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Review

Actinobacillus seminis virulence factors: Clues to understand its pathogenicity and develop control strategies

G. A. Ramírez-Paz y Puente[1], C. Vázquez-Cruz[2], P. Sánchez-Alonso[2], V. Rosales-Islas[1] and E. Negrete-Abascal[1,*]

[1]Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (UNAM), Av. de los Barrios 1, Los Reyes Iztacala, C.P. 54090 Tlalnepantla de Baz, Estado de México, Mexico;
[2]Centro de Investigaciones en Ciencias Microbiológicas, Benemérita Universidad Autónoma de Puebla, Edif. DCyTIC - 1. Calle SIU esq. Calle Biblioteca Blvd. Valsequillo, Puerta 3, Ciudad Universitaria Col. Jardines de San Manuel, C.P. 72570 Puebla, Mexico.

ABSTRACT

Actinobacillus seminis is a microorganism that infects the reproductive tracts of different ruminants, leading to economic losses in livestock due to low fertility, infertility, or abortions in female-infected animals. The lack of secure diagnostic tools, pathognomonic disease characteristics, and vaccines makes it challenging to control those infections. Knowledge of pathogenicity mechanisms and virulence factors could permit us to count on valuable tools to control diseases caused by this bacterium.

KEYWORDS: *Actinobacillus seminis*, virulence factors, pathogenicity, identification test, immune response.

1. Introduction

Actinobacillus seminis was reported as a new species for the first time in 1960 in Australia after confirming that this microorganism, isolated from the seminal liquid of goats with infectious epididymitis, was the causal agent of this disease in ruminants non-infected with *Brucella ovis*; *B. ovis* was considered in this epoque as the primary pathogen associated to infectious epididymitis [1].

*Corresponding author: negretee@yahoo.com

Since then, *A. seminis* isolates have been reported from goat-producing areas in all the continents: United States of America (1964), South Africa (1977), New Zealand (1977), Hungary (1987), Argentine (1990), United Kingdom (1991), Turkey (1991), Kenya (1996), Mexico (1999), Spain (2000) and Brazil (2000) [2, 3].

The presence of *A. seminis* in rams around the world causes economic problems due to infertility problems and abortions. Unfortunately, there is a lack of efficient tools to identify and diagnose the infections caused by this microorganism, and symptoms related to the reproductive tract could be absent. Actinobacillosis can develop as an asymptomatic disease since the beginning of the infection; epididymitis can be asymptomatic; therefore, the disease caused by this microorganism is often not diagnosed. However, diseases caused by *B. ovis* are diagnosed because it produces signs when infecting ruminants. Most of the time, when symptoms are evident (inflammation and fever in genital zones), the disease would have attained a chronic status. Consequently, ovine actinobacillosis is a critical problem for ovine producers due to the difficulty in early detection.

The present manuscript considers the biological characteristics of *A. seminis*, the pathology caused by this microorganism, and the virulence factors described nowadays. It also considers the immune

response against *A. seminis* and proposes a putative evasion mechanism based on bibliographic information on other pathogens causing epididymitis in their hosts.

2. Biochemical characteristics and growth conditions of *A. seminis*

A. seminis is a pleomorphic coccobacillus of 1 x 1 - 4 µm, Gram-negative, non-motile, non-sporulated member of the *Pasteurellaceae* family, and part of the preputial microbiome in male and vaginal lips and mammary gland of different female ruminants, including ovines, goats, and bovines.

The biochemical characteristics of *A. seminis* include catalase positive and oxidase negative reactions, nitrate and indole negatives, and weak glucose fermentation reactions. There is the absence of spores, capsules, or pili as evidenced by electron microscopy observations [4]. *A. seminis* has been reported as sensitive to penicillin, streptomycin, chloramphenicol, tetracyclines, erythromycin, sulfonamides, and novobiocin, but resistant to bacitracin and partially resistant to neomycin [1, 5]. Currently, susceptibility or resistance patterns remain unknown.

The growth of this microorganism is optimal in a 10% CO_2 atmosphere, at 37 °C temperature, in Brain heart infusion (BHI) or Tryptic soy broth (TSB) media; there is no growth on MacConkey agar, but its growth is improved by the addition of bovine serum or blood to the culture medium. After 24 h, tiny colonies are observed; at 48 h, colonies are 1-2 mm in diameter, convex, round with an entire margin, and greyish white [1].

Recently, Ramírez-Paz y Puente *et al*. [6] described those steroid sexual hormones (testosterone and estradiol) influence the growth of *A. seminis*. They reported that testosterone, in ovine physiological concentrations (1-5 ng/ml), duplicates the growth of *A. seminis*, whereas estradiol does not have that effect. This result suggests the presence of molecules (putative receptors) on the surface of this bacterium that could detect the presence of sexual hormones, and those molecules could induce a genetic regulation favoring the bacterial growth or the expression of virulence factors. These components are considered later in this work.

3. *A. seminis* pathology

A. seminis is a common cause of ovine epididymitis, infertility, and sterility of rams worldwide [7]. Its pathology is defined when rams get sexual maturity because as an opportunistic pathogen (commonly associated with *Brucella ovis* e *Histophilus somni*), this microorganism ascends to the tip of pennis to epididymis when luteinizing and/or follicle-stimulant hormone concentration increases [8]. Similarly, it has been described as the cause of spontaneous abortions in pregnant ewes [9]; those infections implicate economic losses to producers in countries involved in the production of those ruminants.

Epididymis inflammation is due to damage in epididymal conduits, which induces adverse effects in spermatozooids, the presence of spermatic granulomas, and, subsequently, chronic epididymitis. Exceptions have been described in pre-pubescent animals with no spermatozoid production; in those cases, a true abscess is formed. Epididymitis with no spermatic granulomas is possible in adult animals, but this is generally an early subclinical event.

Macroscopic lesions are limited to the epididymis tail independently of the bacterial causal agent. The epididymis tail can increase its size up to 10-fold; it could be more prominent by the presence of spermatic granulomas. Microscopically, spermatozoids, neutrophils, macrophages, and multinucleated giant cells can fill the conduits. The epithelium changes from simple columnar to pseudo-stratified columnar and cubic with focal hyperplasia. Lymphocytes, plasmatic cells, edema, and fibrin are present in the duct smooth muscle wall and interstice [10].

Fibrous tissue develops rapidly, beginning in granulation tissue (generated by fibroblast secreting type III collagen necessary to tissue repair) and eventually forming fibrous mature tissue. Interstitial abscesses and spermatic granulomas form after tissue death or duct rupture, and there is spermatocele formation (spermatic cyst painless and non-cancerous). Spermatocele can break in the vaginal tunic cavity and produces periorchitis grave. After some time, depending on the damage, tunics can present an increase in size with edema and fibrin deposition, inducing granular tissue formation and, finally, fibrosis [11].

4. *A. seminis* natural infection

This microorganism is transmitted mainly by venereal means. However, *A. seminis* has been isolated from rams without a history of previous mating, and transmission can happen in other ways. *A. seminis* has been isolated in 13.8% of rams without clinical lesions or seminal alterations in epididymis and testis; those animals are asymptomatic carriers [4]. The most accepted alternative transmission method is lamb infection during the release of pregnant ewes [2, 12].

Baynes and Simmons [13] reported the clinical signs of rams naturally infected with *A. seminis* for the first time. Their experiment included 27 rams between 6 and 12 months old with no previous reproduction mating. Rams were divided into three groups: Group 1: rams with previous infection and clinical signs in the genital zone; Group 2: rams with palpable epididymis damage and clinical signs; Group 3: rams without clinical signs. In this study, *A. seminis* was isolated from the epididymis, vaginal tunics, or kidney, finding minor to severe lesions depending on the rams' age; besides this, they discovered that epididymis tail presented an eminent inflammation, interstitial fibrosis, and macrophage and neutrophil grouping. From six rams selected randomly, seminal samples presented low spermatic quality and count, leading to the conclusion that the presence of *A. seminis* induces damage in the seminiferous epithelium, causing sterility in infected rams.

In 1970, Watt *et al*. [14] described that *A. seminis* naturally infected rams presented purulent polyarthritis associated with the presence of the bacteria. Furthermore, they presented severe myocarditis, and *A. seminis* was isolated from purulent abscesses. Besides that, rams with *A. seminis* positively presented posthitis in different affectation levels. In the same work, the authors reported mastitis in lactating ewes. They also suggest that the death of lambs associated with *B. ovis* or *H. ovis* was due to *A. seminis*. Based on all the evidence, it is essential to correctly identify the bacteria causing damage independently of the genre of the animal affected (male or female).

Walker and LeaMaster [15] described the isolation of *A. seminis* from ovines of 6 months to two years old. They found *A. seminis* in the presence of *Histophilus ovis* (now *Histophilus somni*) in prepuce and vaginal cavity of young ovines with a higher percent to that reported in rams or ewes. Those microorganisms could be considered a transitory component of the ovine genital microbiome; their prevalence could be associated more with the animal genre's age. An additional evaluation on the recovery of *H. ovis* and *A. seminis* from prepuce in just-born to one-year-old rams indicated the isolation of the bacteria in rams of 12 weeks of age or higher. A higher prevalence was observed at 20 weeks of age; after that, isolation of *H. ovis* or *A. seminis* diminishes constantly until one year old. The age at which those microorganisms were isolated is related to the epididymis development period; also, immaturity of tissues could be essential to epididymitis development.

5. *A. seminis* experimental infection

Al-Katib and Dennis [16] performed the first study in which they inoculated *A. seminis* through nine different ways (intra-conjunctiva, intranasal, oral, intravenous, intramuscular, intra-epididymal, deferent conduits, intra-urethral or intra-preputial) in 18 to 24 months old rams, to determine the ways of transmission of this microorganism and the affected target organs. They found that clinical systemic signs were minimal, and damage was limited to the inoculation zone and scrotum bag. Epididymitis with different severity levels was observed in all rams except those inoculated intra-conjunctival or intranasal ways. Damage is more severe in rams directly inoculated in reproductive track, particularly in those inoculated intra epididymal. In rams inoculated intra-epididymal or intra-urethral, the authors observed an ascendant urethral infection, with epididymis as the final target organ. In contrast, inoculation by deferent ducts produced a descendent urethral infection, where the target organs were accessory sexual glands (ampule and seminal vesicle). In this study, the authors showed that *A. seminis* could be transmitted in a venereal way by urethral ascending or by non-venereal way: intravenous, intra-muscular, or oral; however, the main target organ is the epididymis, where the bacterium remains and produces tissue damage.

In 2006, Acosta-Dibarrat *et al*. [17] inoculated 18 six-month-old rams through intra-urethral or intra-epididymal routes. The authors tried to test that

previous inoculation of rams with gonadotropin-releasing hormone favored bacterial pathogenicity. They found that hormone administration produced more severe testicular damage, epididymis tissue lymphocyte infiltration, spermatic granuloma formation, and damage to epididymis tissue integrity, finally presenting hyperplasia. The presence of *A. seminis* was found in ampules, deferent ducts, and seminal vesicles, considering that those sites could be reservoirs of the bacterium. However, due to the heterogenicity of the disease (tissue inflammation, epididymis epithelial rupture with lymphocytes infiltration, and spermatic granuloma formation, which were different in the rams infected), the authors concluded that their results are not conclusive, and there is a need for more studies in this topic.

In 2008, Al-Katib and Dennis [18] evaluated the sequential pathological changes in the epididymis and testis of young rams after intra-epididymal inoculation with *A. seminis*. They found that all the rams presented epididymis inflammation at 24 h post-infection (PI), granuloma formation at 72 h PI, and a gradual increase in lymphocyte epididymis infiltration. Also, they observed testis atrophy and severe inflammation in the epididymis tail, head medium, and corps of epididymis. After 24 h PI, the authors reported epididymis eosinophil infiltration and disruption of epididymis tissue wall after 72 h PI, with spermatozoid extravasation to interstitial tissue. With all those observations, the authors concluded that *A. seminis* was able to persist in young rams' genitals after an experimental inoculation. Suppurative epididymitis was observed at 24 h PI and spermatic granuloma at 72 h PI (Figure 1). Eosinophile infiltration could be an early host response to bacteria and be significant in epididymitis pathogenesis.

The same authors [18, 19] reported the susceptibility, lesions, and dissemination *via* accessory sexual organs of young rams experimentally infected with *A. seminis* through the nine different inoculation routes evaluated previously in 2005. They found that vesicular adenitis was the more frequent lesion, but ampules, deferents, urethritis, and bulbourethral lesions were also observed. Prepuce and prostate (tissue and gland, respectively) were the least

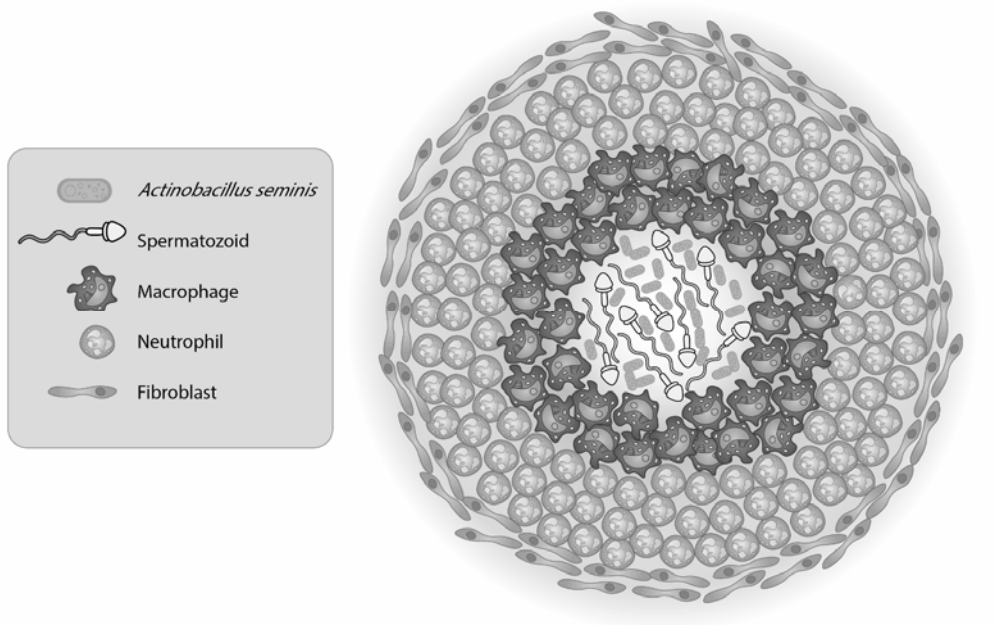

Figure 1. Spermatic granuloma. Schematic representation showing the spermatic granuloma constitution, which contains spermatozoids and bacterial cells (*A. seminis*). This representation shows the constituting granuloma cellular lines (macrophages and neutrophils) and fibroblast cells covering the outer layer (All images were prepared using Adobe Illustrator).

affected, probably by the presence of IgA, an immunoglobulin that could be important in resisting the infection by *A. seminis*. In this work, the authors confirmed that the target organ of *A. seminis* is epididymis, and accessory sexual glands act as bacterial reservoirs. Also, they concluded that *A. seminis* is closely related to ovine infertility evidenced by spermatic degradation and accessory sexual gland infections and that early immune response is due to eosinophils action, which could induce a tissue-exacerbated inflammation.

In 2014, Moustacas and collaborators [20] experimentally inoculated rams 18 to 24 months old with *A. seminis*, *H. somni*, or *B. ovis*, slaughtering them at 42 dpi. In this study, the authors describe that tissue damage in the testicle, epididymis, seminal vesicle, and other accessory sexual glands were very similar, generating testicular atrophy, damage to the seminiferous epithelium, and inflammation. The authors highlight that the histopathological differentiation between the damage caused by any of the three microorganisms would only be possible to distinguish with specific diagnostic tests for each pathogen.

Despite all the knowledge of the bacterium's etiology and pathogenesis, there are no tests or kits for rapidly detecting *A. seminis*. All this makes the identification of the epididymitis etiological agent difficult. Thus, detecting and identifying the epididymitis causal agent is essential due to the economic losses that infections cause in the ruminant industry.

6. *Actinobacillus seminis* diagnostic

Actinobacillus seminis clinical diagnosis is not enough to identify ruminants infected with this bacterium because, in most cases, animals do not present clinical signs (they are asymptomatic). More than one test (serologic, bacteriologic, or molecular) could be necessary to secure and precisely identify the causal agent. In the absence of clinical signs, it could be appropriate to conduct regular screening, especially in those animals involved in reproduction, to diminish or control the pathogen dissemination [8].

Biochemical tests cannot specifically identify the causal agents of epididymitis [7]. Besides, *A. seminis* isolation requires time, and another microorganism with faster growth in prepuce samples makes its isolation difficult. The diagnosis of epididymitis due to *A. seminis* should include clinical signs, anatomical alterations, and bacteriological evaluations of semen.

7. Serologic tests

Different serologic tests have been proposed to detect *A. seminis* as the cause of epididymitis; however, until now, there is no infallible test to diagnose the presence of *A. seminis* in symptomatic or asymptomatic animals. Next, we describe the different diagnostic tests that have been reported until now.

Rahaley [21] developed a complement fixation test using *H. somni*, *B. ovis*, or *A. seminis* total cell extract samples and sera from animals presenting epididymitis clinical signs. Under those conditions, he could not distinguish between *H. somnii* or *A. seminis* infections. The test could only give differential titles when each antigen was tested individually. However, he also found cross-reactivity among the three antigens, concluding that more specific antigens are necessary.

Ajai *et al*. [22] proposed the use of direct or indirect immune fluorescence tests for the diagnosis of ovine epididymitis due to *A. seminis*, *B. ovis*, or *Corynebacterium pseudotuberculosis*, and the use of cell lysate and goat's specific antiserum as antigens against each of those microorganisms. Their results indicate that the immune fluorescence test could identify each of the microorganisms evaluated, showing the poly morphology of *A. seminis* and the differentiation between *B. ovis* and *C. pseudotuberculosis* by stain characteristics because the last one is Gram-positive. Despite the specificity of this test, it must be performed in specialized laboratories, and a long time is necessary to develop it specifically.

Cárdenas and Maki [23] developed an ELISA test using a cell lysate as an antigen. They attempted to detect the presence of specific antibodies against *B. ovis, A. seminis,* or *A. seminis*-like in sheep with epididymitis. They found that all sera presented cross-reactivity between the three pathogens, and there was no possibility of making a difference between the samples analyzed.

With this, they conclude that the ELISA test is not specific for detecting *A. seminis* infections.

Other authors proposed using molecular tests to identify the ruminants' epididymitis causal agents due to the lack of specificity.

8. *Actinobacillus seminis* molecular identification

A secure identification of *A. seminis* has been done using PCR techniques. Appuhamy *et al*. [7], using 24 *A. seminis* isolates, proposed the identification of this microorganism by using PCR ribotype, a PCR technique based on the detection of repeats extragenic palindromic (REP) elements or PCR based on the detection of enterobacteria repetitive intergenic consensus (ERIC) sequences. PCR ribotyping produced a straightforward pattern that could confirm *A. seminis* from other related species. REP and ERIC-PCR techniques produced complex patterns, but they also showed common markers for all the isolates, indicating a genetic homogeneity among the evaluated strains. Those results could help in looking at *A. seminis* in epidemiological studies and identify it specifically.

In the same year, Appuhamy *et al*. [24] evaluated the presence of *A. seminis* in ram-preserved semen; in this assay, they used two ribosome operons, rrnA, and rrnB, present in the *A. seminis* genome, containing one and two ARNt, respectively, in the intergenic region of the ARNr 16S and 23S genes. The authors designed *A. seminis*-specific primers for the rrnB intergenic region to identify and detect *A. seminis* by PCR. Using those conditions, the authors reported a specific *A. seminis* identification with no amplification products to *H. sommi*, a phenotypically related microorganism. The authors also reported that the solution used for a long-time preserved semen inhibited PCR reactions. By this, they recommend performing the assay after primary isolation or using fresh semen before storing it if a suspect of contamination exists.

At this point, the damages that *A. seminis* could produce in rams naturally or experimentally infected and the detection tests to diagnose ovine epididymitis have been described. However, the mechanisms, molecules, or bacterial components in the developing lesions described are yet to be included. In the following paragraphs, virulence factors described until now will be considered.

9. Virulence factors

The way bacteria colonize new niches has been studied for a long time based on their behavior: as a pathogen, opportunist, or symbiotic microorganisms interacting with their host or other organisms in their environment. To get any of those relationships, microorganisms must count on an array of components or molecules that let them survive in those new environments, "fight" with other organisms present in that environment, or resist the immune defensive mechanism in a host. Different pathogens species "optimize" their genetic content using multifunctional proteins, commonly known as moonlighting proteins: proteins whose original functions have been described to include taking part in routes or metabolic processes, acting as chaperone proteins, enzymes, ion channels, taking part in secretion systems or protein synthesis, among other functions [25]. Those proteins could also act as virulence factors functioning as adhesins or toxins [25]. *A. seminis* is a bacterium that uses this moonlighting protein as a virulence factor (Figure 2).

9.1. Cellular adhesion

Healey *et al*. [26] described for the first time the ability of *A semis* to attach to bovine kidney epithelial cells. A prior incubation with a polyclonal antiserum against this bacterium inhibits this binding. However, the molecules involved in this process have not been previously identified. This manuscript suggests that *A. seminis* could infect other organs besides the epididymis or testis. The ability of this microorganism to attach to kidney epithelial cells indicates infections in the urinary tract, symptoms commonly associated with chronic diseases. Chronic diseases are frequently related to microorganisms producing biofilms; *A. seminis* possesses that capability [27]. Biofilm formation lets microorganisms resist the effect of different stresses and evade the host's immune response. Healey *et al*. [26] propose identifying and characterizing those molecules and using them as vaccinal components and control strategies.

9.2. Identification of two *Actinobacillus seminis* adhesins

In 2018 (27 years after Healey's report), Montes García *et al*. [28] described the identification and characterization of two non-fimbrial adhesions

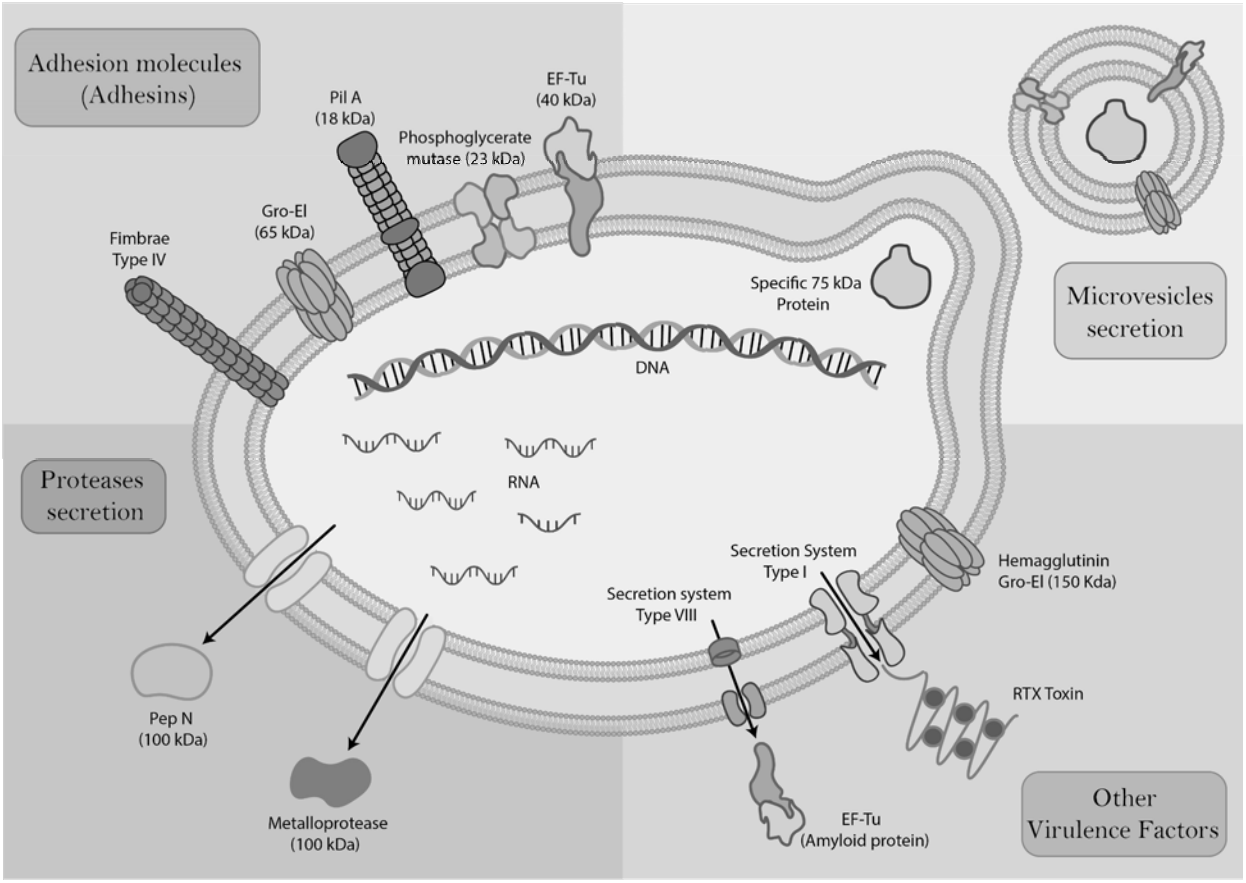

Figure 2. *A. seminis* virulence factors. Schematic representation showing the different virulence factors of *A. seminis* described in the study (some unpublished).

(two moonlighting proteins). One of these moonlighting proteins was the elongation factor Tu (EF-Tu). This cytoplasmic protein participates in protein synthesis and bacterial adhesion as a biofilm component [28].

EF-Tu protein has also been described as an adhesin in other microbial models such as *Acinetobacter baumannii* (binding to fibrinogen and plasminogen) [29, 30], *Gallibacterium anatis* (interacting with fibronectin and fibrinogen) [31], *Lactobacillus delbrueckii* (binding to mucin) [32], different species of *Mycobacterium* genus (binding to fibronectin, heparin, fibrinogen, plasminogen, and collagen (*M. tuberculosis*, *M. bovis*, *M. avium*)) [33]. In the case of *A. seminis*, this protein binds to fibrinogen and fibronectin [28], essential elements of the cell's extracellular matrix.

As EF-Tu is a cytoplasmic protein, knowledge about how it attaches to the outer membrane is needed. Different hypotheses have been proposed, including the use of secretion systems or participation of chaperone proteins; the more accepted hypothesis is through microvesicles containing this protein [33].

Another adhesin described for this model is Phosphoglycerate mutase (PGM), a cytoplasmic bacterial protein that participates in the glycolysis route. PGM has been described as an adhesin in other microbial pathogens, such as *Mycoplasma pneumoniae* [34], *Streptococcus suis* [35], and *Pasteurella multocida* [36], interacting with cell-extracellular matrix components, including fibrinogen, fibronectin, collagen, laminin, and elastin.

9.3. Fimbrial adhesins

Pili type IV are usually filament structures in the extreme of the bacterial cells of different bacterial pathogens, including *Neisseria gonorrhoeae*, *Moraxella bovis*, *Dichelobacter nodosus*, or *Pseudomonas aeruginosa* [37]. They comprise a small subunit highly conserved among different bacterial species and participate in cell adhesion and translocation of bacterial cells in epithelial surfaces through a phenomenon known as "spasmodic motility." Those pili are essential in host tissue colonization and important immunogen antigens conferring protection when used as vaccinal components [38]. PilA is a protein expressed in bacterial surfaces that participates in cell attachment. It is a good immunogen proposed as a vaccinal component against different bacterial infections. The monomeric protein is ensembled in the periplasm internal membrane after processing by a specific peptidase, PilD, and secreted through a channel formed by the secretin PilQ in an ATP-dependent process [39].

The expression of fimbrial adhesins in *A. seminis* has not been described until now, despite this microorganism possessing in its genome the genes *pil*A (codifying an approximately 18 kDa protein) and *pil*F type IV pili (codifying an approximately 21 kDa protein) [40] necessary to express it. Besides this, other genes (*pil*Q, *pil*M, or *pil*T) could participate in biosynthesis, ensemble, and secretion of the components of those fimbrial adhesins.

However, until now, we have not been able to visualize those fimbrial structures by electron microscopy in a similar manner as was described by Acosta-Dibarrat and Tortora [4] and Healey *et al*. [26]. This indicates that there are specific *A. seminis* growth conditions to their expression. The expression of bacterial fimbrial adhesins is essential for cell adhesion. However, they are also immunogenic proteins [37]. Their expression could generate an immune response by the host; for this reason, the expression of *A. seminis* alternative adhesins, such as moonlighting proteins, could evade this recognition system and let it have successful colonization.

In the study of identification and characterization of *A. seminis* adhesins by Montes-García *et al.* [28], cell adhesion assays were performed using HTB5 human bladder cancer cell line; it was described that bacterial adhesion can be inhibited until 40% by previous bacterial incubation with hyperimmune rabbit polyclonal serum performed against each protein, EF-Tu or PGM, emphasizing their importance, in epithelial cell adhesion, of those proteins. *A. seminis* interaction with this human bladder cancer cell line suggests this bacterium could have a putative zoonotic potential.

9.4. Chaperone Gro-EL as *A. seminis* adhesin

Gro-EL is a heat-shock polymeric protein expressed by prokaryotes and eukaryotes. This protein participates in different cellular aspects, such as stress, adherence, inflammatory response induction, or intracellular signaling molecules; however, its primary function in association with GroES is to generate the correct conformation of proteins that have suffered changes in their tridimensional structure [41].

In 2019, Montes García *et al*. [42] described that *A. seminis* Gro-EL homologous protein functions as an adhesin and a hemagglutinin protein interacting with sheep red blood cells. *A. seminis* Gro-EL protein could be found as a 65 kDa protein or a dimer associated with the membrane. Both protein forms conserve the ability to interact with red blood cells.

Like EF-Tu or PGM proteins, this heat shock protein can interact with fibronectin or fibrinogen. In cell adhesion assays using human bladder cancer cell lines, bacteria can attach, and previous incubation of the bacteria with a hyperimmune rabbit polyclonal serum against Gro-EL induces a 50% cell adhesion inhibition, supporting its importance in the interaction with host epithelial cells.

9.5. Host-bacteria interaction

Continuing with this topic, Ramírez-Paz y Puente *et al*. [6], working with *A. seminis* grown in the presence of testosterone or estradiol, observed that those sexual hormones induced an up-expression of EF-Tu and PGM adhesins, suggesting that those proteins could participate in adhesion to other tissues, on the basis that when *A. seminis* detects changes in the sexual hormone concentration,

this could be considered as a signal that induces an ascending signal from the tip of penis to epididymis or other tissues such as bladder epithelial cells. They also described that both proteins were immune recognized by a sheep's serum with epididymitis. They were infected with *A. seminis* but were negative to *B. ovis*. This result indicates an *in vivo* expression of those proteins and that they could be considered suitable immunogens, helpful in preparing a vaccine.

9.6. Proteases

Secreted proteases are other *A. seminis* virulence factors reported, particularly a 100 kDa secreted metalloprotease degrading sheep fibrinogen and bovine IgG, described by de la Cruz-Montoya *et al*. [43]. Fibrinogen is a central element in homeostasis and blood coagulation and takes an integral part in inflammatory response, modifying leukocyte functions, including cell adhesion, migration, and cytokine expression; fibrinogen can depolymerize in α and γ subunits, that, in coordination with thrombin, are constituents of host cellular membranes [44]. Damage to cellular membranes could be due to the secretion of 100-kDa metalloprotease by *A. seminis* that degrades fibrinogen.

Fibrinogen degradation could also affect the opsonization or phagocytosis processes by affecting complement. Fibrinogen fragments can inhibit complement components and affect innate immune response [45]. Fibrinogen fragments can also be used to "cover" the bacterial surface; in this way, *A. seminis* could cause the host's mucosal surface colonization, evade the immune response, and get nutrients.

Other molecules used to avoid bacterial infections are immunoglobulins (Igs): IgG and IgA are the primary Igs participating in this process. These Igs are components of the humoral immune response. The degradation of any or both Igs limits the host humoral response [46]. As described above, the *A. seminis*-secreted metalloprotease can degrade IgG, eliminating a primary participant in the general immune response [43]. However, as a mucosal pathogen, *A. seminis* could express other proteases that degrade the IgA molecule. *Mannheimia haemolytica* [47, 48] and *A. pleuropneumoniae*, both respiratory mucosal pathogens of ruminants or pigs, respectively, and both *Pasteurellaceae* family members, express different secreted metalloproteases degrading IgA, IgG, and other molecules [48, 49].

9.7. Microvesicles secretion

Bacterial cells communicate with host cells and other bacteria by releasing membrane vesicles known as bacterial extracellular microvesicles. Bacterial microvesicles are mediators of intracellular signaling, stress tolerance, horizontal gene transfer, immune stimulation, and pathogenicity [50]. Microvesicles of 20 to 100 nm dimensions associated with the *A. seminis* cell surface were described by Núñez del Arco *et al*. [51] in early stationary phase cultures using scanning electron and transmission microscopies. Morphologically and structurally, those *A. seminis* vesicles were similar to that described for *A. actinomycetemcomitans* [52] or *A. pleuropneumoniae* [49], other members of the same *Pasteurellaceae* family. The *A. seminis* microvesicle content analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed the presence of an immunogenic 75 kDa protein specific to this bacterial species. Unfortunately, this protein was unidentified or more characterized.

Microvesicles could contain genetic material, toxins, and proteases, among other intracellular or in-transit molecules [53]. Microvesicles released by bacterial pathogens could damage different issues or organs infected by the pathogens. Unfortunately, the participation of *A. seminis* microvesicles in pathogenesis is unknown.

9.8. RTX toxins

Proteins of the Repeats in Toxins (RTX) family are secreted to the extracellular medium and are virulence factors of essential pathogens. RTX proteins are characterized by glycine-rich nonapeptide repeats with the consensus sequence GGxGxDxU (x—any amino acid, U—large hydrophobic amino acid), representing the so-called GG-repeats [54]. The presence of a putative *A. seminis* RTX secreted protein was described by cross-reactivity using sera against *A. pleuropneumoniae* Apx toxin [55]; however, until now, this protein has not been isolated nor

characterized, but by *in silico* analysis, the presence of a putative gene encoding an RTX transporter protein D has been confirmed [40], which in coordination with an RTX transported protein B constitute the type I secretion system, a specific secretion system of this kind of proteins [54]. *A. seminis* is a non-hemolytic bacterium; for this reason, the participation of the putative RTX protein in the pathogenesis of this microorganism will not be related to red blood cell hemolysis. RTX proteins could have different biochemical activities, including hemolytic, proteolytic, adhesion, or cytotoxic [55, 56].

In contact with the target host cell, RTX proteins could insert themselves in the lipid bilayer of cell membranes, producing ionic channels that transport ions essential to each protein's biological activity and cell lysis. Those channels have a short proper lifetime and are selective in releasing cations by punctual negative charges in or close to the channel.

9.9. Amyloid-like proteins

Amyloid proteins are associated with abnormal aggregation and accumulation of neurotoxic proteins in humans and other mammals. They are associated mainly with neurodegenerative diseases such as Alzheimer's, Parkinson's, or Creutzfeldt-Jacob disease [57, 58]. In bacteria, it has been described as a similar kind of protein. Still, prokaryotes are "functional" multimeric proteins. A representative of this kind of protein, "curli," is an *E. coli* protein forming fibers that promote surface adhesion and cell aggregation and participate in biofilm formation. Amyloid aggregation, described as an assembled defect in mammals and a cause of illness, has a functional role in prokaryotes [59]. Amyloid-like proteins found in prokaryotes, described until now, participate in physiological activities in bacterial cell surfaces, constituting up to 40% of the biofilm biomass [60].

Congo red (CR) dye is a hydrophobic compound that can join to amyloid proteins or certain carbohydrates. Bundles of fiber stained with CR dye indicate the presence of amyloid fibers. Those protein aggregates are resistant to chemical, acid, or boiling denaturalization. Also, they resist denaturalization by SDS or digestion by K proteinase [61]. Amyloid proteins have been described for different prokaryotes, including *Pasteurellaceae* family members, such as *Mannheimia haemolytica* [62] or *Gallibacterium anatis* [31].

In *A. seminis*, the presence of amyloid proteins was described by Ramírez-Paz y Puente *et al*. [6]. They described the presence of proteins of 30, 39, or 48 kDa that presented identity with EF-Tu protein by spectrometry masses analysis and cross-reacted with a rabbit polyclonal hyperimmune serum against the *G. anatis* amyloid-like protein also identified as EF-Tu [31]. This protein also participates in cell adhesion, forming part of the *A. seminis* biofilm components. The authors also reported that *A. seminis* grown in the presence of estradiol 5-20 pg/ml or 4 ng/ml testosterone presented an increase in its CR dye binding, but 3 or 5 ng/ml testosterone diminished this binding; the authors concluded that steroid hormones could modify the expression of amyloid proteins or carbohydrates involved in this interaction.

10. Host immune response and its evasion by *A. seminis*

The immune system is constituted by different cellular and molecular elements whose main objective is to protect and defend the host against invaders. Coordination among those elements lets us recognize, control, and eliminate invaders' agents. Sometimes, an exacerbated and uncontrolled immune response could damage the host. The immune response is not homogeneously distributed throughout an organism; different tissues present specific actions to defend against possible pathogens. Next, we will briefly summarize cells and immune molecules that are taking part in the defense of the ram reproductive tract.

10.1. Immune response of rams' reproductive tract

2014 Acosta-Dibarrat *et al*. [63] described cells and immune molecules along the ram's reproductive tract. IgA, IgG, and IgM-producing immune cells were found in high numbers in prepuce; besides those cells, CD4, CD8, CD45R0, WC1, CD14 (macrophages), and CD1b (dendritic) cells were also present in prepuce. This is

considered the first humoral immune response controlling or limiting cell invasion of opportunists or pathogenic bacterial cells that are part of the prepuce microbiome. Notably, the presence of IgA was associated with mucosal immunity. The presence of CD4+ type T lymphocytes was related to the activation of B lymphocytes, cells responsible for the production and secretion of antibodies. In the urethra of sheep experimentally infected with *A. seminis,* a higher concentration of CD8+ T lymphocytes, cytotoxic lymphocytes that secrete papules containing pro-apoptotic enzymes (granzymes) and perforins, was described. In the urethral epithelium, the mucosa was the only tissue in which high concentrations of IgA were found.

Acosta-Dibarrat *et al.* [64] experimentally inoculated one-year-old rams with *A. seminis via* intra-epididymal or intraurethral routes; they evaluated IgM, IgA, and IgG concentrations in blood serum, smegma, and seminal plasma. They found that secreted IgA concentration did not vary during the study period (five weeks). However, through intra-epididymal, starting from the first week of post-inoculation (PI) until euthanasia, the IgG concentrations increased in blood serum, smegma, and seminal plasma of experimentally infected rams. IgG concentration in smegma diminished weekly until basal levels at five weeks PI. Immunoglobulin M concentrations showed a decrease after the first week PI in rams inoculated intra-epididymally, reaching basal levels before the second week PI; IgM concentrations also increased in blood serum of intraurethral-infected sheep until the animals were euthanized.

Immunoglobulin M is the first antibody produced during an infection. This Ig is considered a marker of acute-phase immunity. IgM is the molecule that activates the immune complement system, a complex group of proteins that helps Ig and immune cells eliminate an infectious invader. After IgM expression occurs, the expression of other specialized Igs, such as IgA, the main Ig present in mucosae or IgG as a blood serum molecule, take place; this last molecule will be responsible for host protection in future contacts with the same microorganism. By the immune response specificity and adaptability, microorganisms (pathogens or not) have developed different mechanisms to evade or resist the host, clearing immune mechanisms that permit them to colonize and, consequently, produce an illness, mechanisms fundamental to pathogen microorganisms.

10.2. Host immune system putative *A. seminis* evasion mechanisms

Bacteria have diverse strategies to evade the host immune system. Some possess protective capsules that avoid cell phagocytosis and their recognition and the effect of complement or Igs. Others express inhibitory immune response molecules; others modify their cell surface, making recognition difficult. Those strategies let bacteria elude the action of macrophages, avoiding their destruction, contributing to their persistence, and provoking an infection. Some bacteria express toxins affecting immune cells, debilitating or eliminating their functions. Bacteria can also produce biofilms, biological ultra-structures containing bacterial cells or other microorganisms, immersed in an auto-produced matrix composed of DNA, proteins, and carbohydrates that let them survive different stressing conditions and the effect of environment, antimicrobials, or immune response [65]. Those complex strategies avoid bacteria detection or elimination by host-clearing defensive mechanisms, letting the opportunity persist in the host as a chronic infection. Three putative *A. seminis* mechanisms that persist in its host will be mentioned ahead.

10.3. Fibrinogen degradation

Fibrinogen is a central element of homeostasis and coagulation, essential in inflammatory response [66]. Fibrinogen degradation affects opsonization and phagocytosis induced by complement components because fibrinogen fragments inhibit them. A fibrinogen deficiency increases the possibility of severe thrombosis or mortal hemorrhage [45]. Fibrinogen fragments can be attached to bacterial surfaces, allowing bacteria to adhere to mucosal surfaces, evade immune response, or get nutriments [46].

In 2016, Ko and Flick [67] described that some bacteria bind to fibrinogen fragments on their surface, evading phagocytosis. Due to that, those fragments are recognized as own by the immune system. *Staphylococcus aureus*, a Gram-positive

bacterium that causes different respiratory or skin infections, produces fibrinogen and fibronectin-binding proteins; those proteins favor the attaching of *S. aureus* to cytoskeleton host cells or fragments released to extracellular space. Fibrinogen binding proteins can form fibrine oligomers, covering the bacterium and favoring and prolonging an infection.

10.4. Bacteriospermia

As previously commented, diverse bacteria cover their surface with cellular components, including fibrinogen, collagen, and elastin, generating a structure like a capsule to evade the host immune system. A similar mechanism was described for *E. coli* and other microorganisms that attach to spermatozoids through specific adhesins joining spermatozoid heads or tails, forming a similar structure to spermatic granuloma, as an evasion of the immune response. *Salmonella enterica* spp. Typhimurium induces lysis of spermatozoids; next, it uses those fragments to cover its cellular membrane or flagellum to evade the immune response of testis or epididymis [68]. *A. seminis* infection can induce epididymitis and infertility in ruminants; also, spermatic degradation and infection of sexual accessory glandules have been observed. This microorganism has also been detected in the semen of infected males. However, it is unknown if *A. seminis* can interact directly with spermatic cells or their components, as they have been described as pathogens affecting humans and other hosts (Figures 3 and 4).

10.5. Biofilm formation

Microorganisms can live in isolated form (planktonic) or associated with other organisms (sessile), conforming to a biological structure called biofilm. In this structure, microorganisms behave like that presented by multicellular organisms [69]. This growth requires a communication mechanism to coordinate the behavior of the inhabitants. This communication way is known as *Quorum Sensing* (QS). QS is a communication method that coordinates the behavior of the biofilm members through populational density. Biofilm members produce, secrete, and detect small molecules called autoinducers (AI). When population density is high and AI gets a density threshold, those molecules control, in a coordinated way, the expression of their genetic content. In this way, genetic regulation expression lets biofilm members act as multicellular organisms [69].

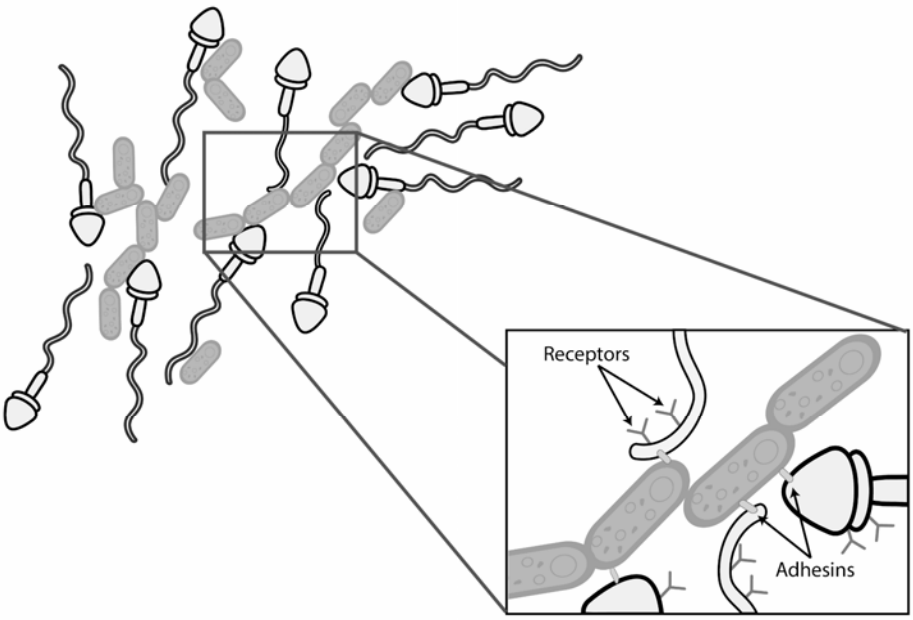

Figure 3. Spermatic agglutination. Putative interaction of *A. seminis* with ram spermatozoids through adhesion molecules.

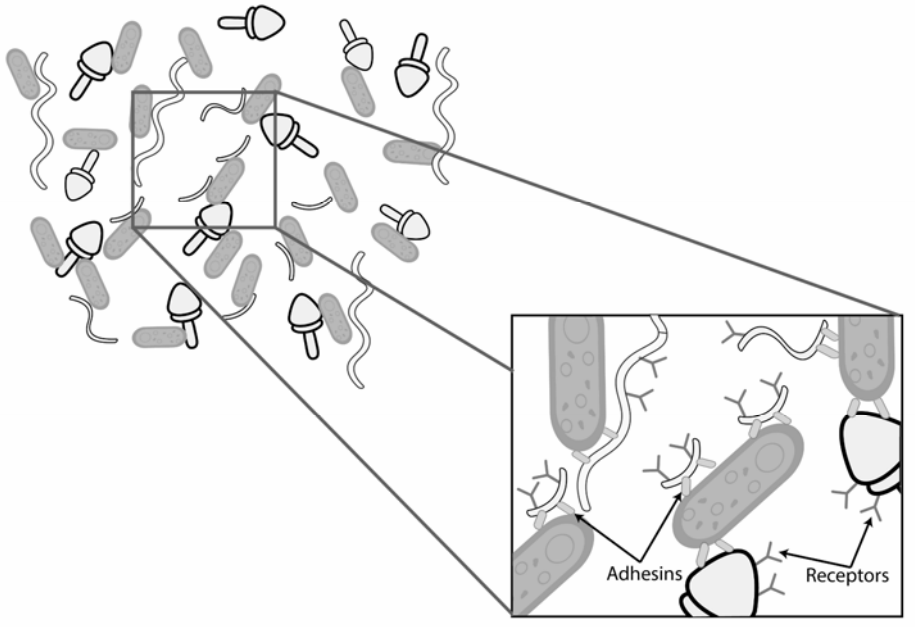

Figure 4. Spermatic lysis. Schematic representation of the putative spermatozoid lysis by *A. seminis*. Generated fragments could cover the bacterial surface and evade the immune response.

Biofilms resist different stress forms (osmotic, saline, pH, temperature, hydric) and evade immune response and the effect of antimicrobials. QS can also regulate bacterial growth and the expression of different virulence factors, favoring the microorganism's pathogenic potential [27].

In 2020, García *et al*. [27] described *A. seminis* biofilm formation and characterization. They showed that *A. seminis* biofilm was constituted mainly by proteins, followed by carbohydrates and, in minor concentration, extracellular DNA. This biofilm was sensitive to calcium concentration changes, which could suggest that if those structures form in the epididymis, biofilm inhabitants can detect calcium changes occurring during spermatozoid maturation, and this could constitute a signal to disperse them to other tissues (Figure 5).

In the same study [27], authors described that Furanona and LED-209, QS inhibitors, inhibit biofilm formation without affecting growth and cell viability, suggesting the presence of a specific receptor to those molecules. LED-209 has been described as a specific inhibitor; QseC protein, in coordination with QseB, conforms to the two-component signaling system QseBC, in which QseC functions as a receptor of AI molecules and not only detects changes in concentration of type III AI molecules but also can detect changes in host catecholamine (epinephrine or norepinephrine) concentrations [70]. Furanona inhibits the function of LuxR, a protein receptor of the two-component signaling system LuxIR, a phosphorylation system involved in detecting homoserine lactones. These molecules function as type II AI. This system regulates different phenotypes, such as bioluminescence, virulence, sporulation adhesion, and biofilm formation of different microorganisms [71]. Those results suggest the presence of both types of two-component signaling systems; however, *in silico* search, looking by LuxIR or QseC receptor, into *A. seminis* genome did not indicate the presence of any of the two systems. This could be due to a low homology in nucleotide sequences or other proteins being responsible for these functions.

In 2023, Ramírez-Paz y Puente *et al*. [6] described that testosterone at high concentrations (15 to 25 ng/ml; testosterone physiological concentrations for rams are 3 to 6 ng/ml [72]), induces biofilm dispersion. A similar effect was observed with estradiol. This result suggests that *A. seminis* can

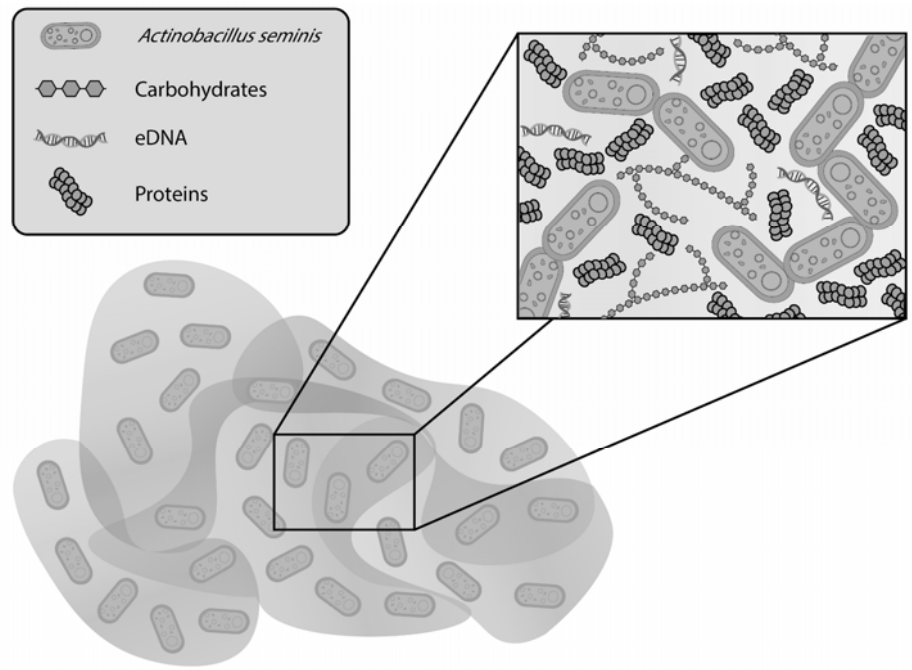

Figure 5. *A. seminis* biofilm representation. Schematic representation showing the *A. seminis* biofilm extracellular matrix constituted by different polymers. The abundance of proteins, carbohydrates, and DNA biofilm components as in García *et al*. (2020).

detect changes in sexual hormone concentrations in the reproductive tracts of female or male ruminants, and biofilm dispersion could favor *A. seminis* dissemination through the genitourinary tract. This could also be due to testosterone suppressing the defensive immune mechanism [73]. Biofilm formation *in vivo* could let *A. seminis* remain permanently in reproductive tissues without being detected by the host immune system. Future research could provide information on how this bacterium gets to colonize the different reproductive tract tissues and get access through anatomical limits present in the reproductive tract.

11. Conclusion

In conclusion, *Actinobacillus seminis* expresses different virulence factors (that could be modified in their expression by the presence of steroidal hormones) that could be essential to colonize, infect, maintain inside its host, or produce tissular damage to testis or epididymis. However, the lack of knowledge of pathogenicity mechanisms and pathognomonic characteristics of epididymitis makes early diagnosis and its control difficult. A better understanding of its virulence mechanisms and evasion immune response mechanisms could help develop prevention strategies or functional vaccines to control infections caused in ovines, goats, or bovines, the primary hosts of this bacterium.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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