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ACTINOBACILLUS SEMINIS INFECTION IN SHEEP IN THE REPUBLIC OF SOUTH AFRICA. I. IDENTIFICATION OF THE PROBLEM

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ABSTRACT

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A clinical palpation and semen smear examination of 647 rams submitted to the Regional Veterinary Laboratory during 1967 revealed that 42 (6,5%) of these animals had clinical epididymitis or orchitis, 6 (0,9%) showed other types of genital lesions and 98 (15,1%) suffered from subclinical genital infection. *A. seminis* and *A. seminis*-like organisms were isolated from semen specimens of 18 out of 35 rams with clinical epididymitis or orchitis, 25 out of 33 rams with subclinical infection and none out of 13 rams which showed no neutrophils in their semen.

On 4 stud farms where Elberg Rev. 1 vaccine was meticulously applied and the complete absence of *Brucella ovis* infection was established, of a total of 327 rams examined, 10 (3,6%) were found to be clinically and 72 (22,0%) subclinically affected. *A. seminis* was isolated from 5 out of 6 of these rams with clinical lesions and 10 out of 15 of those which showed evidence of subclinical infection.

Résumé

INFECTION À ACTINOBACILLUS SEMINIS *CHEZ LE MOUTON EN RÉPUBLIQUE SUD-AFRICAINE. I. RECONNAISSANCE DU PROBLÈME*

La palpation clinique et l'examen de frottis séminals chez 647 *béliers présentés au Laboratoire Vétérinaire Régional en* 1967 *ont montré que* 42 (6,5%) *de ces animaux étaient atteints de l'épididymite clinique ou de l'orchite,* 6 (0,9%) *présentaient d'autres types de lésions génitales et* 98 (15,1%) *souffraient d'une infection génitale sub-clinique. On a isolé* A. seminis *et des organismes de type* A. seminis *dans des échantillons seminals chez* 18 *béliers sur* 35 *atteints d'épididymite clinique ou d'orchite et chez* 25 *béliers sur* 33 *souffrant d'infection sub-clinique. On n'a rien trouvé chez* 13 *béliers qui n'avaient pas de neutrophiles dans leur sperme.*

Chez quatre élevages où l'on avait méticuleusement administré du vaccin Elberg Rev. 1 *et où l'on avait démontré l'absence complète d'infection à* Brucella ovis, *on a trouvé, sur un total de* 327 *béliers examinés, que* 10 (3,6%) *étaient cliniquement affectés et* 72 (22,0%) *sub-cliniquement.* A. seminis *a été isolé chez* 5 *sur* 6 *de ces béliers qui avaient des lésions cliniques et chez* 10 *sur* 15 *de ceux qui montraient des signes d'infection sub-clinique.*

INTRODUCTION

Despite the effective control of brucellosis in rams by the general application of *Brucella melitensis* Rev. 1 vaccine, epididymitis and resultant infertility remain a serious problem in the major sheep raising areas in South Africa. Although various bacteria, including *Corynebacterium pseudotuberculosis* (Belonje, 1951; Simmons & Hall, 1953; Van Rensburg, 1953, 1955; Clapp, Symons & Doolette, 1955; McGowan & Schultz, 1956; Van Rensburg, Van Heerden, Le Roux & Snyders, 1958; Baynes & Simmons, 1960; Van Heerden, 1964; Watt 1966, 1970; Ekdahl, Money & Martin, 1968; Hughes & Claxton, 1968), *Corynebacterium pyogenes* (Watt, 1966; Gamcik, cited by Hughes & Claxton, 1968; Hughes & Claxton 1968; Ekdahl *et al.*, 1968), *Actinobacillus lignieresii* (Verdes, Vasilescu, Galusca & Constantin, cited by Laws & Elder, 1969), *Staphylococcus* and *Streptococcus* spp. (Ekdahl *et al.*, 1968; Watt, 1970) and *Pasteurella pseudotuberculosis* (Jamieson & Soltys, 1947; Ekdahl *et al.*, 1968) *Pasteurella haemolytica, Pasteurella multocida, Brucella abortus* and *Bacteroides* spp. (Ekdahl *et al.*, 1968) have been associated with epididymitis in sheep and have been well documented, their precise role has never really been assessed.

The incidence and role of a variety of Gram-negative non-acid-fast, pleomorphic organisms has, however, been less well studied. In New Zealand, Dodd & Hartley (1955) described a suppurative epididymitis in rams caused by an organism which is still referred to as a "Gram-negative pleomorph", while in Australia Baynes & Simmons (1960) isolated a similar organism from 3 cases of epididymitis and named it *Actinobacillus seminis*. *A. seminis* was also isolated from the genitalia, foetal membranes and stomach of the male foetus of a ewe which had been experimentally infected in the advanced stages of pregnancy and which delivered 2 still-born lambs 4 days later (Baynes & Simmons, 1960).

In a report of an outbreak of traumatic epididymitis in Dorset Rams in South Australia, Pulsford, Eastick, Clapp & Roberts (1967) also recorded the isolation of an unidentified Gram-negative organism from a few of these cases. More recently, however, Watt (1970) isolated an organism closely resembling *A. siminis* from 3 out of 10 rams with chronic epididymitis in Western Australia. *A. seminis* has since been reported in the same country as a cause of polyarthritis and posthitis in sheep by Watt, Bamford & Nairn (1970), who produced acute gangrenous mastitis by injecting cultures into the teat canals of 2 lactating ewes. They assumed that *Histophilus ovis* recovered by Roberts (1956) from a case of ovine mastitis could have been *A. seminis*. This assumption appears to be reasonable, as *H. ovis* has since been recovered from cases of suppurative synovitis and pyaemia in lambs in New Zealand (Kater, Marshall & Hartley, 1962) and in Australia (Hughes, Hartley, Haughey & McFarlane, 1964; Dennis, 1974; Rahaley & White, 1977), and from the semen and, on autopsy, from the ampullae of a ram in Australia with clinical epididymitis (Claxton & Everett, 1966). Infection caused by *A. seminis* has also been reported by Livingston & Hardy (1964) in a Rambouilliet ram in the United States of America.

A. seminis has not yet been isolated in New Zealand. In addition to the Gram-negative pleomorph (Dodd & Hartley, 1955), Ekdahl *et al.* (1968) reported the isolation of *Actinobacillus*-like organisms from epididymal lesions in rams. These organisms did not have identical properties and could not be classified as *A. seminis*. It appears from their report that prior to 1965 isolates subsequently assigned to the genus *Actinobacillus* were classified as Gram-negative pleomorphs. These authors concluded that 3 different

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species or groups of organisms, namely *B. ovis*, *Actinobacillus* spp. and the Gram-negative pleomorphs, predominate in the aetiology of epididymitis of rams in New Zealand.

In the light of the above it was considered desirable to undertake an investigation relating to the incidence and importance of *A. seminis* infection in rams in the Republic of South Africa.

Materials and Methods

Animals

The animals used in this preliminary investigation included all rams submitted to the Regional Veterinary Laboratory, Middelburg, Cape Province, during 1967, as well as all rams intended for sale during that year on 4 stud farms where sale rams were tested anually and where Rev. 1 vaccine was consistently used.

These rams were of different ages and the affected animals were representative of different stages of the disease.

Examination of the genitalia

Examination of the genitalia was conducted on all the rams by visual inspection and palpation.

Biological specimens

Specimens of semen were collected from all rams for microscopic examination. Bacteriological cultures were made from the semen of 35 of the rams with obvious lesions sent to the laboratory, as well as from 33 of the rams whose semen contained neutrophils and from 13 that appeared to be uninfected on smear examination. Bacteriological cultures were also made from the semen of 6 of the 10 rams with clinical lesions and 15 of the 72 rams which showed neutrophils on semen smear examination on the 4 stud farms.

Method of collection

The standard method for semen collection was electrical stimulation as devised by Gunn (1936), with certain modifications as described by Van Tonder, Bolton, Robertson & Greeff (1973). Similarly, the methods of restraint, extrusion and fixation of the penis and actual collection of semen as described by Van Tonder *et al.* (1973) were followed. Before each collection the penis, and especially the glans and urethral process, were cleaned by being flushed with distilled water containing 0,1% cetrimide B,P[1] and then wiped with sterile cotton wool. To avoid the frequent opening of containers and the resultant contamination of the specimen, it became a standard practice to collect 2 specimens at intervals of up to 1 hour when the semen was to be examined bacteriologically. This practice proved beneficial, since the first ejaculate cleared the urethra of contaminants and the concentration of these organisms was greatly reduced when the second specimen was cultured.

Preparation and staining of semen smears

Duplicate smears were prepared by placing a small drop of semen with the aid of a platinum loop onto a glass slide. The semen was then spread across the slide in a band and in such a manner that the smear consisted of areas of varying density. By this method smears from up to 4 rams could be made on the same slide. The denser portions of the smears were suitable for the detection of neutrophils, while the thinner parts were examined for the presence of bacteria.

[1] Cetavlon; Imperial Chemical Industries (Ltd.) Alderley Park, Macclesfield, Cheshire

All the smears were air-dried and lightly fixed over a flame. One of each pair of smears was stained by Stamp's modification of the Ziehl-Neelsen technique (Stamp, McEwen, Watt & Nisbet, 1950) and the other with Preston and Morrell's modification of the Gram's method (Preston & Morrell, 1962).

Bacteriological cultivation

Standard methods and techniques for the cultivation, isolation and identification of bacteria were employed. *B. ovis* was identified according to the methods and description of Simmons & Hall (1953) and Buddle & Boyes (1953), while *A. seminis* was identified by the methods described by Baynes & Simmons (1960).

Primary cultivation was conducted on *Brucella* agar and 5% tryptose agar plates containing 5% horse blood. One plate of each medium was incubated aerobically and the other in 15% CO_2 at 37 °C. All plates were examined daily and discarded as negative when no bacterial growth was observed after 4 days.

Results

Differentiation between clinical and subclinical infection of the genitalia

When gross lesions could be detected by visual inspection and palpation, the animal was classified as a clinical case, while infection was regarded as subclinical when neutrophils were demonstrated in Ziehl-Neelson stained semen smears (Stamp *et al.* 1950).

Since *B. ovis* is the only known pathogen occurring in ram semen that resists decolororization by a weak acid, the presence of neutrophils in semen smears in the absence of *B. ovis* was therefore considered indicative of genital infection attributable to some other organism. Non-acid-fast pleomorphic bacilli were often encountered in semen smears and the majority of cases examined in detail proved to be *A. seminis*, as the identity of these organisms could only be established with certainty by cultural methods. As the majority of bacteria stained negative with this staining method, the presence of neutrophils rather than non-acid-fast bacteria was considered to be a more reliable criterion for the detection of subclinical genital infection.

Clinical and subclinical infection in rams examined during 1967

The incidence of clinical abnormalities and subclinical infection as determined by the above criteria is given in Table 1.

From these data it is evident that 6,5% of rams examined suffered from clinical epididymitis and/or orchitis, while 15,1% had subclinical infection of the genitalia. Other genital lesions included unilateral or bilateral hypoplasia and atrophy, while 1 case showed scrotal dermatitis.

On the basis of the modified Ziehl-Neelsen smear examination, only 1 case with an epididymal lesion and 4 cases with subclinical infection could be diagnosed as being due to *B. ovis* infection. The majority of clinical and subclinical cases showed the presence of small pleomorphic, non-acid-fast bacilli, often in close association with polymorphic leucocytes and epithelial cells in the semen.

TABLE 1 The incidence of lesions and subclinical infection in rams examined at the Regional Laboratory during 1967

Rams examined	Rams with clinical lesions				Rams without clinical lesions			
	Orchitis[1] epididymitis		Other[2]		Rams with subclinical infection		Rams not affected	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
647.....	42	6,5	6	0,9	98	15,1	501	77,4

[1] Rams with grossly swollen scrotal contents in which specific structures and lesions could not be palpated were classified as having either orchitis or epididymo-orchitis
[2] Lesions not usually associated with infection

Unevenly stained Gram-positive bacilli were observed in 5 cases and Gram-positive cocci in 3 with chronic epididymitis. In 2 of these cases both types of organism occurred in the same smear. In the subclinical cases, similar Gram-positive bacilli were encountered in 2 rams of which 1 was also positive for *B. ovis*. Gram-positive cocci were observed in the semen smears of 5 rams.

The results of the bacteriological investigations on the semen of some rams submitted to the Regional Laboratory are summarized in Table 2.

No semen could be obtained from 3 rams with bilateral lesions, the semen of another 3 rams with unilateral lesions showed no evidence of infection on smear examination, but *A. seminis* and *A. seminis*-like organisms were isolated from 62,1% of clinical cases which excreted neutrophils in the semen. These organisms were also isolated from 75,8% of rams that showed neutrophils but from none of the rams free from signs of infection on semen smear examination.

The laboratory records of 20 randomly selected rams within the 2 groups presented in Table 3 give a comparison of the results of the various tests conducted on rams that were either clinically or subclinically affected.

From these records it can be seen that no semen could be obtained from 1 ram with bilateral lesions in the clinically affected group. Neutrophils, epithelial cells and organisms were found in Ziehl-Neelsen-stained semen smears of all rams irrespective of whether clinical lesions were present or not, and *A. seminis* was also consistently isolated from both groups. Gram-stained smears proved to be of no value in detecting either the tissue cells or bacteria. It is also evident from these records that *A. seminis* and *A. seminis*-like organisms were the most consistently isolated.

TABLE 2 Bacteria isolated from semen of rams with and without clinical lesions

Isolates	Rams with clinical epididymitis orchitis	Rams without clinical lesions	
		Neutrophils present	Neutrophils absent
A. seminis.....	14	23	0
A. seminis-like organism[1].....	4	2	0
B. ovis.....	1	0	0
C. ovis.....	1	0	0
C. bovis.....	3	1	0
C. renale.....	1	2	0
Corynebacterium sp.....	0	1	0
Streptococcus sp.....	2	0	0
S. epidermidis.....	1	0	0
Micrococcus spp.....	3	3	2
Kurthia sp.....	0	1	0
E. coli.....	0	0	1
Alcaligenes.....	1	0	0
Contaminants.....	2	0	1
Negative.....	6	4	9
Total number of rams examined.....	35[2]	33[2]	13

[1] Isolates that showed minor differences from the original description were classified as *A. seminis*-like
[2] The discrepancy between the number of rams and number of isolates is due to the isolation of 2 types of organisms from the same ram

TABLE 3 Laboratory records of 20 randomly selected rams examined in the preliminary survey

Ram	Clinical lesions	Breed	Description of lesions	Semen smear examination					Bacteria isolated
				Modified Ziehl-Neelsen			Gram+		
				Neutrophils	Epithelial cells	Organisms	Rods	Cocci	
1.....		Merino	LES+	+	+	?	—	—	*A. seminis*
2.....		Merino	R & LES+	++	++	++	—	—	*B. ovis*
3.....		Dorper	R & LES++	No semen					Contaminants
4.....	Present	Merino	RES+++	+++	+	++	—	+	*A. seminis*-like, *Streptococcus* sp.
5.....		Merino	LEK++	++	+	+	—	—	*Streptococcus* sp.
6.....		Merino	LES & B+++	+++	++	+	—	—	*A. seminis*
7.....		Dorper	LES+++	++	+++	++	—	—	*A. seminis*
8.....		Dorper	LEK,++ REK+++	+++	++	+++	—	—	*A. seminis*
9.....		Merino	LES++	+++	+	++	—	—	*A. seminis*
10.....		Corriedale	RES+++	+++	+++	+++	—	—	*Alcaligenes* sp.
1.....		Dorper	N.A.	+	+	+	—	—	*A. seminis* & *Micrococcus* sp.
2.....		Dorper	N.A.	++	+	+	—	—	*A. seminis* & *Micrococcus* sp.
3.....		Dorper	N.A.	++	+	+	—	—	*A. seminis*
4.....		Merino	N.A.	++++	+	++	—	—	*A. seminis*
5.....	Absent	Merino	N.A.	++	+	+	—	—	*A. seminis* & *C. renale*
6.....		Merino	N.A.	++	—	+	—	—	*A. seminis*
7.....		Merino	N.A.	+	+	—	—	—	*Kurthia* spp.
8.....		Merino	N.A.	++	++	+	—	—	Negative
9.....		Merino	N.A.	++	+	+	—	—	*A. seminis*
10.....		Corriedale	N.A.	++	++	?	—	—	*A. seminis*

L=Left
R=Right
T=Testes
E=Epididymis
K=Head of Epididymis
B=Body of Epididymis
S=Tail of Epdidymis

?=Not distinct
+ — +++++=Arbitrary evaluation of concentration or severity of lesions
— =Absent or negative

The results of the examination of the sale rams from the 4 selected stud farms are analysed in Table 4.

TABLE 4 Results of examinations conducted on sale rams from 4 selected stud farms

Item	Farm			
	A	B	C	D
Number of rams examined.....	54	72	94	107
Number of rams clinically affected.....	1	3	2	4
Number of rams without lesions:				
Neutrophils present.....	7	25	26	14
Neutrophils absent.....	46	44	66	89

It can be seen that clinical and subclinical genital infection continues to present a problem on these farms, despite regular vaccination with Rev. 1 vaccine. *B. ovis* organisms could not be demonstrated in the semen smears of any of the clinically and subclinically affected animals. The semen of 21 of the clinically and subclinically affected rams was also examined bacteriologically. *A. seminis* was isolated from 5 of the 6 rams with clinical lesions and from 10 of the 15 rams with no lesions but which showed neutrophils in the semen.

Discussion

There seems to be general agreement that normal semen should not contain any leucocytes and that their presence indicates an inflammatory process of the genital tract. Non-specific local inflammatory conditions of the distal genital tract, independent of inflammatory processes of the scrotal contents and/or specific venereal infections, could also cause the appearance of polymorphonuclear leucocytes in the semen, but it was found that such cases were rare and that the presence of these cells in the semen could be regarded as indicative of specific genital infection (Roberts, 1956; Maximow & Bloom, 1957; Van Rensburg *et al.*, 1958; Zemjanis, 1962; Jubb & Kennedy, 1963; Miller, 1966). While any clinical abnormality was detected by careful palpation of the scrotal contents, the presence of neutrophils in semen smears, regardless of the presence or absence of bacterial organisms, was taken as indicative of subclinical infection.

The modified Ziehl-Neelsen staining method was found to be a satisfactory method whereby neutrophils could be demonstrated in semen smears and it also proved to be reliable for detecting *B. ovis* organisms in the semen, as reported by Edgar, Inkster & McDiarmid (1956), Van Rensburg *et al.* (1958), Biberstein & McGowan (1958), Edgar (1959) and Van Heerden & Van Rensburg (1962). Smears stained by this technique are therefore preferred as the standard preliminary method adopted for the determination of genital infection.

As most of the material in a semen smear does not retain the basic dye and is stained by the counter stain, Gram-stained smears proved to be of little value in determining the presence of Gram-negative organisms, neutrophils or epithelial cells. In subsequent work the Gram staining technique was used

only as a secondary procedure, although it proved of value in clinical and subclinical cases where morphological differences discernible on Ziehl-Neelsen smear examination indicated the possible presence of Gram-positive organisms.

Examination of rams presented at the laboratory during 1967 clearly showed that clinical and subclinical genital infection was a severe problem. The presence of *B. ovis* in semen smears of only a negligible number of these rams not only proved the minor role of this organism but also incriminated other possible aetiological agents. The semen of the majority of the clinical and subclinical cases showed the presence of small pleomorphic, non-acid-fast bacilli, often in close association with neutrophils and epithelial cells. Although in most of these cases *A. seminis* was isolated on culture, smear examination could only be regarded as supportive evidence of infection, since most organisms were non-acid-fast. The bacteriological examination of semen is therefore required to confirm the identity of the aetiological agents in these cases.

Bacteriological examination of semen specimens proved that *A. seminis* and *A. seminis*-like organisms were the most common and the most consistent bacteria, evidence which strongly suggests their possible role in the aetiology of these clinical and subclinical infections.

The sporadic or irregular isolation of a variety of other bacteria and their normal association with the more chronic lesions indicated that these organisms were not of primary importance. This view was also substantiated by the fact that these organisms were seldom isolated in large numbers or in pure culture and were often not assocaited with the presence of inflammatory products in the semen.

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