Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.020

RESEARCH ARTICLE

Actinobacillus seminis Secretes a Metalloprotease That Degrades Bovine Fibrinogen and IgG

De la Cruz Montoya Aldo Hugo[1], Ramírez-Paz Y Puente Gerardo A[1], Vazquez-Cruz Candelario[2], Montes-García J Fernando[1] and Negrete Abascal Erasmo[1]*

[1]Carrera de Biología, Facultad de Estudios Superiores Iztacala, UNAM; Av. de los Barrios # 1, Los Reyes Iztacala, Tlalnepantla, Estado de México 54090, Mexico
[2]Centro de Investigaciones en Ciencias Microbiológicas, BUAP, Apdo. Postal 1622, Puebla 72560, Mexico
*Corresponding author: negretee@yahoo.com

ARTICLE HISTORY (22-417)

Received:	December 09, 2022
Revised:	February 16, 2023
Accepted:	February 24, 2023
Published online:	March 28, 2023

Key words:
Actinobacillus seminis
Secreted proteases
Iron restriction
Calcium
Fibrinogen

A B S T R A C T

Actinobacillus seminis is a Gram-negative bacterium member of the *Pasteurellaceae* family. Even though it is part of the ovine microbiota it is also the causal agent of genital organs affections, particularly epididymitis and orchitis. Infections caused by this microorganism generate economic losses to the ovine industry due to impaired fertility and sterility in affected animals. Knowledge about virulence factors expressed by *A. seminis* is scarce. In the present work, we describe the expression of a metalloprotease secreted by *A. seminis* that can degrade bovine fibrinogen and immunoglobulin G and present homology with a carboxy-terminal protease from *A. seminis*. This metalloprotease presents an optimal activity at a pH between 6 and 7, is stable up to 60°C, inactive at higher temperatures, and completely inhibited by 30 mM EDTA. The expression of this proteolytic activity is controlled by temperature, calcium, and iron. Proteases degrading extracellular matrix components and molecules involved in the immune response could facilitate and improve host colonization and invasion by *A. seminis.*

To Cite This Article: De la Cruz Montoya AH, Ramírez-Paz y Puente GA, Vazquez-Cruz C, Montes-García JF, Negrete-Abascal E, 2023. *Actinobacillus seminis* associated metalloprotease degrades bovine fibrinogen and IgG. Pak Vet J, 43(2): 276-282. http://dx.doi.org/10.29261/pakvetj/2023.020

INTRODUCTION

Ovine epididymitis is a transmissible disease commonly caused by *Brucella ovis*, *Actinobacillus seminis*, or *Histophilus somni*; of them, *B. ovis* is the microorganism most frequently isolated from infected animals. Although *A. seminis* has been considered part of the microbiota of the genital tract, it could be a primary pathogen as demonstrated by its solo inoculation in rams (Moustacas *et al*., 2014). Ovine epididymitis is considered one of the major causes of economic losses to the ovine industry due to impaired fertility and sterility in rams. Because young animals with epididymitis and orchitis caused by *A. seminis* are commonly asymptomatic in the early stages of illness, it is common that affected animals are detected until the disease has already progressed (Al-Katib and Dennis, 2009). This microorganism has also been isolated from the bovine semen of animals with testicular lesions (Santos *et al*., 2019) and uterine discharges from abortions in cows.

A. seminis can attach to bovine kidney epithelial cells and cultured human bladder cells (Healey *et al.,* 1991; Montes-García *et al*., 2018), agglutinate fixed sheep red blood cells (Montes-García *et al*., 2019) and form in vitro biofilms (García *et al*., 2020). The capability of *A. seminis* to express RTX proteins by immune cross-reactivity with anti-Apx sera (Schaller *et al.,* 2000) and to release outer membrane vesicles (Núñez-del Arco *et al*., 2006) has been suggested.

Mucosal pathogens can produce and excrete different kinds of precise nonspecific and specific proteases that degrade substrates involved in many functions, including the immune response, where they let them hide from the host defense mechanisms and take advantage of them (Potempa and Pike, 2009). *A. seminis* has been described as a habitant of the prepuce that ascends to the epididymis when hormonal changes occur during the ram's sexual maturity (Al-Katib and Dennis, 2009), where it is subjected to different clearance mechanisms that it has to evade, including the immune response.

Different members of the *Pasteurellaceae* family, to which *A. seminis* belongs, express and secrete proteases as a mechanism to evade the clearance strategies of the different hosts these microorganisms colonize (Negrete-Abascal *et al*.,1998, 2004), as a means to get nutrients, expose putative receptors, and as an invasive strategy. Despite this knowledge, the virulence factors that participate in the potential pathogenesis of this microorganism are not completely understood. In the

present work, we describe the expression of metalloproteases by *A. seminis*. These proteins can degrade bovine IgG and fibrinogen and their expression is controlled by temperature, the presence of calcium, and the iron concentration. These metalloproteases could be a mechanism to evade the immune response mediated by IgG and induce changes in the ram's physiology during the *A. seminis* pathogenesis.

MATERIALS AND METHODS

Bacterial strains: *A. seminis* ATCC 15768 reference strain was cultured on red blood agar sheep plates to reactivate and determine the purity of trypticase soy broth (TSB) cultures. Plates were incubated at 37°C in candle jars with humidity for 24 h.

Growth conditions: To obtain the putative proteolytic activities, *A. seminis* strains were grown in TSB medium in agitation at 37°C overnight. This culture was used to inoculate TSB media incubated at 37°C or 39-40°C. Cultures were maintained in agitation until an OD600 nm 0.1 was obtained, and 10 mM $CaCl_2$ or 0.25 mM 2,2´dipyridyl were added and incubated overnight. Media without $CaCl_2$ or 2,2´dipyridyl were used as controls. The purity of broth cultures was determined by growth on blood agar sheep plates.

Secreted proteins: Broth cultures were centrifuged at 10500*g* at 8°C for 25 minutes. Cultured supernatants without bacterial cells were precipitated overnight with ammonium sulfate at 60% saturation. Precipitated proteins were recovered by centrifugation at the same conditions and resuspended with PBS. Samples were frozen until their use.

Zymograms: The presence of proteolytic activity in the secreted proteins was determined through zymograms with porcine gelatin as previously described (Negrete-Abascal *et al*., 2004). To determine the optimal pH of the proteolytic activity, 10 µg of protein were loaded per well and, after electrophoretic separation, gels were incubated in three different conditions: i) 50 mM acetate buffer, pH 4 to 6; ii) 50 mM Tris-HCl, pH 7 or 8; and iii) 50 mM glycine-NaOH, pH 9 and 10. The effect of temperature on the proteolytic activity was determined at 4°C, room temperature, 37, 40, 50, 60, or 70°C for 10 minutes before electrophoresis. The effect of different protease inhibitors (10mM p-hydroxy mercury benzoate [PHMB], 5 mM phenyl methyl sulphonyl fluoride [PMSF], or 10-40mM ethylenediaminetetraacetic acid [EDTA]) was determined by previous incubation with each inhibitor and their addition to the activation buffer at the same concentrations.

Degradation of other substrates: To establish the capability to degrade molecules of physiological importance, 20 µg of secreted proteins were mixed with 20 µg of bovine IgG or fibrinogen and incubated at 37°C overnight. Then, samples were separated by electrophoresis in a 10% SDS-PAGE. Bovine IgG or fibrinogen without secreted proteins were used as negative controls.

Proteolytic activity quantification: To know if the differences in proteolytic activity observed in zymograms were significant, we used azocasein as substrate (Sigma-Aldrich, St. Louis, MO, US) as described (Silva *et al*., 2015) and modified it with a 1% azocasein solution and 100 µg of sample in a final volume of 1 mL with PBS. As positive control, 10µg proteinase K (Sigma-Aldrich) was used. To quantify protease activity, one unit of activity was defined as an increase of 0.01 in absorbance units at 405 nm; results are expressed as specific activity units.

Separation of proteases and immune recognition: To separate proteolytic activity at the base of their molecular weight, we separated proteases using centrifugal filter devices (Centriprep®, Millipore, Burlington, MA, US) of 50 kDa or 100 kDa of the nominal molecular weight limit, according to the manufacturer. The samples recovered from Centriprep® devices were separated by electrophoresis, transferred to a nitrocellulose membrane, and incubated with serum against the *Actinobacillus pleuropneumoniae*-secreted metalloprotease (Negrete-Abascal *et al*., 1998) or the serum from sheep infected with *A. seminis* (Montes-García *et al*., 2018).

Mass spectrometry analysis and phylogenetic analysis of the protease sequence: A 50-kDa protein band was cut from the gel and analyzed for peptide mass fingerprints as previously described (Figueroa-Valenzuela *et al*., 2022). The tryptic peptide data were analyzed using Proteome Discoverer 1.4.1.14 (Thermo-Fisher Co). The protease's amino acid sequence was analyzed by BLASTP (https://blast.ncbi.nlm.nih.gov) to know its phylogenetic relationship with other proteases. One hundred seventy-two proteases' sequences were recovered from the GenBank and were aligned using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree was obtained with the online version of Itol (https://itol.embl.de). Identity groups were indicated with color circles.

RESULTS

Detection of secreted proteases by *A. seminis*: *Actinobacillus seminis* secreted proteases of 100 kDa or higher molecular weight to the culture supernatant at 37°C in the presence of calcium or at 39 to 40°C in its absence, after 24 h of incubation (Fig. 1A), as can be observed on zymograms with porcine gelatin.

Protease activity assay: The proteolytic activities were active mainly at a pH of 6 to 7. At a pH of 8, the proteolytic activity diminished by approximately 50%, and no activity was detected at a pH lower than 4 or higher than 9 (data not shown). Proteases were active in the range of temperatures from 4 to 60°C. Higher temperatures inactivated them (Fig. 1B). Proteolytic activity was not affected by 10 mM pHMB, 5 mM PMSF, or 10 mM EDTA; EDTA at 20 mM diminished the activity, which was totally inhibited with 30 mM or higher concentrations (Fig. 1C). Bovine IgG and fibrinogen were both completely degraded after 24 h of incubation with a sample of secreted proteins (Fig. 2).

Quantification of proteolytic activity: The quantification of proteolytic activity using azocasein indicates that the growth of *A. seminis* in iron restriction conditions or in the

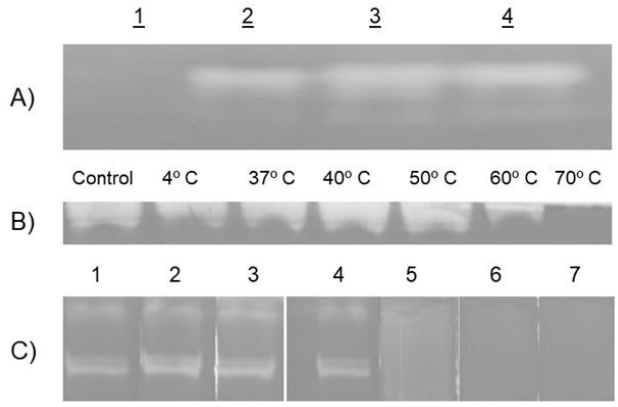


Fig. 1: Zymogram with porcine gelatin. (A) Proteolytic activity of secreted proteins of *A. seminis* grown at 37°C (1 and 2) or 39°C (3 and 4) without (1 and 3) or with 10 mM $CaCL_2$ (2 and 4). (B) *A. seminis* secreted proteins (10 µg/lane) incubated at different temperatures. (C) in the presence of protease inhibitors: 10 mM pHMB (lane 2), 5 mM PMSF (3) or 10, 20, 30, or 40 mM EDTA (lanes 4 to 7). Lane 1, control without any addition.

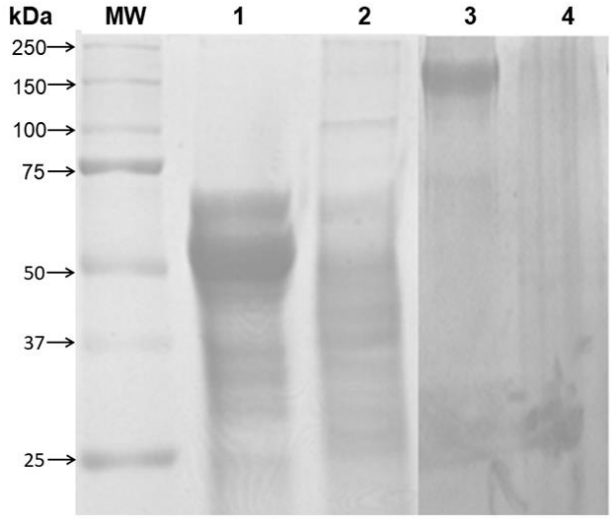


Fig. 2: Bovine fibrinogen and IgG degradation; 10 µg of bovine fibrinogen or IgG, incubated overnight at 37°C with 10 µg of *A. seminis* secreted proteins (lanes 2 and 4, respectively). Lanes 1 and 3 show the bovine fibrinogen and IgG, respectively, incubated overnight without *A. seminis*-secreted proteins, as negative controls

presence of calcium, at 37°C, increased the proteolytic activity in 10% (Fig. 3). However, a diminishing proteolytic activity, at a similar percent, was observed at 39°C.

Electrophoresis separation and immune recognition: When proteases with proteolytic activities were separated by molecular weight, high molecular weight proteolytic activities were missing, but proteolytic activity was mainly observed at around 100, 75, and 50 kDa (Fig. 4), suggesting that higher molecular weight proteolytic activities are aggregates of this last band. Bands of approximately 50 kDa and higher were immune recognized by a polyclonal serum against a secreted metalloprotease from *A. pleuropneumoniae* and by a pool of serum from sheep infected with *A. seminis.*

Protein identity: The mass spectrometry analysis indicates that this protein presented identity with a carboxy-terminal protease from *A. seminis* (access number AOA263HFT9) (Fig. 5). However, it also presented identity with proteases

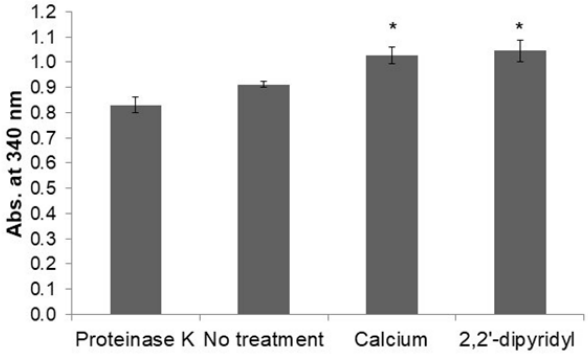


Fig. 3: *A. seminis* proteolytic activity on azocasein at 37°C or 39°C. Proteinase K was used as enzymatic degradation positive control. *Significant difference ($P < 0.05$) compared with the control.

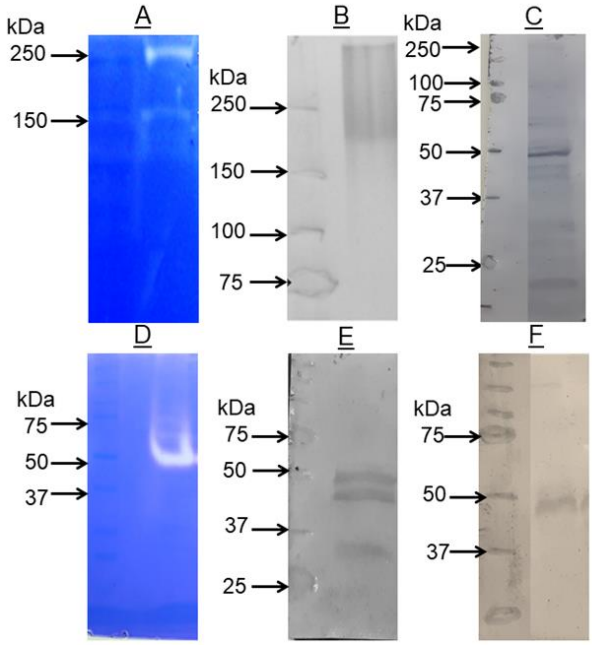


Fig. 4: *A. seminis* proteolytic activity (A and D) and immune recognition (B, C, E, F), of samples previous (A, B, C) and after (D, E, F) separation by Centriprep® devices, using a rabbit polyclonal serum against a secreted protease from *A. pleuropneumoniae* (B and E), or serum of sheep with epididymitis (C and F).

SUU34672.1Carboxy-terminal protease [Actinobacillus seminis]
MKFKLKGYLTALLLGVSFVSSNTALSSEPKIKPADIVLPQPTEEN
MLTKRLTTRLTQSHYRKFQLDEEFSEKIFDRYLKNLDFSHNTFY
QSDVDQMRSKYAKKLGEQLNQGILTAAFEMYDLMMKRRYERY
KYALSLLDTEPNLKENDSIEIDREKAAWPKNEVEANELWAQRVK
NDVINLKLKDKKWSEIKTKLTKRYNLAIRRLTQTKADDIVQVYLN
AFTREIDPHTSYLARTAKSSFNESMNLSLEGIGATLQSEDDETIIK
SLVPGAPADRSKKIKAGK**IIGVGQATGEIEDVVGWR**LDDIVDKIK
GKKGTKVRLEIEPEKGGK**SYIILVRDKVRLED**QAAKLTVEKVNG
ENVAVIKIPSFYLGLTEDVKKLLEEISKKASSVIIDLRENGGGALTE
AVGLSGLFISDGPVVQVRDAYQRIRIHDPDSAQVYAGPLLVMID
RFSASASEIFAAAMQDYNR**AIIVGQNTFGK**TVQQNRSLNFTFDL
NQTPLGNIQYTIQKFYR**INGGSTQLKG**VAPDIKPEVIDANENGE
DKEDNALPWDK**IPPASYSEVGNARE**AVAFLIEKHNEKDPEFIAL
NEDLRIREDRRRARKYLSLNFATRKAENDQDEARYLKDRFKRE
GKKPLKNIDDLPKDYEAPDFFLKEVEK**IVVDYVLFLTKKENNPK**

Fig. 5: Amino acid sequence of the *A. seminis* secreted protease (AOA263HFT9). Peptides identified by mass spectrometry are shown in bold

DegP (access number A0A263HCG5), SobH (access number A0A263HAZ2), and Lon (access number A0A263HCR7), all from the same microorganism. The *A. seminis* protease with access number AOA263HFT9 is

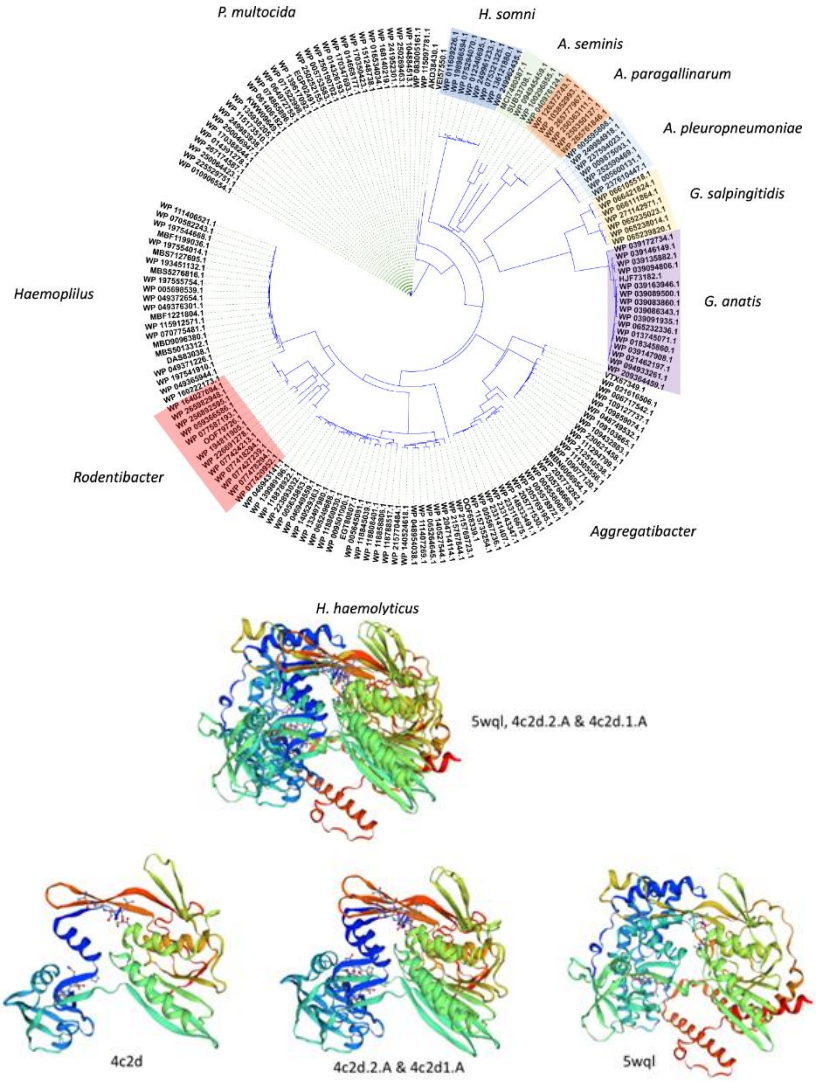


Fig. 6: Phylogenetic analysis of the *A. seminis* secreted protease. Amino acid sequence of the *A. seminis* secreted protease presented 100% identity with SUU34672.1 and WP_094945459.1proteins from the GenBank. The phylogenetic relationship of the *A. seminis* secreted protease with other proteases from *Pasteurellaceae* members: *Pasteurella multocida, Haemophillus influenzae, Histophilus somnii, H. haemoliticus, Rodentibacter, Agregatibacter actinomicetencomitans, Gallibacterium anatis, G. salpingitidis Avibacterium paragallinarum, and Actinobacillus pleuropneumoniae* was obtained using GenBank access codes. The *A. seminis* secreted protease clade includes *P. mairii, P. aerogenes, Necropsobacter massiliensis* and *Cavibacterium pharyngcola*; access numbers of these microorganisms are WP_094945459, MCI7480587, SUB33708, WP_040976124, WP_100296855.

Supplemental Fig. X: *A. seminis* protease (WP_094945459.1) structural homology using PDB. Identity of 5wql.2.B, a PDZ protease with WP_094945459.1, was of 50.89% with QMEAN global of 0.79 ± 0.05; identity of 4c2d and 4c2e with WP_094945459.1 was of 36.04% with QMEAN global of 0.65 ± 0.05. Overlap of 4c2d and 4c2e 3D structures and 5wql.2.B, 4c2d, and 4c2e, showing the multidomain spatial distribution corresponding to alignment of Supplemental figure Z.

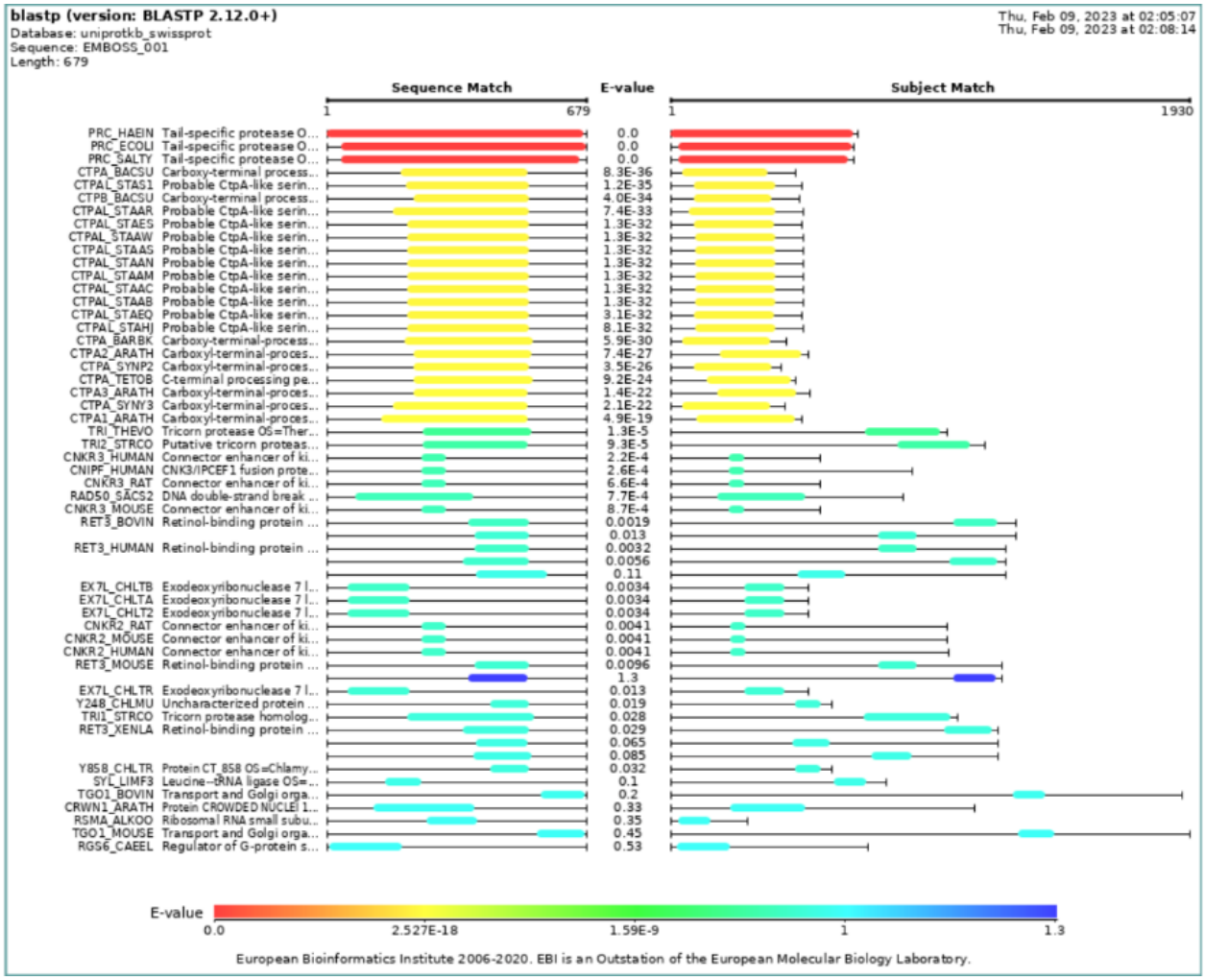
homologous to WP_094945459.1 sequence of the GenBank. For this reason, this last sequence was used for the analysis. This sequence presented structural identity with protein structural models 4C2D (CBP protease) and 5WQL (AAA+ tail-protease) in the Protein Data Bank (PBD) (Supplemental Fig. X) reported as monomers or dimers subunits, indicating polymerizing properties. Comparison among *A. seminis* protease and *B. subtilis* 4C2D_1_ChainsABC presented a 136 score, with 44% coverage, 33% identity, and E value of 6e-39; comparison with *E. coli* 5WQL_2_ChainsC exhibited a 676 score with 92% coverage, 54.71% identity, and E value of 0.0. Both proteases, 4C2D and 5WQL, present a common amino acid sequence located in the central domain (Supplemental Fig. Y). This domain is ubicated among amino acids T238 al Q486 of *A. seminis* protease (Supplemental Fig. Z). A search in the BLASTP database, identified 171 sequences related to the *A. seminis* protease. Sequences were from: *Haemophillus influenzae*, *Histophilus somni*, *H. haemolyticus*, *Rodentibacter*, *Aggregatibacter actinomycetemcomitans*, *Gallibacterium anatis*, *G. salpingitidis*, *Avibacterium paragallinarum*, *A. pleuropneumoniae*, *Pasteurella multocida*, *P. mairii*, *P. aerogenes*, *Necropsobacter massiliensis*, and *Cavibacterium pharyngcola*. Sequences' phylogenetic association indicates 4 clades with 11 different subclades: clade 1.- *P. multocida*; clade 2.- *H. somni*, *A. seminis*, *A. paragallinarum*, *A. pleuropneumoniae*, *G. salpingitidis* and *G. anatis*; clade 3.- *Agregatibacter sp* and clade 4.- *H. haemolyticus*, *Rodentibacter* sp., and *Haemophilus* sp. (Fig. 6).

DISCUSSION

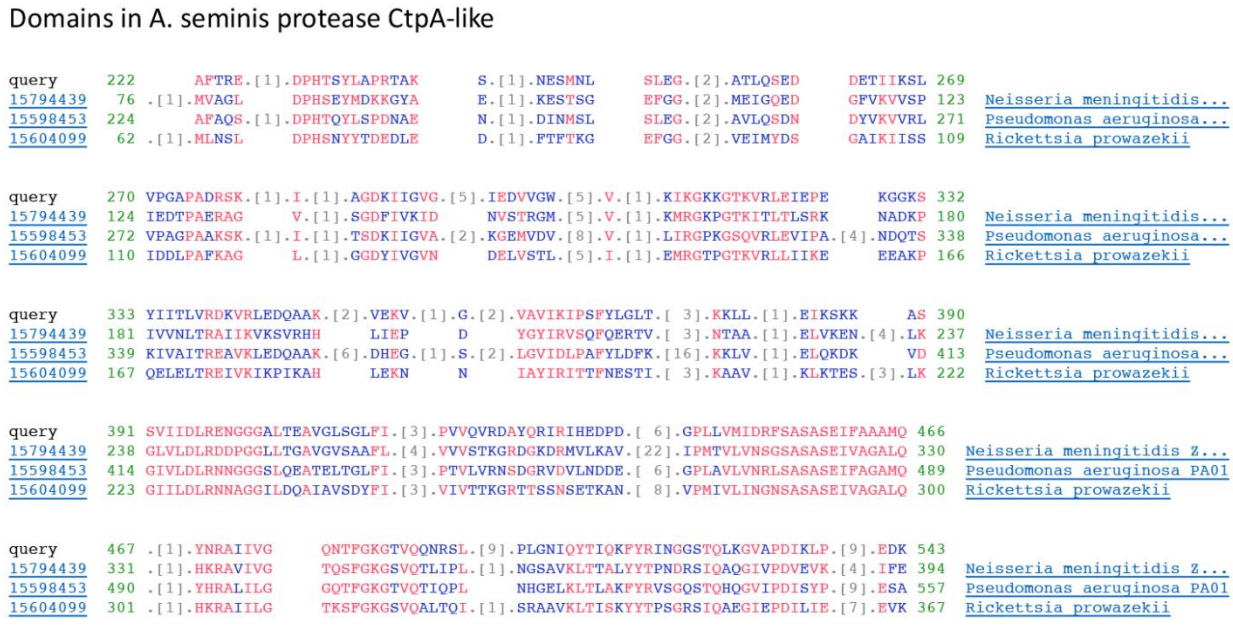
Coagulation of blood is a process that prevents the loss of body fluids after an injury. During this event, soluble fibrinogen is transformed into insoluble fibrin (Dubin *et al*., 2013). Coagulation is not only a physiological process; it is also an important part of the innate immune response that allows higher organisms to contain and kill invading microorganisms. However, diverse microbial proteases can degrade fibrinogen molecules and enable pathogens to escape from these host immune responses. Also, other microorganisms can degrade host fibrinogen and cover themselves with it, creating a pseudo capsule and, thereby, being unchecked by the host's defensive mechanisms (Guggenberger *et al*., 2012).

A. seminis ram's infection can be subacute, acute, or chronic and lesions can be purulent or necrotizing with fibrinous inflammation of the tunica vaginalis (Al-Katib and Dennis, 2009; Moustacas *et al*., 2014).

We describe the expression of proteases secreted by *A. seminis* that can degrade bovine IgG and fibrinogen. This expression is controlled by temperature, the presence of calcium and iron concentration. Fibrinogen degradation by *A. seminis* proteases could contribute to the evasion of the



Supplemental Fig. Y: *A. seminis* protease (WP_094945459.1) analysis with MEROPS. Analysis of protein families with GENE3D, PANTHER, PFAM, PIRSF, PRINTS, PRODOM, PROFILE, PROSITE, SMART, SSF, TIGERFAMs and Unclassified shows possible homologues, in higher identity with Tail-specific proteases and next with carboxy terminal proteases (CTP). Common identity to both kinds of proteases is located among amino acids T238 to Q486 of WP_094945459.1 protease.



Supplemental Fig. Z: Search of domains in WP_094945459.1 sequence and its comparison with three multidomain PDZ type proteins. Alignment showing homologous domains present in CtpA proteases of the super family c134043 (COG0793, in https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi) present in pathogenic bacteria. Numbers in green indicate the position of amino acids containing the analyzed domains of three Gram-negative bacteria expressing CTP proteases.

host clearance mechanisms, inducing the observed lesions; these processes could also be promoted and enhanced by IgG degradation.

A. seminis proteases were active in a pH range of 6 to 7, and the proteolytic activity diminished considerably at a pH lower than 5 or higher than 8. Proteases secreted by *A. pleuropneumoniae and Actinobacillus suis*, which are swine pathogens and members of the *Pasteurellaceae* family, like *A. seminis*, have proteolytic activity at a wide range of pH, 4 to 8 and 5 to 10, respectively (Negrete-Abascal *et al*., 1998, 2004), suggesting a complex structure and high stability even with drastic pH changes. Previous reports on *A. pleuropneumoniae*-secreted proteases have demonstrated their activity in the presence of 0.25 M urea or after heating to 90°C for 5 min (Negrete-Abascal *et al*., 1998). *A. seminis* proteases diminish their proteolytic activity in the presence of 0.25 M urea (data not shown) and this activity was not observed at temperatures higher than 60ºC.

The limited range of pH and temperature in which *A. seminis* proteases present activity suggests specific sites or tissues in which they could be acting. It has been described that the ductus epididymis has a pH of 7.2 and the region of sperm storage at the cauda has a pH of 6 (Rodriguez-Martinez *et al*., 1990). *A. seminis* secreted proteases were observed in zymograms at 40°C or 37°C in the presence of calcium. The normal temperature of goats and sheep is 38.5 to 40°C and the preputial temperature in bulls is considered to be 37.81°C ± 1.76, with an average pH of 8.45 (Koziol *et al*., 2017). This information supports why these proteases do not present proteolytic activity in cultures incubated at 37°C without calcium, and probably explains why they activate at higher temperatures.

The proteolytic activity of proteases secreted in *A. seminis* cultures is favored by temperature, the presence of calcium, and iron availability. The proteolytic activity *A. seminis* expression at 40ºC could be the response of this microorganism to host defensive mechanisms through immunoglobulins when this microorganism is invading its host. Calcium could be a signal that indicates to this microorganism its ubication and protease expression could be needed by *A. seminis* for tissues colonization, evasion of the host defenses or to obtain nutriments when it is colonizing the epididymis. Calcium is present in the epididymis because it participates in spermatozoids maturation and activation of their motility. On the other hand, iron-restricted conditions have been described as a "nutritional immunity" that a host can use as a growth-restricting strategy. However, pathogens can take advantage of it by promoting the expression of different bacterial proteins to overcome this restriction. *Bacillus anthracis* over-expresses InhA1, a secreted metalloprotease that mediates the proteolysis of human hemoglobin to release iron and amino acids that enhance its growth (Terwilliger *et al*., 2015). *Aeromonas hydrophila* presented an increase in the expression of different virulence factors such as protease activity, hemolytic activity, lipase activity, and swimming ability when it is grown in iron-restricted conditions (Teng *et al*., 2018).

The separation of proteases with proteolytic activities in 75 kDa and 50 kDa by Centriprep® devices suggests that they can aggregate and give rise to proteolytic activities of higher molecular weights similarly to secreted proteases from other *Pasteurellaceae* members (Negrete-Abascal *et al*., 1998, 2004). The aggregation capability of the *A. seminis* protease to form high molecular oligomers could be due to the presence of other molecules, such as LPS. Association of LPS with a *Mannheimia hemolytica* leukotoxin allows this protein to associate with the membrane and form high molecular weight masses oligomers (Li and Chickenbeard, 1999). The presence of LPS could also explain how this protein resists denaturalization by temperature or urea. If this is the case in the *A. seminis* protease, it could be possible that its separation by Centriprep® devices removes LPS because the *A. seminis* protease is inactivated by temperature or urea. The possibility of the *A. seminis* protease to form higher molecular masses oligomers is supported by the immune recognition of bands of 50, 75 kDa, or higher molecular weight with polyclonal serum against the *A. pleuropneumoniae* secreted metalloprotease.

The identity of this *A. seminis* protease as carboxy-terminal protease (CTP) is not conclusive, due to the sharing homology with other proteases and that those are mainly serine proteases; the *A. seminis* protease identified here was identified as a metalloprotease. This conclusion will need more work; however, CTPs have been implicated in diverse processes such as regulation, stress, and virulence (Sommerfield and Darwin, 2022).

Proteases identified as related to the *A. seminis* protease were evaluated by sequence similarity according to the protease's characteristics indicated in the MEROPS database that considers structural characteristics GENE3D, PANTHER, PFAM, PIRSF, PRINTS, PRODOM, PROFILE, PROSITE, SMART, SSF, TIGERFAMs, and Unclassified. Higher identity relates this *A. seminis* protease with AAA+ type proteases and next with carboxypeptidases. Structural search in the PDB indicates identity values of 50% with QMEAN Global of 0.79 ± 0.05 and 0.65 ± 0.05 with PDZ-protease, and Prc-protease, respectively, suggesting that the *A. seminis* protease is a PDZ protease or multidomain protein (Supplemental Fig. Z). These proteases form oligomers to process efficiently their substrates and can be compartmentalized in any of the cellular membranes. They seem to contain AAA+ domains necessary to use ATP to act on their substrates. Ex situ activity (on zimograms) suggests a dual effect; to function as an ATP-dependent protease in cells, processing the proteins needed for cell function and as an ATP-independent protease in direct contact with a partially degraded substrate (gelatin) with an unspecific effect.

The expression of proteases secreted by *A. seminis* that can degrade bovine IgG and fibrinogen is controlled by temperature, the presence of calcium, and the iron concentration. The latter could make these proteases a relevant virulence factor that can act in different adverse situations in response to the host immune system and explain part of the *A. seminis* pathogenesis.

Funding: This study was funded by a grant from UNAM, DGAPA-PAPIIT IN204122.

Authors contribution: CVC and ENA designed the experiments, AHDCM, GARPP, and FMG performed the experiments. AHDCM, ENA, and CVC wrote the draft of the article. AHDCM, ENA, FMG, and CVC revised this last version of the manuscript.

Availability of data and materials: In this study, all data generated or analyzed are included in the article. Code Availability. Not applicable**.**

Declarations: Conflict of interest. The authors declare that there is no conflict of interest.

REFERENCES

Al-Katib WA and Dennis SM, 2009. Ovine genital actinobacillosis: a review. NZ Vet J 57:352–8.

Dubin G, Koziel J, Pyrc K, *et al*., 2013. Bacterial proteases in disease - role in intracellular survival, evasion of coagulation/ fibrinolysis innate defenses, toxicoses and viral infections. Curr Pharm Des 19:1090-113. doi: 10.2174/1381612811319060011.

Ferreira Silva FM, Martins Mota C, dos Santos Miranda V, *et al*., 2015. Biological and enzymatic characterization of proteases from crude venom of the ant *Odontomachus bauri*. Toxins (Basel) 7:5114-28. doi: 10.3390/toxins7124869.

Figueroa-Valenzuela C, Montes-García JF, Vazquez-Cruz C, *et al*., 2022. *Mannheimia haemolytica* OmpH binds fibrinogen and fibronectin and participates in biofilm formation. Microb Pathog 172:105788. doi: 10.1016/j.micpath.2022.105788

García JFM, Rojas L, Zenteno E, *et al*., 2020. Characterization of *Actinobacillus seminis* biofilm formation. Antonie Van Leeuwenhoek. 113:1371-83. doi: 10.1007/s10482-020-01447-w.

Guggenberger C, Wolz C, Morrissey JA, *et al*., 2012. Two distinct coagulase-dependent barriers protect *Staphylococcus aureus* from neutrophils in a three-dimensional in vitro infection model. PLoS Pathog 8(1):e1002434. doi: 10.1371/journal.ppat.1002434.

Healey MC, Hwang HH, Elsner YY, *et al*., 1991. A model for demonstrating the adhesion of *Actinobacillus seminis* to epithelial cells. Can J Vet Res 55:121–7.

Koziol JH, Fraser NS, Passler T, *et al*., 2017. Initial steps in defining the environment of the prepuce of the bull by measuring pH and temperature. Aust Vet J 95:480-2. doi: 10.1111/avj.12650.

Li J and Clinkenbeard KD, 1999. Lipopolysaccharide complexes with *Pasteurella haemolytica* leukotoxin. Infect Immun 67: 2920–7.

Montes-García JF, Chincoya Martinez DA, Vaca Pacheco S, *et al*., 2018. Identification of two adhesins of *Actinobacillus seminis*. Small Rum Res 167:100–3. https://doi.org/10.1016/j.smallrumres.2018.08.013

Montes-García JF, Delgado-Tapia WA, Vazquez-Cruz C, *et al*., 2019. *Actinobacillus seminis* GroEL-homologous protein agglutinates sheep erythrocytes. Antonie Van Leeuwenhoek 112:1655–62. https://doi.org/10.1007/s10482-019-01292-6

Moustacas VS, Silva TM, Costa LF, *et al*., 2014. Clinical and pathological changes in rams experimentally infected with Actinobacillus seminis and Histophilus somni. Sci World J 2014:241452. https://doi.org/10.1155/2014/241452

Negrete-Abascal E, Tenorio RV, Guerrero AL, *et al*., 1998. Purification of a protease from *Actinobacillus pleuropneumoniae* serotype 1, an antigen common to all the serotypes. Ca J Vet Res 62:183-90.

Negrete-Abascal E, Vaca Pacheco S, Paniagua GL, *et al*., 2004. Metalloproteases secreted by *Actinobacillus suis*. Curr Microbiol 49:55-8. doi: 10.1007/s00284-004-4279-6.

Núñez-del Arco A, Salas-Téllez E, de la Garza M, *et al*., 2006. Identification of an immunogenic protein of *Actinobacillus seminis* that is present in microvesicles. Can J Vet Res 70:43-9.

Potempa J and Pike RN, 2009. Corruption of innate immunity by bacterial proteases. J Innate Immun 1:70–87. DOI: 10.1159/000181144

Rodriguez-Martinez H, Ekstedt E and Einarsson S, 1990. Acidification of epididymal fluid in the boar. Int J Androl 13:238-43.

Santos FA, Figueiredo da Costa D, Ferreira da Silva A, *et al*., 2019. Microbiological, molecular, and histopathological findings in goats experimentally infected with *Actinobacillus seminis*. Microb Pathog 133:103555. doi: 10.1016/j.micpath.2019.103555.

Schaller A, Kuhnert P, de la Puente-Redondo VA, *et al*., 2000. Apx toxins in Pasteurellaceae species from animals. Vet Microbiol 74:365-76.

Sommerfield AG and Darwin AJ, 2022. Bacterial carboxyl-terminal processing proteases play critical roles in the cell envelope and beyond. J Bacteriol. 204:e0062821. doi: 10.1128/jb.00628-21.

Teng T, Xi B, Chen K, *et al*., 2018. Comparative transcriptomic and proteomic analyses reveal upregulated expression of virulence and iron transport factors of *Aeromonas hydrophila* under iron limitation. BMC Microbiol. 4;18:52.

Terwilliger A, Swick MC, Pflughoeft KJ, *et al*., 2015. *Bacillus anthracis* overcomes an amino acid auxotrophy by cleaving host serum proteins. J Bacteriol 19:2400-11. doi: 10.1128/JB.00073-15.