DOI: 10.5433/1679-0359.2020v41n6Supl2p3165

Study of metalloproteinases in the blood of goats experimentally infected with caprine encephalitis arthritis virus[1]

Estudo de metaloproteinases no sangue de caprinos experimentalmente infectados pelo vírus da artrite encefalite caprina

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Highlights:
MMPs is a new approach to complement the diagnosis of caprine arthritis encephalitis.
Presence of proMMP-13 may be indicative of seroconversion in acute infection.
ProMMP-9 at post-seroconversion stage can facilitate control of the disease.
MMP-2 at post-seroconversion stage indicates the presence of infection.

Abstract

Caprine arthritis encephalitis is a lentiviral disease that leads to considerable losses in goat farming. In the acute phase of viral infection, though antiviral antibodies are produced by the host's immune system, they are not sufficient to be detected by serological tests. Acute infections begin with an incubation period, during which the viral genome replicates and host innate responses are initiated. Matrix metalloproteinases (MMPs) are enzymes that play an important role in the physiological and pathological processes of tissue remodeling. The present study aimed to evaluate the expression of MMPs and their activity in the blood serum of male goats experimentally infected with caprine arthritis encephalitis virus (CAEV). Five dairy male goats, aged 3-4 years, were intravenously inoculated with CAEV Cork strain (titer: 10^{5-6} $TCID_{50}$/mL) after being tested negative for CAEV thrice at consecutive intervals of 30 days using western blot analysis and nested-PCR. The study included three stages: S1 or pre-infection stage; S2 or seroconversion stage, corresponding to the occurrence of first seroconversion; and S3 or post-seroconversion stage, corresponding to 23 weeks after seroconversion. Zymography was performed for the samples using gelatin zymography gels (12.5%), which were subjected to electrophoresis at 170V, 1A, and 300W for 50-70 min. The density of MMP-2 was found to be lower at

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Received: Nov. 29, 2019 - Approved: Aug. 07, 2020

S1 (1456.20 pixels) than that at S2 and S3 (1943.80 and 2104.40 pixels, respectively) ($P < 0.05$); and the density of MMP-9 was found to be lower at S3 (133.60 pixels) than that at S1 and S2 (359.60 and 370.60 pixels, respectively). The density of proMMP-2 was low at S1 and S3 (130.45 and 145.20 pixels, respectively). On the other hand, the density of proMMP-9 was statistically different between S1 and S3 (89.22 *vs*. 415.60 pixels). Both proMMP-2 and proMMP-9 were absent at S2. Thus, MMP-2 and MMP-9 exhibited opposite behaviors depending on the stage of infection. As the greatest activity of MMP-2 was detected at stage S3, we suggest that MMP-2 can be used as a biomarker for complementary diagnosis of acute CAEV infection. In addition, the presence of proMMP-13 can be used to indicate active viral infection.
Key words: Lentivirus. MMPs. Proteases. Zymography.

Resumo

A encefalite por artrite caprina é uma doença lentiviral que leva a perdas consideráveis na criação de caprinos. Na fase aguda da infecção viral, embora os anticorpos antivirais sejam produzidos pelo sistema imunológico do hospedeiro, eles não são suficientes para serem detectados por testes sorológicos. As infecções agudas começam com um período de incubação, durante o qual o genoma viral se replica e as respostas inatas do hospedeiro são iniciadas. As metaloproteinases da matriz (MMPs) são enzimas que desempenham um papel importante nos processos fisiológicos e patológicos da remodelação tecidual. O presente estudo teve como objetivo avaliar a expressão de MMPs e sua atividade no soro sanguíneo de reprodutores caprinos infectados experimentalmente pelo vírus da encefalite por artrite caprina (CAEV). Cinco machos caprinos, com idades entre 3-4 anos, foram inoculadas por via intravenosa com a cepa de CAEV Cork (título: 10^{5-6} $TCID_{50}$/mL) após serem testados negativamente para CAEV três vezes em intervalos consecutivos de 30 dias usando a análise de western blot e nested-PCR. O estudo incluiu três etapas: estágio S1 ou pré-infecção; S2 ou estágio de soroconversão, correspondente à ocorrência de primeira soroconversão; e S3 ou pós-soroconversão, correspondendo a 23 semanas após a soroconversão. A zimografia foi realizada para as amostras utilizando gel de gelatina (12,5%), que foram submetidos à eletroforese em 170V, 1A e 300W por 50-70 min. Verificou-se que a densidade de MMP-2 era menor em S1 (1456,20 pixels) do que em S2 e S3 (1943,80 e 2104,40 pixels, respectivamente) ($P < 0,05$); e a densidade de MMP-9 foi menor em S3 (133,60 pixels) do que em S1 e S2 (359,60 e 370,60 pixels, respectivamente). A densidade do proMMP-2 era baixa em S1 e S3 (130,45 e 145,20 pixels, respectivamente). Por outro lado, a densidade do proMMP-9 foi estatisticamente diferente entre S1 e S3 (89,22 vs. 415,60 pixels). ProMMP-2 e proMMP-9 estavam ausentes no S2. Assim, MMP-2 e MMP-9 exibiram comportamentos opostos, dependendo do estágio da infecção. Como a maior atividade da MMP-2 foi detectada no estágio S3, sugerimos que a MMP-2 possa ser usada como biomarcador para o diagnóstico complementar de infecção aguda por CAEV. Além disso, a presença de proMMP-13 pode ser usada para indicar infecção viral ativa.
Palavras-chave: Lentivírus. MMPs. Proteases. Zimografia.

Introduction

Caprine arthritis encephalitis leads to great economic losses in goat production, particularly when breeders and matrices of high genetic value are affected by the disease; importantly, asymptomatic animals represent a serious threat to herd health (Pinheiro, Gouveia, Alves, & Andrioli, 2004). Although animals infected with caprine arthritis encephalitis virus (CAEV) develop specific cellular and humoral immune responses against the virus, they are not capable of preventing viral transmission among animals (Dawson, 1987). Some infected animals exhibit delayed the production of detectable antibodies (Rimstad et al., 1993), which leads to false negative results in serological tests. The false negative results can also be attributed to the antigenic variation between the viral strain used in the test and the circulating strain (Jones, 2014). In addition, production of antibodies and their

concentration in the blood are influenced by several factors, including stress, nutritional condition, age of the animal, and presence of other diseases (Franke, 1998).

The time interval between infection and seroconversion varies among animals, ranging from weeks to months or even years, with the possibility of seroreversion making it even more difficult to control the disease (Hanson, Hydbring, & Olsson, 1996). In most cases, at third week post infection, immune response against the capsid protein (p25 or p28) (Houwers & Nauta, 1989) can be detected; and at fifth week post infection, antibodies against other proteins can be detected (Concha-Bermejillo, Brodie, Magnus-Corral, Browen, & DeMartini, 1995).

Proteomics has emerged as an advanced tool to circumvent and promote effective control of infections because it facilitates the study of molecules involved in innate immune response to bacterial and viral infections (Aslam, Basit, Nisar, Khurshid, & Rasool, 2017).

Among the expressed proteases, matrix metalloproteinases (MMPs), which play important roles as inflammatory mediators, enable the monitoring and prognosis of diseases and can serve as potential biomarkers for diagnosis of viral infections, subsequently facilitating the discovery of antiviral compounds (Pereira, 2014). MMPs are calcium-dependent zinc-containing endopeptidases secreted by polymorphonuclear leukocytes, keratinocytes, monocytes, macrophages, fibroblasts, and mesenchymal cells as proenzymes (Navarro, Nelson, Silva, & Freitas, 2006). A total of 24 MMPs have been identified in mammals, and they are classified into five subfamilies: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), elastases (MMP-7 and MMP-12), and membrane-like MMPs (MT-MMP-1 to MT-MMP-5) (Visse & Nagase, 2003; Lopez-Avila & Spencer, 2008). MMPs participate in several biological activities, including determination of the structure of extracellular matrix, embryonic development, implantation of blastocysts, organ morphogenesis, development of the nervous system, ovulation, cervical dilation, postpartum uterine regression, development and remodeling of oral tissue, tissue healing, angiogenesis, and apoptosis (Verma & Hansch, 2007). Therefore, MMPs are recognized as important regulatory enzymes exhibiting beneficial and harmful activities in the inflammatory pathways, where they may contribute to the regulation of host defense and inflammatory diseases (Concha, 2010). To evaluate the active and latent forms of proteolytic enzymes and to determine the number and approximate molecular mass of enzymes in biological samples, zymography, a method based on the degradation of a copolymerized substrate, is commonly employed (Eloy, Bezerra, Pinheiro, & Andrioli, 2015).

In medicine, studies related to the application of MMPs in monitoring, control, and diagnosis of bacterial and viral infections, diabetes, cancer and its progression, and other diseases, are at an advanced stage. On the contrary, in veterinary medicine, studies on MMPs are still incipient, especially in goats. Studies related to the role of MMPs in goat diseases are scarce. However, Bezerra et al. (2015) described the high activity of MMPs (proMMP-2 and MMP-2) in goats naturally infected with CAEV, both in the presence and absence of joint symptoms. Considering the important roles of MMPs in the physiological processes of animals, particularly CAEV-infected animals, and the lack of information regarding the performance of these enzymes in goats experimentally infected with CAEV, the present study aimed to acquire a comprehensive understanding of the functions of MMPs in the blood serum of male goats experimentally infected with CAEV.

Materials and Methods

A total of five males goats Saanen and Anglo-Nubian goats, aged 3-4 years, belonging to the herd of EMBRAPA Goats and Sheep, located in the municipality of Sobral, Ceará, Brazil, were used in the present study. The animals were tested for CAEV thrice at consecutive intervals of 30 days using western blotting (WB) and nested-PCR, and it was observed that all animals tested negative for CAEV. All animals were healthy according to a general clinical examination performed following the recommendations of Diffay, Mckenzie, Wolf and Pugh (2005). The animals were fed elephant grass (*Pennisetum purpureum*) supplemented with 300 g concentrate containing 70 % corn, 27 % soybean meal, 2 % limestone, and 1 % mineral salt *ad libitum*. The study was carried out in accordance with the ethical principles of animal experimentation and according to the protocol submitted to and subsequently approved by the Institutional Animal Care and Use Committee of the Universidade Estadual Vale do Acaraú (UVA) on December 17, 2014, under protocol number 001.04.013.UVA.505.01.

The five CAEV-free animals were inoculated with 1 mL of minimum essential medium containing CAEV Cork strain (titer: $10^{5\text{-}6}$ $TCID_{50}$/mL) intravenously, followed by weekly follow-up with WB until seroconversion and monthly follow-up thereafter. Thus, the study included three stages: S1 or pre-infection stage, corresponding to one week before viral inoculation; S2 or seroconversion stage, corresponding to third week after viral inoculation i.e., the occurrence of first seroconversion; and S3 or post-seroconversion stage, corresponding to 23 weeks after seroconversion.

Blood samples were collected by jugular venipuncture using a vacutainer® system in 10 mL tubes without anticoagulant, and centrifuged at 3000 *g* for 10 min for separation of serum. The serum was stored in a 2.5 mL microtube at 20 °C until further analysis.

Protein concentration was determined using Bradford method (Bradford, 1976). Zymography was performed using 12.5 % polyacrylamide gels copolymerized with sodium dodecyl sulfate (SDS), with gelatin (2 mg/mL gelatin) as the substrate, according to the protocol of Kupai et al. (2010). Electrophoresis was carried out at 170 V, 1 A, and 300 W for 50-70 min (Laemmli, 1970). The gels were prepared in duplicates for each stage (S1, S2, and S3), and were scanned and analyzed using Doc-IT® LS Image Analysis Software 6.0. The *LMW-SDS* marker kit (*GE Healthcare, Chicago, IL, United States), containing* phosphorylase B (97.4 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa), was used as protein molecular weight standard. Active MMP-9 (86 kDa) and MMP-2 (66 kDa) and their respective latent forms, proMMP-9 (92 kDa) and pro proMMP-2 (72 kDa), as well as other MMPs are expected to have an affinity towards gelatin. Recently, these MMPS were found in the blood serum and seminal plasma samples of goats chronically infected with CAEV. (Bezerra et al., 2015, 2017; Pinto et al., 2019; Santos et al., 2019).

Statistical analysis for this study was descriptive, analyzing the presence or absence of bands corresponding to the respective enzymes in the gels. The mean volume and intensity of each enzyme was calculated using Gel Analyzer version 2010®. The band intensity data were subjected to Shapiro-Wilk and Bartlett tests for verification of the assumptions of normality and homogeneity of variances. Logarithmic transformation of base 10 was applied to guarantee such assumptions. F test was performed using analysis of variance, followed by Tukey's posthoc test, using a 5 % significance level. All statistical analyses were performed using the R software.

Results and Discussion

Seroconversion was detected using WB in all animals at third week post infection. In the post-seroconversion stage i.e., 23 weeks after infection, only one animal tested negative for CAEV, while the others remained seropositive (Table 1). Lara, Birgel, Fernandes and Birgel (2003) injected infected goat kids with blood and colostrum from infected animals, and found that the titer of specific antibodies reached a maximum level between 45 and 60 days post-infection, and decreased thereafter. Our results corroborate the work of Spivak et al. (2010), who demonstrated that seroconversion occurred in human immunodeficiency virus-infected individuals within 3 weeks to one year. The variation in the seroconversion period can be attributed to the tropism of CAEV to monocytes and macrophages, which play important roles in viral replication and diffusion (Blacklaws, 2012). According to Pugh (2004), restricted replication allows the virus to remain latent in host monocytes, making them go undetected by the immune system.

Table 1
Results of western blot analysis for detection of CAEV at pre-infection (one week before infection), seroconversion (third week post infection), and post-seroconversion (twenty-third week post infection) stages in the blood serum of goats experimentally infected with CAEV

Stage	Animals				
	1	2	3	4	5
Pre-infection (S1)	−	−	−	−	−
Seroconversion (S2)	+	+	+	+	+
Post-seroconversion (S3)	+	−	+	+	+

(+) seropositive for CAEV; (−) seronegative for CAEV.

At S1, latent proMMP-2 (72-75 kDa) and proMMP-9 (92 kDa) as well as active MMP-2 (64-66 kDa) and MMP-9 (80-84 kDa) were detected; at S2, only active MMP-2 and MMP-9 and latent proMMP-13 (48 kDa) were detected; and at S3, the MMP profile was similar to that observed at S1 (Figure 1). High molecular weight proteins were detected in all animals; however, these were not the focus of this study.

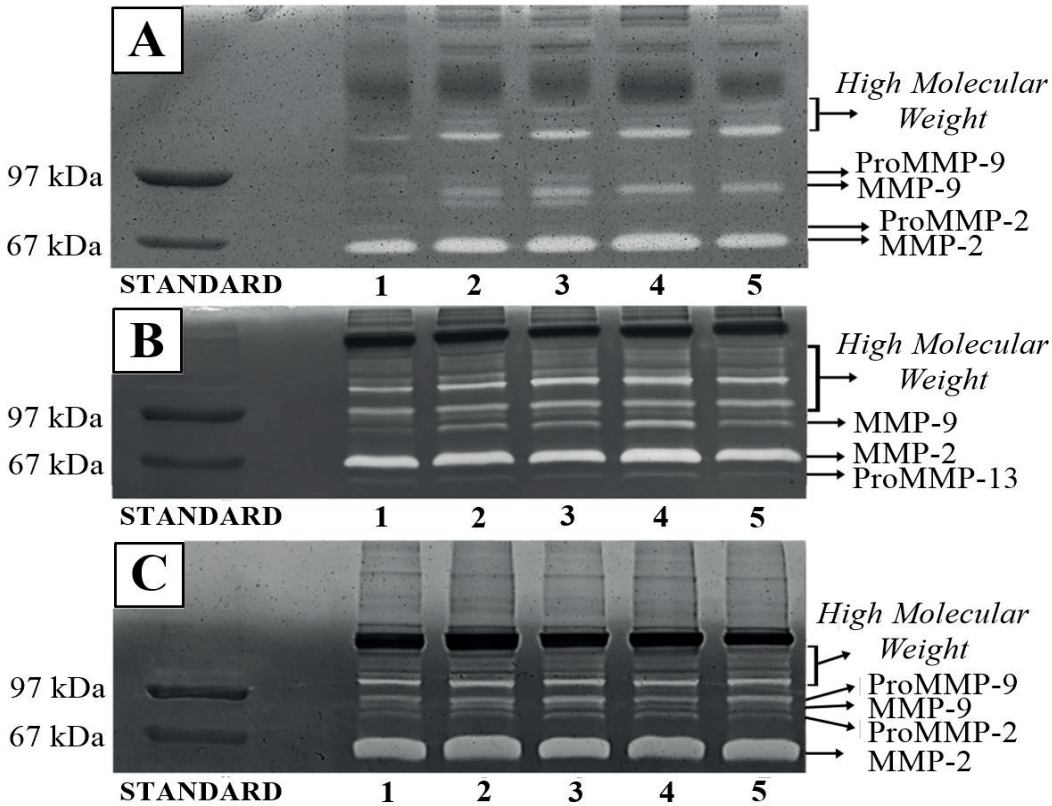

Figure 1. Enzymatic activity of metalloproteinases in the blood serum of goats experimentally infected with caprine arthritis encephalitis virus. Animals (1-5); (A) pre-infection, (B) seroconversion, and (C) post-seroconversion.

Bezerra et al. (2015) found that the activity of proMMP-2 ($P = 0.0025$) and MMP-2 ($P = 0.0001$) was greater in breeders naturally infected with CAEV than that in animals of the control group (negative), suggesting that proteases play an important role in chronic inflammatory processes in goats. An increase in the activity of these enzymes and a greater number of peaks have also been demonstrated among seropositive animals. Using a two-dimensional electrophoresis technique and matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, Bezerra et al. (2017) identified arisultadase A, a protease involved in the regulation of sulfate metabolism and subsequent control of viral infections, in the seminal plasma of goat breeders infected with CAEV.

MMPs are secreted as proenzymes or zymogens, therefore, they must be proteolytically activated by removal of the propeptide. This process is accompanied by breaking of the bond between the cysteine thiol group in the propeptide and the catalytic zinc in the peptide (Geurts, Opdenakker, & Steen, 2012; Mannello & Medda, 2012).

Analysis of band intensity in pixels (Figure 1) revealed that the density of MMP2 was greater at S3 than that at S1 and S2, suggesting a positive regulation. On the contrary, the density of MMP-9 was lesser at S1 and S3 than that at S2, suggesting a negative regulation.

Latent proMMP-2 and proMMP-9 were produced only at S1 and S3. Considering S1, this is possibly because they are required for the physiological

processes of healthy animals but at low activities (130.45 *vs.* 89.22 pixels). At S2, the latent forms were transformed into their active forms i.e., MMP-2 and MMP-9; and at this stage, MMP-2 had greater activity (1943.80 pixels) than MMP-9 (370.60 pixels). The presence of proMMP-13 in all animals undergoing seroconversion indicates active viral infection.

Thus, MMP-2 and MMP-9 exhibited opposite behaviors according to the stage of infection. In addition, MMP-2 exhibited a difference ($P < 0.05$) between S1 and S3, while MMP-9 exhibited no statistical difference between the two stages. In healthy animals, the density of proMMP-9 was below 90 pixels at S1and above 400 pixels at S3 ($P < 0.05$). ProMMP-2 did not present statistically significant differences between S1 (130 pixels) and S3 (145.20 pixels) (Table 2).

Table 2
MMP density (mean ± SD) observed at pre-infection (one week before infection), seroconversion (third week post infection), and post-seroconversion (twenty-third week post infection) stages in the blood serum samples of goats experimentally infected with CAEV using zymography

Treatment	MMP density (Pixels)			
	MMP-2	MMP-9	ProMMP-2	ProMMP-9
Pre-infection (S1)	$1456.20^{b} \pm 236.8$	$359.60^{a} \pm 209.1$	$130.45^{a} \pm 73$	$89.22^{b} \pm 51.6$
Seroconversion (S2)	$1943.80^{a} \pm 242$	$370.60^{a} \pm 247.8$	Absent	Absent
Post-seroconversion (S3)	$2104.40^{a} \pm 139.5$	$133.00^{a} \pm 50.7$	$145.20^{a} \pm 42.3$	$415.60^{a} \pm 114.7$

Different lower-case superscript letters in the same column indicate that the values differ statistically at 5 % significance.

The low intensity of most MMPs at S1 indicates that they are present in healthy animals and perform physiological functions inherent to the organism's development. Proteolytic enzymes are regulated by different mechanisms and are responsible for the degradation and remodeling of extracellular matrix components, which are involved in normal physiological processes and are associated with various diseases (Matrisian, 1990; Woessner, 1991).

The proteases MMP-2, MMP-9, and proMMP-13 were found in all animals at S2. The absence of latent proMMP-2 and proMMP-9 at S2 suggests that they were transformed into their respective active forms i.e., MMP-2 and MMP-9 due to immune response induced by the virus.

Elkington, White, Addington-Hall, Higgs and Edmonds (2005) demonstrated that MMP-2 and MMP-9 are required for the migration of inflammatory cells *in vitro*. ProMMP-13 is required for the activation of most of the MMPs.

It is worth mentioning that CAEV infection induces a strong humoral and cellular immune response (Lofstedt, 2014). Owing to the role of MMPs in inflammatory processes, it is suggested that enzyme activity of MMPs in the blood serum can serve as a marker of recent infection in animals. The presence of MMPs in the blood serum is attributed to the secretion of these enzymes by components of the circulatory and immune systems, including neutrophils, monocytes, macrophages, and fibroblasts, as well as by tumor cells in response to a variety of stimuli (Woessner, 1991). MMPs play a fundamental role in the inflammatory process and are involved in the pathophysiological remodeling of the vascular wall (Kuzuya & Iguchi, 2003; Hu, Steen, Sang, & Opdenakker, 2007).

Studies have revealed that MMP-9 is responsible for the release of the biologically active form of vascular endothelial growth factor, which plays an essential role in angiogenesis (Bergers et al., 2000; Klein & Bischoff, 2011). Moreover, MMP-9 has the ability to proteolytically degrade the basement membrane, which indicates the important role of MMP-9 in the formation of new blood vessels (Klein & Bischoff, 2011). MMP-9 is also expressed in conditions that require tissue remodeling, such as wound healing and tumor invasion (Stamenkovic, 2000; Klein & Bischoff, 2011). Bezerra et al. (2015) reported that the levels of proMMP-9 and MMP-9 were higher, though variable, in the seropositive group than those in the control (uninfected) group. MMP-2 can degrade gelatin, collagens (type I, IV, and V), elastin, and vibronectin, and is widely expressed by cells of the connective tissue (Stamenkovic, 2000). In addition, MMP-2 is involved in cellular migration, and is responsible for direct degradation of the basement membrane (Klein & Bischoff, 2011). Though both these MMPs work in different ways, they complement each other.

The presence of proMMP-13 in all animals at S2 suggests that it can be used as a biomarker of active CAEV infection. ProMMP-13 is involved in the degradation of proteoglycans and collagens, which are the main components of cartilages (Takahashi, Sasaki, Tsouderos, & Suda, 2003). It has been reported that patients with joint cartilage destruction exhibit high MMP-13 expression (Roach et al., 2005), suggesting the role of MMP-13 in cartilage degradation (Wang et al., 2013). Knäuper, López-Otín, Smith, Knight, & Murphy et al. (1996, 1997) observed that MMP-13 is overexpressed in joints and articular cartilage in patients with early osteoarthritis but hardly detected in normal adult tissues. MMP-13 can modify a large amount of extracellular proteins and cause degradation and remodeling of damaged tissues, acting mainly on the layers of synovial lining and synovial tissues (Iwamoto et al., 2011). In addition, MMP-13 is involved in several diseases, including rheumatoid arthritis (Ye et al., 2007), coronary artery disease (Vasku et al., 2012), and abdominal aortic disease (Saracini et al., 2012).

Knäuper et al. (2002) demonstrated that, in fibrosarcoma cell line (HT1080), proMMP-13 was activated to a 48-kDa active form i.e., MMP-13 via a 56-kDa intermediate. In the present study, the 48-kDa form of MMP-13 was detected at S2, suggesting that it is the active form in animals infected with CAEV. In addition, MMP-9 was expressed at a high level at S2, probably because MMP-13 induced greater activation of this protease. *In vitro* studies have shown that MMP-13 induces activation of proMMP-9 and self-activation of MMP-13. (Hernández Rios et al., 2009). These results suggest that active MMP-13 plays an important role in the activation of other MMPs, and is crucial for bone metabolism and homeostasis (Vincenti & Brinckerhoff, 2002).

At S3, MMP-2 and MMP-9, together with their respective latent forms, were detected. It has been suggested that the expression of these enzymes varies depending on the type and stage of disease, presence of neoplasms and inflammations, and cell type (Parks & Mecham, 1998). Thus, these proteases occur naturally in healthy animals as well as in infected animals; however, their expression is increased in infected animals to facilitate control of the disease. The absence of proMMP-13 at S3 is probably because after the first seroconversion, when there is a decrease in antibody production, activation of proteases by proMMP-13 is not required. The presence of proMMP-13 at S2 stage active viral infection. In addition, though the animals tested negative for CAEV at S3, the MMP profile at S3 was similar to that at S2.

The observed variations in the MMP profile can be attributed to the fact that enzyme profiles vary according to the type and stage of disease, presence of neoplasms and inflammation, and cell type; as the extracellular matrix, present in all tissues, requires MMPs for physiological processes and tissue remodeling (Yo & Werb, 1998).

Conclusion

At all stages (S1, S2 and S3), MMP-2 exhibited greater activity than MMP-9. The greatest activity of MMP-2 was observed in S3, suggesting that MMP-2 can be used as in monitoringinfection. In addition, proMMP-13, detected at seroconversion, indicates active viral infection. MMPs can diagnose viral infections even during viral latency, when serological tests show negative results. This is the first study reporting the expression profile of MMPs in male goats with experimentally infected with CAEV.

Acknowledgments

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for funding this research, Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) and Embrapa Caprinos e Ovinos (CNPCO) for providing the physical space and materials necessary for conducting this study.

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