_1 -acid glycoprotein (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6)

Groups	Moments (days of admission)					
	M1	M2	M3	M4	M5	M6
Healthy goats	7.4±2.7Ba	9.2±2.2Ba	9.0±2.03Ba	8.6±1.93Ba	9.5±2.09Ba	8.3±1.97Ba
Mastitis goats	38.1±4.8Aa	33.9±5.3Aab	37.2±5.0Aa	31.8±4.7Ab	30.2±4.9Ab	25.6±4.0Ac
Healthy ewes	8.7±1.2Ba	10.1±1.8Ba	9.6±0.95Ba	8.3±1.2Ba	10.0±1.4Ba	9.2±0.93Ba
Mastitis ewes	33.4±5.2Aa	36.1±6.1Aa	35.3±5.4Aa	28.6±5.5Ab	26.3±4.3Ab	21.7±4.2Ac

Means followed by different capital letters in the same column and different small letters in the same line indicate significant difference by the Tukey's test (P>0.05).

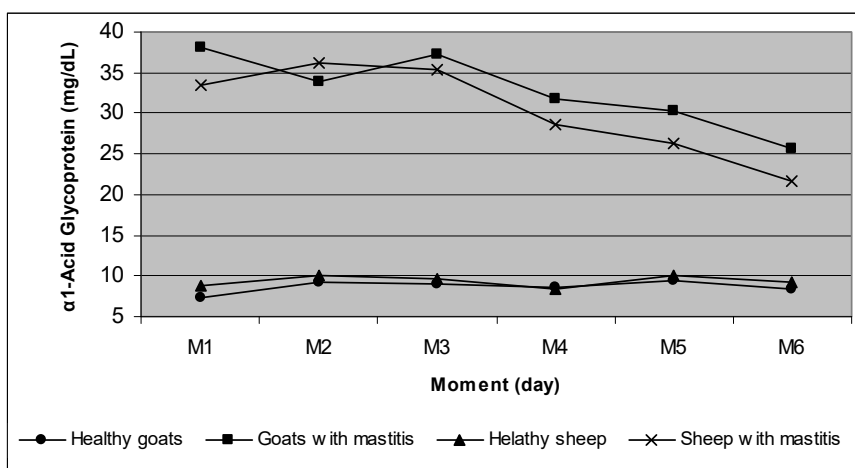


Figure 5. Means of serum α_1 -acid glycoprotein (mg/dL) in both healthy and ill (staphylococcal mastitis) goats and ewes from admission (M1) to the end of the experiment (M6).

Results by Rinaldi *et al.* (2008), showed that α_1 -acid glycoprotein had immunomodulatory properties acting on leucocytes and cytokines during inflammation, which justifies the increase of this APP along with the acute phase response. Recent studies in sheep and other species revealed that α_1 -acid glycoprotein may be an indicator of chronic inflammation, due to its long plasma half-life compared with other APPs (Eckersall *et al.*, 2007; González *et al.*, 2008). However, the early detection and significant high level of this protein at the beginning of disease, associated with its decrease by the fourth day in hospital (M4) in both goats and ewes, confirm its characteristic of positive APP, acting early in the acute phase response in females with staphylococcal mastitis.

CONCLUSIONS

Detection and quantification of APPs by SDS-PAGE was useful and reliable as a complementary test in the diagnosis of mastitis caused by *Staphylococcus aureus* in goats and

ewes. Moreover, the APPs ceruloplasmin, haptoglobin, and α_1 -acid glycoprotein were early and sensitive indicators of mammary inflammation in both species.

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