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Determination of Pathological Changes in the Reproductive Track, IgG, IgM and IgA Antibodies in Blood, Seminal Plasma and Smegma of Rams Inoculated with *Actinobacillus seminis*

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Abstract: In experimentally infected rams with *Actiobacillus seminis,* pathological changes (reproductive organs) and antibodies (IgM, IgG and IgA) in serum, seminal plasma and smegma were evaluated. Twelve 1-year old rams were infected via intraurethal IU group (n = 4) and intraepididymal IE group (n = 4) with *A. seminis*; four animals were kept as Controls (CON) and inoculated both ways (intraepididymal and intraurethal) with saline solution. Blood, seminal plasma and smegma were collected, during 9 weeks: 4 before and 5 after inoculation. Bacteriological analysis of semen was performed. Animals were euthanized 35 days postinoculation; reproductive organs were collected for bacteriological and histopathological analysis. IE animals developed epididymitis. Ampullae and seminal vesicles in the IE and IU showed interstitial lymphoplasmacytic infiltrates. Thus, the bacteria were not re-isolated. Two controls developed epididymal granuloma. *A. seminis* was re-isolated in most of the semen samples and reproductive organ of IE animals, but not in those of the IU. Postinoculation mean IgG titer were different between CON and IE in serum weeks 1 to 5 ($p<0.01$), seminal plasma 1, 3, 4 and 5 weeks ($p<0.05$) and smegma 2, 3, 4 weeks ($p<0.05$), likewise between the IU and CON in serum IgG titers in the 2, 3 and 5 weeks ($p<0.05$). Postinculation mean IgM titers were different between CON and IE in the first week ($p<0.05$). No differences were observed in the IgA means. Serological response (IgG/IgM) and. pathological changes (reproductive organs) were induced by IE and IU inoculation of *A. seminis*.

Key words: Rams, epididymitis, *Actinobacillus seminis*, histopathology, seminal plasma, smegma, IgA, IgG, IgM

INTRODUCTION

Infectious epididymitis in rams is considered an economically important pathology because of its adverse effects on the male and herd fertility (Walker *et al*., 1986; Appuhamy *et al*., 1998). This pathology has been primarily associated with *Brucella ovis and Actinobacillus seminis* infections (Walker *et al*., 1986; De Long *et al*., 1979; Genetzby, 1995). *Actinobacillus. seminis (*family *Pasteurelaceae)* has been isolated in epididymitis cases from semen samples in Australia (Baynes and Sinumons, 1960), New Zealand (Ekdahel *et al*., 1968) United States of America (Livingston and Hardy, 1964), South Africa (Tonder, 1973), Mexico (Trejo *et al*., 1986), United Kingdom (Heath *et al*., 1991), Kenya (Mbai *et al*., 1996) and more recently, in Spain (Puente *et al*., 2000).

Higher incidence of this pathology is present when rams reach sexual maturity (Simmons *et al*., 1966; Bagvey *et al*., 1985), but it has also been found in adult rams; in both cases, causing alterations in semen quality and low fertility(Mbai *et al*., 1996; Low *et al*., 1995). The pathogenesis of epididymitis due to *A. seminis* is unclear; it has been suggested that this is an opportunistic microorganism normally found in the genital flora of young males and females, which, for undetermined reasons, is capable of causing epididymitis in some individuals (Jansen, 1980; Walker and Leamaster, 1985). The attempts to experimentally reproduce the pathology are not always successful and reasonable results are only obtained with direct inoculation of the bacteria into the epididymis (Al-Katib and Dennis, 2005), a condition that is far from the possible natural occurrence mechanisms of the pathology.

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The relationship of *A. seminis* and its pathologies in the ram reproductive tract with local and systemic immune response has not been determined. On the other hand, the presence and changes in immunoglobulin isotypes in the ruminant reproductive tract has been scarcely studied; it has been described in normal rams and rams infected with *B. ovis* (Fostee *et al*., 1988a, 1988b), as well as in bulls infected with *Tritrichomonas foetus* (Rhyan *et al*., 1999). The aim of the present study was to reproduce epididymitis due to *A. seminis* by intraurethral and intraepididymal inoculation and to analyze its relationship with changes in IgA, IgG and IgM levels in serum, seminal plasma and smegma of infected rams.

MATERIALS AND METHODS

Animals: Twelve one-year old male Pelibuey sheep from a herd with no clinical background of the disease were used. A clinical exam, Double Immunodiffusion (DID) serological test for *A. seminis* and *B. ovis* and semen bacteriological exam were performed to confirm that the animals did not present epididymitis (Mendez *et al*., 1999). Animals were distributed in 3 groups of four animals: Intraurethral (IU) and Iintraepididymal (IE) both inoculated with *A. seminis* and Control (CON) with intraepididymal and intraurethral administration of physiological saline solution.

Preparation of challenge inoculum: Blood agar plates were plated with *A. seminis* ATCC 15768 reference strain and incubated at 37°C for 48 h in an atmosphere with 10% CO_2 and harvested with saline solution. Colony-forming units (CFU) were counted in the inoculum by the method of Miles *et al*. (1938) and this was standardized at a $2.3x10^9$ CFU mL^{-1} concentration.

The IU inoculation was performed with 2 mL of the bacterial suspension with previous ablation of the urethral process under sedation with 1 mL of 2% xylazine using 3 $^1/_2$ F.r Tom Cat Catheters (Sherwood-Medical, St. Louis, USA) that was introduced approximately 10 cm into the urethra. The IE inoculation was performed, with previous cleansing and disinfection of the scrotum, with 1.5 mL of the bacterial suspension in the epididymis left tail and 0.5 mL in the right tail, the differences between inoculated aliquots were done to evaluated, possible variation in the re-isolation of the bacteria. The four control animals were inoculated with physiological saline solution; 2 mL intraurethrally plus 1.5 mL intraepididymally with of in the right epididymis. The animals were maintained isolated in individual pens to avoid the possible transmission of the challenge pathogen among them.

Clinical exam: A clinical exam of the genital tract was performed weekly recording alterations and their anatomic location. Changes in the epididymis were set out in subjective degrees from 0 to 4 crosses according to their severity. Scrotal circumference was measured in the major diameter of testes. Observations were recorded always by the same two observers and the data were averaged.

Sample collection and semen examination: Serum, semen and smegma samples were collected during 9 weeks; 4 weeks before the experimental inoculation and 5 weeks after inoculation. Semen extraction was performed using an electroejaculator (Hafez, 1993), with a 12 VCT, 500 mA transformer with previous cleansing of the sheath with a 0.1% benzalkonium chloride solution and dried with sterile cotton (Tonder, 1979). Ejaculated volume, spermatic concentration (hemocytometer count), number of abnormalities and total sperm count per ejaculation were determined from semen samples. Smears were obtained and stained with Giemsa stain to determine spermatic abnormalities and presence of inflammatory cells (Hafez, 1993). Finally, semen was centrifuged at 1500 xg for 15 min to separate seminal plasma from packed cells. Seminal plasma was frozen at -80°C until its use.

Smegma samples were collected by intrauterine lavage catheter for bovines, which was introduced up to the deep portion of the sheath and by creating a vacuum with a syringe. The contents collected in the catheter were emptied into PBS, pH 7.4, with 0.01% sodium azide as bacteriostatic agent (Rhyan *et al*., 1999). Smegma samples were centrifuged at 1500 xg for 10 min to eliminate cell debris and other contaminants.

Animals were euthanized 35 days post inoculation to obtain the seminal vesicles, ampulla of vas deferens, bulbourethral glands, testes and epididymes tail and head for the histopathology and bacteriology analyzes. Samples were fixed in 10% formaldehyde buffered at pH 7.4. For histopathological analysis, samples were embedded in paraffin and 5 μm sections were obtained and stained with hematoxylin and eosin.

Bacteriological analysis: A 10% sheep blood agar medium was used for the bacteriological exam of semen samples. Plate incubation was performed at 37°C in an atmosphere with 10% CO_2 up to for 3 days (Heth *et al*., 1991; Simmons *et al*., 1996). Cultures presumed to contain *A. seminis* were Gram stained. Isolations were biochemically characterized with the commercial system API 20 E (bioMériux, Marcy l´Etoile, Francia) using the ATCC 15768 strain (Erasmus, 1983) as control. Tissue samples were macerated with sterile saline solution;

subsequently, these were plated and incubated and suspicious colonies were identified in the same way as described for semen samples.

Preparation of *A. seminis* complete antigen for ELISA: A. seminis (ATCC 15768) was plated on blood agar plates, incubated for 48 h and harvested with isotonic saline solution. Bacteria were washed 3 times by centrifugation at 15000 xg for 15 min and then resuspended in sterile PBS and centrifuged again. Cells were then broken by freezing and thawing in 8 cycles; protease inhibitors PMSF and EDTA (Sigma-Aldrich, St Louis, USA) were added 3 times during the process. Cell rupture was verified by Gram staining. Protein was quantified by dilutions with the Micro BCA ™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, USA).

ELISA of serum, seminal plasma and smegma: The antigen was bound to 96-well flat-bottomed microplates Immuno Plate MaxiSorp (Nalge Nunc Internacional, Rochester, USA). Antigen dilution was performed using calcium chloride, pH 9.0; 100 μL of the dilution were added to each well with 4 μg of antigen; samples were incubated overnight at 37°C and washed 3 times with PBS-TW 20. Subsequently, plates were blocked with 1% Bovine Serum Albumin (BSA) for 1 h at 37°C.

Dilutions were standardized for each type of sample and these were made in duplicate. Table 1 summarizes ELISA conditions of each sample. The dilution of each sample was incubated for 1 h, washed 3 times, incubated again with 100 μL of rabbit anti-sheep peroxidase-conjugated IgG, IgM or IgA antibody (Lab. Bethyl, Montgomery, USA) for 1 h, developed with ABTS (Sigma-Aldrich, St Louis, USA) and read at 405 nm in an ELISA reader EIA Multiwell Reader (Sigma Diagnostics, St Louis, USA) (Nunez *et al*., 1977). ELISA plates control included: No *A. seminis* antigen, No antibodies (serum, plasma or sgmegma, corresponding to each case), No anti-IgG, IgM or IgA in accordance to each case. One positive serum and other negative control serum were included by duplicate.

Statistical analysis: Scrotal circumference, percentages of spermatic alterations and total spermatozoid counts from the ejaculate were measured. The results were pooled in three study groups, for the statistical proposes; Previous (weeks -3, -2, -1 and 0 previous inoculation), P1 (weeks 1 and 2 post inoculation), P2 (weeks 3, 4, 5 post inoculation). The ELISA results for each of the immunoglobulin isotypes (IgA, IgG e IgM) in each sample type (serum, seminal plasma and sgmema). All data were analyzed by ANOVA for repeated measurements and two factors; groups and weeks (repeated measurement) using the software STATISTICA 6.0 (Stat Soft, Inc., Oklahoma, USA).

Table 1: ELISA conditions

Antibody type	Sample type	Sample dilution	Antibody dilution	Reading time
IgG	Serum	1:800	1:10000	50 min
	Seminal plasma	1:100	1:2000	20 min
	Smegma	1:100	1:2000	30 min
IgM	Serum	1:400	1:10000	30 min
	Seminal plasma	1:10	1:4000	30 min
	Smegma	1:10	1:2000	40 min
IgA	Serum	1:100	1:1000	30 min
	Seminal plasma	1:10	1:2000	20 min
	Smegma	1:10	1:1000	40 min

RESULTS

Clinical exam and seminal analysis: Summary of the clinical and seminal findings are presented in (Table 2). Animals challenged by IE presented an increase in the scrotal circumference (swelling and adhesions) during the P1 (first two weeks PI, $p = 0.022$) and decreased by the P2 (third week PI, $p = 0.0077$). Postinoculation palpable alterations were detected in the epididymis tails, the size increased and/or change during the five weeks of the experiment. Animals in the IU group did not presented changes in epididymis and testes. Two of the 4 control animals presented an increase in the size in the right epididymis tail from the third week postinoculation remaining until the end of the experiment. One control animal presented a small induration (0.5 cm) that remained all along experiment and the others presented no changes in the inoculated epididymis. Spermatic alterations in the IE group in both P1 and P2 increased ($p = 0.015$ y $p = 0.030$, respectively). Spermatozoid counts from the ejaculate did not show statistical differences

Gross pathology: Animals of the IU group two did not showed gross alterations in testes, epididymes or accessory glands; one animal (12730) presented a small granuloma (0.5× 0.5 cm) in the right epididymis head with caseous contents, which was not detected by palpation and small adhesions between the testis and the tunica albuginea at testicular level. Another animal (14758) presented weak adhesions in both epididymes tails. Thus, these lesions were detected through the clinical examination.

All the animals of the IE group showed alterations in the epididymes tails (left and right) with adhesions in the tunica albuginea, increase of consistence and presence of a pearly-walled cavity with content of a whitish-yellowish contents in cut sections.

Table 2: Clinical exam and seminal analysis in rams inoculated with *A.seminis*

	Group	Previous	P1	P2
#Scrotal circumference (cm)	IU	32.1±1.28	32.0±0.53	31.5±1.12
	IE	32.9±1.38	34.5±1.36[a,c]	32.1±1.04[c]
	CON	32.2±1.60	32.4±1.01	32.8±0.71
*Epididymal lesions	IU	0	0	0
	IE	0	2.0 (1-3)	2.8 (2-4)
	CON	0	0	1.1 (0-2)
# Spermatic alterations (%)	IU	5.1±3.0	9.4±0.9	15.1±8.4
	IE	6.1±2.9	25.6±11.1[a]	34.7±11.8[a]
	CON	7.1±3.0	7.1±4.4	8.1±3.3
# Total spermatozoid counts from the ejaculate ($X10^9$)	IU	3.0±0.9	3.8±2.4	3.9±2.2
	IE	3.8±1.7	1.8±3.1	0.8±1.1
	CON	2.2±1.3	2.1±1.3	1.5±0.9
? Inflammatory cells in semen	IU	-	-	+/-
	IE	-	+++	++
	CON	-	-	-

IU Intraurethral group, IE Intraepididymal group, CON controls, Previous (weeks -3, -2, -1 and 0 previous inoculation samples), P1 (weeks 1 and 2 post inoculation), P2 (weeks 3, 4, 5 ,post inoculation). *Average of the ranges (maximum - minimum). ? +/-<1, ++ 10 a 30, +++ = 30 cells for field (X40)., # a $p<0.05$ are the result of the application of an ANOVA for repeated samples where the previous were compared to the P1 and P2. c $p<0.05$ these are the result of the comparison within each group and between P1 and P2. Mean±SE

Table 3: Histopathological analyses of reproductive organs of rams inoculated with *A. seminis*

		Left			Right						
			Epididymis			Epididymis					
Group	Number	Testis	Head	Tail	Testis	Head	Tail	Ampulla	Seminal vesicle	Disseminate prostate	Bulbourethral
IU	864	N	N	N	N	N	N	N	LI	N	N
	12611	N	N	N	N	N	N	N	N	N	N
	12730	N	LI	EA LI+	N	LI	EA LI+	LI	LI	N	N
	14758	N	N	N	N	N	N	LI	N	N	N
IE	584	N	LI+	EA LI++ Gr	TD	EA	EA LI++ Gr	LI+	LI++	LI	N
	866	TD	N	EA LI++ Gr	N	N	EA LI++ Gr	LI	LI++	LI	N
	867	TD-LI	N	EA LI++ Gr	TD-LI	LI	EA LI++ Gr	LI+	N	LI	N
	12724	N	N	EA LI++ Gr	N	N	EA LI++ Gr	LI+	LI	N	N
CON	368	N	N	N	N	N	EA LI+SGr	N	N	N	N
	531	N	N	N	N	N	EA	N	N	N	N
	576	N	N	EA	N	N	EA LI	N	N	N	N
	12514	TD	N	N	TD	N	EA LI+SGr	N	N	N	N

IU Intraurethral, inoculation, IE Intraepididymal inoculation, CON controls, N normal, TD testicular degeneration; TD-LI testicular degeneration with lymphocytic and plasmacytic infiltration; LI presence of inflammatory infiltrates (scarce, + abundant, ++ very abundant); Gr abscedative granuloma, SGr spermatic granuloma; EA presence of epithelial alterations (hyperplasia, cystic structures, metaplasia)

Animals of the control group showed alterations in the epididymes inoculated with saline solution (right); two revealed adhesions between the epididymis tail and the tunica albuginea and the other two presented, in addition to the adhesions, intense yellowish contents in cut sections.

Microscopic pathology: Table 3 summarizes the histopathological findings. In the IU group, one animal revealed alteration in both epididymis tails with interstitial lymphoplasmacytic infiltrate; cells were clustered mainly around blood vessels (perivascular cuffings) and a slight hyperplasia of the epididymal epithelium was found with intraepithelial cysts. The epididymes heads presented scarce lymphoid infiltration. Two animals, showed lymphoplasmacytic infiltration in the ampulla and in the seminal vesicles and one of them also presented epididymis lesion (Table 3).

Animals in the IE group showed alterations in all the tails of epididymes inoculated with *A. seminis* presenting an increase of interstitial fibrous tissue and epithelial hyperplasia with intraepithelial cystic structures limited by hypertrophic epithelial cells. All animals showed encapsulated granulomas with lymphocytes, plasmacytes, macrophages, epithelioid cells and multinucleated cells around a central mass of sperms and abundant neutrophils; in three of these animals, the challenge bacteria were recovered. In the epididymes heads, scarce interstitial lymphoid infiltration was found and one animal revealed epithelial hyperplasia in the epididymal duct. The three cases showed, at testicular level, decrease of seminiferous epithelium at testicular level, which in one case was accompanied by an increase in interstitial fibrous tissue with focal lymphoid infiltration (Table 3).

Animals in the IE group in all cases showed, abundant lymphoid infiltrates in the ampulla of vas

Table 4: Bacteriological of semen and reproductive organs of rams inoculated with *A. seminis*

		Bacteriology of semen weeks Pos inoculation					Bacteriology of reproductive organs									
								Left Epididymis			Right Epididymis					
Group	Number	1	2	3	4	5	Testis	Head	Tail	Testis	Head	Tail	Ampulla	Seminal vesicle	Disseminate prostate	Bulbourethral
IU	864	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	12611	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	12730	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14758	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IE	584	-	+	+	+	+	-	-	+	-	-	-	+	-	-	-
	866	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	867	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+
	12724	+	+	-	-	+	-	-	+	-	-	-	+	-	-	-
CON	368	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	531	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	576	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	12514	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

IU Intraurethral inoculation, IE Intraepididymal inoculation, CON Controls. + *A. seminis* isolation, - negative *A. seminis* isolation

deferens, mainly in the central lumen mucosa or infiltrating the trabeculae or the gland capsular connective tissue. In three animals, the challenge bacteria were isolated from the ampulla. In three of the 4 animals, seminal vesicles revealed abundant interstitial lymphoplasmacytic infiltration and hyperplasia with infiltration of glandular epithelium neutrophils. The disseminated prostate in three animals presented interstitial lymphoid infiltrate. The bulbourethral gland showed no histological changes, in spite of the fact that challenge bacterium was recovered from one animal.

In the control group, all the epididymes inoculated with saline solution presented alterations. Two animals showed an increase of interstitial fibrous tissue, epididymal epithelium hyperplasia and discontinuity of tubular basement membrane with epithelial evaginations presenting interstitial lymphoplasmacytic infiltration and perivascular cuffings, but with no neutrophils in cut sections. Both animals showed granuloma with a thin fibrous layer, macrophages, epithelioid cells and multinucleated cells, with abundant sperm compacted in the center, but in this case, without neutrophils, in contrast with animals challenged via IE. One case revealed alterations at testicular level with decrease of germinal epithelium. The other two controls presented slight lesions characterized by sperm compacting in the tubular lumen, which suggested sperm stasis with slight alterations of mucosa and scarce perivascular lymphoid infiltrate.

Bacteriology: In the samples from the IE group *Actinobacillus seminis* was re-isolated from semen and genital organs, isolates were obtained form the four animals, in 80% of times that samples were taken. In the IE group animals received different aliquot of bacterial inoculum for each epididymis; 1.5 mL (left) and 0.5 mL (right), the bacteria were re-isolated in three of the four animals and in one of the four animals respectively. In the accessory glands re-isolation from the ampulla was positive in three of four animals and in one of four animals from bulbourethral, disseminated prostate and seminal vesicle. No isolations were obtained from IU or control groups (Table 4). Biochemical profiles (API 20 E) of isolations agreed with those of the challenge bacteria used as test control.

ELISA of serum seminal plasma and smegma: Mean IgG titers in blood serum in the IE group, before inoculation, were 0.850±0.108 OD and increased significantly in average from the first week PI to 1.541±0.196 OD. These rose of the titers from the first week postinoculation to the fith week, was statistically significant, when CON group was compared with the IE (week 1 $p<0.05$, weeks 2, 3 and 5 $p<0.0001$, week 4 $p<0.001$). Blood serum in the IU group showed significant differences in titers compared with the CON group (weeks 2, 3 and 5 $p<0.05$). Statistical differences were also obtained between IE and IU groups from week one to five (week 1 $p<0.05$, weeks 2, 3, 4 $p<0.001$and week 5 $p<0.0001$) (Fig. 1 a).

IgG antibodies againt *A. seminis* in the seminal plasma: had a mean titer of 0.528±0.126 OD and rose to 1.154±0.673 OD PI. The results showed large variations among animals and were not uniform with in the experiment, significant differences observed between the IE and Con group in the weeks 1, 3, 4, 5 (weeks 1 and 3 $p<0.01$, weeks 4 and 5 $p<0.05$). Differences between the results from IE and IU group were observed in week 3 ($p<0.01$), 4 and 5 ($p<0.01$) (Fig. 1 b).

The IgG antibodies againt *A. seminis* in the sgmema showed large variations among animals. Differences were

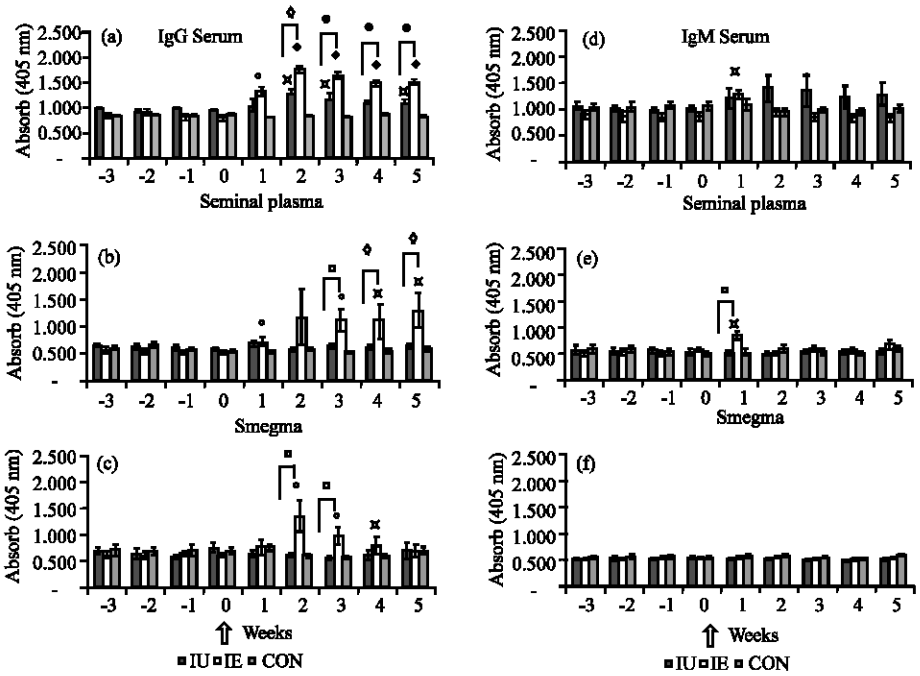

Fig. 1: ELISAs: serum, seminal plasma and smegma immunoglobulins (IgG, IgM) against *A. seminis,*IU Intraurethral inoculation, IE Intraepididymal, inoculation, CON controls, The statistical differences were analyzed by ANOVA for repeated samples, where the groups were compared: IE-CON, IU-CON and IE-IU in each one of the weeks postinoculation (1, 2, 3, 4, 5), against the of previous weeks to the inoculation (-3, -2, -1, 0) within each group. Statistical differences: IE or IU with the group CON ¤ ($p < 0.05$), o ($p < 0.01$), ◆ ($p<0.001$ or less), between IE and IU ◊($p < 0.05$), ◘ ($p < 0.01$), ● ($p < 0.001$ or less). Mean± SE

observed between the IE and Con groups in the weeks 2 and 3($p<0.01$) and week 4 ($p<0.05$). The results from IE and CON group showed differences in weeks 2 and 3 ($p<0.01$) Fig. 1 c.

IgM titers in blood serum in the IE group rose from the first week PI and were statically different from the CON group ($p<0.05$). Two animals of the IU group showed and increase in the seric IgM (1611, 14758), but they did not had an effect in the statistical results Fig. 1 d.

In the IE group the in the seminal plasma IgM titers increased from the first week PI and presented statistical differences with the IU and CON groups ($p<0.05$). However, the titers rises in the animals of IU group was not observed in the IgM values from the smegma and seminal plasma samples Fig. 1e. No differences were observed in the smegma Fig. 1f.

The IgA titers showed no differences among the groups or the samples.

DISCUSSION

The clinical sing shown by the rams were minimal and were confined primarily to the scrotal contents and the inoculation site (Al-Katib and Dennis, 2005). The scrotal circumference in IE group increased in the first period post inoculation, this effect was due to the intense scrotal edema, which latter disappeared.

A reduction in the total spermatozoid counts could be observed in some animals of the IE group, this effect could be link to the infection with *A. seminis* or to the electroeyaculation technique. Mbai *et al.* (1996) reported a similar effect in naturally infected rams with *A. seminis.* Mattner and Voglmayr (1962) reported large variation between samples within each animal, in concentration and volume of the ejaculate when electro-ejaculation was used.

The presence of secundary spermatic alterations like lose tails and heads or bend tails showed a significant difference in the IE group. These kinds of alterations in the sperm correspond to changes in the maturation process in the epididymis and have been described previously in the *Brucella ovis* infection (Kott *et al.*, 1988). Furthermore, the increase in the spermatic alterations had been reported when the ampulla and seminal vesicle are affected, similar to the results of the present study (Barth and Oko, 1989).

In the IE group, the bacterium was isolated constantly from semen, but recovery from the inoculation sites in epididymes and accessory glands was variable; however, the examined organs presented similar lesions (Table 3). The impossibility or inconsistency to receive the challenge bacteria from semen or genitalia, in spite of the presence of lesions indicating its effect on the organs, has been previously reported by other authors while trying to reproduce the pathology through experimental inoculations (Bayres and Simmons, 1960; Livingston and Hardy, 1964; Watt *et al*., 1970). Negative isolations can be explained by the intensity of the inflammatory and immune response, which eventually sterilizes the lesion or inhibits bacterial reproduction (Acosta, 2001).

Clinicopathological findings in animals of the IE group also coincide with those reported in field and experimental infection cases, including IE injection of *A. seminis*, by other authors (Baynes and Simmons, 1960; Livingston and Hardy, 1964; Puenle *et al*., 2000; Al-Katib and Dennis, 2005; Baynes and Simmons, 1968; Erasmus *et al*., 1982). The presence of inflammatory infiltrates surrounding the central channel of the ampulla of vas deferens in the three animals in which *A. seminis* was recovered, as well as the histological findings in seminal vesicles, suggest the existence of a local response against the challenge bacterium or its antigens present in the ductal or acinar lumen, such as has been proposed for *B. ovis* (Foster *et al*., 1987). The bulbourethral glands presented no histopathological lesions, although *A. seminis* was isolated in one case. In field studies of epididymitis due to *B. ovis*, 4 isolations were obtained in these glands against 41 from the ampulla of vas deferens and 28 from seminal vesicles of 49 seropositive rams (Searson, 1986). The histopathological findings in accessory glands coincide with those described by Jansen (1980), in cases of epididymitis caused by agents different from *B. ovis*.

Jansen (1980) explained the presence of *A. seminis* or other bacteria in different sectors of the reproductive tract in absence of bacteremia ascending from the sheath and urethra by backflow of semen. On the other hand, isolation of the bacteria from accessory glands supports the possibility that these act as a reservoir for *A. seminis* in the male genital tract (Al-Katib and Dennis, 2005; Acosta, 2001).

The ELISA tests revealed a great variability of the seminal plasma and smegma results Fig. 1 among the animals with IE treatment, while blood serum results were homogeneous. This can be partly explained by the variability in volume and sperm concentration in the used semen samples collected by electroejaculation, which in some cases, may contain urine, as well as because of the variation found in protein concentration of smegma samples. In all treated animals' serum IgG and IgM PI increase Fig. 1. In the IE group seminal plasma and smegma IgGs and IgMs were increased PI, in IE group IgG was increased at this time, suggesting that this type of immunoglobulins found in the samples were exuded from the inflammatory vascular stage, induced by the intraepididymal inoculation. The clinical observations and the presence of abundant inflammatory cells in the semen of animals inoculated by this pathway support these results Table 2. A previous study determining immunoglobulins in seminal plasma and in different parts of the sheep reproductive tract (cannulating at various levels or extracting and squeezing the accessory glands), also revealed high variability among individuals (Foster *et al*., 1988a). The presence of antibodies in seminal plasma and smegma did not interfered the isolation of *A. seminis* from semen and genital organs, which was only possible in animals of the IE group suggesting that these Igs did not affect the bacterial presence in the genital tract Table 4.

The IU group serum IgG did showed a significant increase where as serum IgM presented variable values (2 animals increased). The variation similar to those observed in the serum IgG and IgM were not detected in the seminal plasma and smegma samples. The animals in the UI group did not presented clinical changes or severe pathological lesions in the reproductive organs.

An increase in the IgA total concentration in seminal plasma of naturally infected animals with *B. ovis* presenting or not slight lesions in the epididymis has been described, although the specific antibodies were not determined (Foster *et al*., 1988b). Jansen (1983) could not identify specific antibodies in semen of animals from which they isolated microorganisms similar to *A. seminis* and *Pasteurella (Mannheimia) haemolytica*; in contrast, they identified Igs in cases of *Streptococcus faecalis* with lesions in seminal vesicle and ampulla of vas deferens. These observations and the results of the present study could demonstrate the significance of the presence of lesions in genital tract to stimulate antibody response.

The absence of a local IgA response in these sheep reinforces the notion of serum exudation as the origin of IgG and IgM in seminal plasma and smegma and may suggest that this bacterium, considered opportunistic by most authors (Jansen, 1980; Walker and Leamaster, 1986), is a poor IgA responses stimulant. Similar results has been reported in bulls inoculates I ntratesticular with *B. abortus* (Campero *et al*., 1990). Nevertheless, the proteases presence in the semen that can breakdown IgA

(Tjokronegoro and Sirisinha, 1974) or the direct proteases production by *A. seminis* like in other related microorganisms (Negrete *et al*., 1933) could have influenced the results.

Granulomas induced in control animals by administration of saline solution corroborate the elements of the autoimmune response induced by the lesion at the site of injection. These granulomas were distinguishable from those induced by the IE inoculation of *A. seminis* only by the almost null presence of neutrophils. The intraepididymal inoculation did produced lesions similar to those observed on the natural of the occurrence of the infection. However, the inoculation techniques appears not to be adequate to reproduce the natural infection

CONCLUSION

The intraepididymal inoculation with *A seminis* produced lesions in the inoculation site and the accessory glands of the reproductive track. The intraepididymal inoculation produced lesions mainly in accessory glands. Histopathological and bacteriological results suggested that accessories glands could act as a reservoir for *A. seminis*. IE and IU bacterial inoculation induced a humoral IgG response. IE group demonstrated a IgG response in seminal plasma and smegma too, suggested a epididymal vascular exudation.

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