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MYCOPLASMAS RECOVERED FROM BOVINE GENITALIA, ABORTED FOETUSES AND PLACENTAS IN THE REPUBLIC OF SOUTH AFRICA

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ABSTRACT

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A total of 917 *Mycoplasma* isolations were made from 4 092 specimens originating from 2 874 cattle in private herds and at AI stations. The percentages of isolations from the different sources were: cervico-vaginal mucus 14,6 %, semen 43 %, preputial wash 25 %, foetuses 3,3 % and placentas 15 %. *Mycoplasma bovigenitalium*, the most common isolate, was recovered from 39 % of males, 47 % of females, 25 % of foetuses and 11 % of placentas. A wide spectrum of mycoplasmas was present, and varying combinations were common. The possible pathogenic significance of the isolates is discussed.

INTRODUCTION

Although most major infectious diseases of domestic ruminants in the Republic of South Africa have been eliminated or brought under control, some economically important bovine reproductive diseases of uncertain aetiology still exist. Bovine genital mycoplasmosis is an important condition engaging world-wide attention, and because of the lack of information on the situation in the Republic of South Africa, we decided to examine all the specimens from cattle submitted for the diagnosis of campylobacteriosis and trichomoniasis for mycoplasmas as well. All aborted foetuses and placentas received at the Section of Reproduction as well as specimens specifically collected were included in a survey conducted over 4 years. Moreover, mycoplasmologists in several countries have proved that mycoplasmas could be associated with lowered reproduction in spite of the apparently normal appearance of the internal reproductive tract harbouring large numbers of these organisms (Al-Aubaidi, McEntee, Lein & Roberts, 1972; Cottew, 1970; Erno, Plastridge & Tourtellotte, 1967; Hoare, 1969; Hirth, Nielsen & Plastridge, 1966).

MATERIALS AND METHODS

Specimens were obtained from ranching, dairying and stud animals, and from AI stations.

Specimens from females

The survey included 151 herds with conception rates varying between 30 and 62 %. Cervical mucus samples were taken from an average of 7 cows per herd. Cows returning to oestrus after service were selected.

Cervico-vaginal mucus samples were collected by aspiration into plastic insemination pipettes. These were then sealed off at either end with plasticine before being placed in a cool bag containing one or more prefrozen freezer packs. The samples were delivered at the laboratory on the day of collection at temperatures in transit which varied from 1 °C to 7 °C. Cervical biopsy samples were collected and placed in the same cool bag as was used for the cervico-vaginal mucus samples.

Although these samples were taken primarily for *Campylobacter* and *Trichomonas* isolations, they were well-suited to our *Mycoplasma* survey.

Where samples were taken for *Mycoplasma* isolation *per se*, the following technique was used. Firstly, the perineum was washed with water, thoroughly cleaned and dried with a disposable paper towel. A cotton-wool swab (cotton bud) was fitted onto a plastic inseminating pipette by pushing the stem of the cotton bud down the pipette. The vulva lips of the animal were then parted and a sterile speculum inserted into the vagina. In the light of a headlamp a swab was taken from the vaginal fornix. Strict care was taken to prevent the swabs from coming into contact with any other surface. A volume of 0,5 mℓ of sterile 0,01 M phosphate buffer pH 7,2 containing 4,2 % sodium chloride was deposited in the plastic test tube, whereafter the cotton bud was replaced and the buffer solution allowed to be absorbed by it. The test tube was sealed, using the plastic stopper provided, placed in a cool bag and transported to the laboratory.

Specimens from males

Sheath washes were done as follows:

The preputial hair was clipped and the orifice thoroughly cleaned with water, and dried. A separate sterile rubber tube 1 m in length was used for each bull sampled, one end of which was placed in the preputial sheath and firmly held. Fifty mℓ of phosphate buffer solution was poured into a funnel attached to the free end of the tube. The fluid was allowed to gravitate into the sheath. The sheath was then vigorously massaged with about 80 strokes and the fluid was finally allowed to drain back into the original 50 mℓ bottle. The samples were immediately packed in the cool bag containing the prefrozen freezer packs, and delivered to the laboratory within 6 h.

After the sheath was washed and care taken to ensure that the samples were not contaminated by urine, semen was collected either by means of an artificial vagina or by electro-ejaculation, depending on circumstances. The tubes containing the semen were packed in a cool bag and delivered to the laboratory on the same day.

Specimens from foetuses and placentas

Lung tissue and abomasal contents were collected with sterile precautions from aborted foetuses and placentas regularly received at the Section of Reproduction at the Veterinary Research Institute. Most placentas were unfortunately badly soiled.

Media employed

The following *Mycoplasma* culture media were used throughout this study.

Hayflick's agar	(Hayflick, 1965)
Hayflick's broth	(Hayflick, 1965)
Gourlay's glucose serum broth	(Gourlay, 1976)
Gourlay's glucose serum agar	(Gourlay, 1976)
Cholquest's agar	(Cholquest, 1961)
Cholquest's broth	(vide infra)
Ureaplasma agar	(Morgan & Lewis, 1977)
Ureaplasma broth	(Morgan & lewis, 1977)

It was decided to use Cholquest's medium both as agar and as broth.

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TABLE 1 Mycoplasmas isolated from bovine cervico-vaginal mucus

Mycoplasma species and combinations	Herds sampled	Herds with single infections	Herds with multiple infections	Individuals sampled	Individuals with single infections	Individuals with multiple infections
A. laidlawii					1	
M. bovigenitalium		24			61	
M. arginini					6	
M. species Group 7		1			4	
M. alkalescens					1	
M. bovigenitalium Untypable *Mycoplasma* sp.			4			
M. bovigenitalium *M. arginini*			2			
M. bovigenitalium *M.* species Group 7			15			41
M. bovigenitalium *M. alkalescens*						3
M. bovigenitalium *M. canadense*			1			1
M. bovigenitalium *M.* species Group 7 *M. alkalescens*			3			1
M. bovigenitalium *M.* species Group 7 *Ureaplasma* sp			4			1
Ureaplasma		9			20	
Untypable *Mycoplasma* sp.		10			15	
Totals:	151	44	29	1 053	108	47
%		29 %	19 %		10 %	5 %

The following formula was used for the broth media:

PPLO broth (Difco)[1]	19,34 g
Trypticase 5 % (BBL)[2]	10 mℓ
Starch (BDH)[3]	5 g
Thallium acetate 10 %	2,5 mℓ
Distilled water	900 mℓ
Pig serum (inactivated)	100 mℓ
Penicillin (Beecham)[4]	200 000 IU
Nicotinamide-adenine dinucleotide (reduced) disodium salt Grade 1	
NADH (BDH)[3]	10 mℓ

The starch was mixed with 5 mℓ of cold distilled water and stirred well until a paste had formed. While being stirred, water to 100 mℓ was added to the paste. The solution was then heated and shaken over a gas flame until it was quite clear.

Eight hundred mℓ of distilled water was poured into a 2 litre flask which already contained the broth. The mixture was shaken until dissolved, and starch, trypticase and thallium acetate were added.

The pig serum, penicillin and NADH were mixed and added to the above, and the pH set at 7,8.

The mixture was filtered through a Seitz EKS filter and dispensed in volumes of 2 mℓ into sterile broth tubes.

Processing of samples

Semen. With the aid of a wire loop a drop of the semen was deposited directly on agar and into broth.

Sheath wash. Samples were centrifuged at 1500 *g* for 10 min. Five mℓ of the supernatant fluid was taken and passed through an 0,65 millipore filter. Four drops was then deposited on an agar plate and 0,5 mℓ in a test tube which contained 2 mℓ culture broth.

Vaginal mucus. After collection in a plastic insemination pipette, the mucus was deposited in a 5 mℓ sterile bottle. On the arrival of the specimen at the laboratory, a sterile cotton bud was introduced into the bottle. This was rotated until enough mucus to cover its surface attached to it. This material was then plated, firstly on agar and then in broth. Where samples were taken initially for *Mycoplasma* isolations, i.e. using the cotton bud technique, they were directly plated on agar and thereafter immersed in culture broth.

Identification of isolates

Mycoplasma isolates were identified by the direct fluorescent antibody technique (Baas & Jasper, 1972), using conjugated gamma-globulin and, where possible, backed up by the well growth inhibition test (Dighero & Bradstreet, 1970). Wells, 5 mm in diameter, were made in the agar and a drop of hyperimmune serum was deposited in each.

The following monospecific hyperimmune rabbit antisera were used: *Mycoplasma bovigenitalium* PG11, *Mycoplasma bovirhinis* PG43, *Mycoplasma bovis* Donetta, *Mycoplasma bovoculi* M165/69, *Mycoplasma alkalescens* D12, *Mycoplasma verecundum* 107, *Mycoplasma* species Group 7, *Mycoplasma canadense* 275C, *Mycoplasma arginini* G230, *Acholeplasma axanthum* S743, *Acholeplasma laidlawwii* PG8 (sewage A) and *Acholeplasma modicum* PG49 (Squire). These type cultures were obtained from the National Collection of Type Cultures, London.

RESULTS

Females

Nine per cent of the herds proved positive for mycoplasmas only, while 38 % were positive for mycoplasmas plus additional organisms such as *Brucella, Chlamydia* and *Corynebacterium pyogenes*.

The spectrum of mycoplasma isolates and, in particular, their combinations proved most interesting. The species isolated and the incidence of recovery was *M. bovigenitalium* 6 %; *M. bovigenitalium* plus *M.* species

[1] Difco Laboratories, Detroit, Michigan, U.S.A.

[2] BBL, Cockeysville, Maryland, 21030, U.S.A.

[3] BDH, Chemicals Ltd, Poole, England

[4] Beecham Research Laboratories, Beecham Pharmaceuticals (Pty) Ltd, Sandton, R.S.A.

TABLE 2 Mycoplasmas isolated from bovine preputial washes

Origin	AI centres					Private herds
Individuals sampled	A	B	C	D	E	119
	115	37	10	21	34	882
Mycoplasma species and combinations						
A. laidlawii	2	1				5
M. bovirhinis				2	1	1
M. bovigenitalium	3	13		2	2	74
M. arginini					3	
M. bovis		1				5
M. species Group 7						12
M. alkalescens	2	2		1		7
M. canadense						1
Ureaplasma sp.	8	4			1	60
Untypable *Mycoplasma* sp.	4	2		1	4	27
M. bovigenitalium						
M. bovis						3
M. arginini						
M. bovis					2	
M. species Group 7						
M. alkalescens						1
A. laidlawii						
M. bovigenitalium						1
M. bovigenitalium						
M. bovirhinis						3
M. bovigenitalium						
M. species Group 7						16
Total positives	19	23		6	13	216
% positive	16	62		29	38	24
% single infections	16	62		29	32	21
% multiple infections	0	0		0	6	3

Group 7, 4 %; *Ureaplasma* sp. 2 %; *M. arginini* 0,6 %; *M.* species Group 7 0,4 %; *M. bovigenitalium* plus *M. alkalescens* 0,3 %; Untypable *Mycoplasma* sp. 1,4 %. One cow harboured *M. bovigenitalium; M.* species Group 7 and *M. alkalescens,* and one harboured *M. bovigenitalium; M.* species Group 7 and *Ureaplasma.*

Males

The 1 099 preputial samples, originating from 5 AI centres and 119 private herds (Table 2), were from both dairy and beef animals. Most bulls were sampled as often as 3 times during the annual testing of bulls at the AI stations.

Recoveries from AI centres varied from 16–62 %, and from private herds an average of 24 %. The frequency of recoveries was: *M. bovigenitalium* 9 %, *Ureaplasma* sp. 7 %, *M. bovigenitalium* plus *M.* species Group 7 1,4 %; *M.* species Group 7 1 %, *M. alkalescens* 1 %, *A. laidlawii* 0,7 % *M. bovis* 0,5 %, and untypable *Mycoplasma* sp. 3 %.

Semen samples. A total of 986 samples from 4 AI centres and 112 private herds were processed (Table 3).

Most bulls at AI centres were sampled 3 times. Recoveries from these stations varied from 33–52 %, and from private herds 41 %. The frequency of recoveries was: *M. bovigenitalium* 16 %, *Ureaplasma sp.* 10 %, *M. bovigenitalium* plus *M.* species Group 7 7 %, *A. laidlawii* 2 %, *M. alkalescens* 1 %, *M. bovis* 0,5 %, *A. modicum* 0,4 % and untypable *Mycoplasma* sp. 5 %.

Table 4 presents mycoplasmas isolated from the prepuce and semen of the bulls sampled. The percentage of positive specimens illustrates the variability of incidence at the different AI stations and in private herds.

Table 5 reflects the species identification of isolations from 100 bulls chosen at random. These bulls had been sampled annually for 3 consecutive years. A total of 49 % of sheath wash specimens were positive, and 89 % of semen samples.

M. bovigenitalium and *Ureaplasma* sp. were the most common isolates from both prepuce and semen samples. Six identified species were isolated from preputial specimens and 9 from semen, *M. conjunctivae* being the least common isolate.

By and large, *Mycoplasma* isolates from preputial washings were unrelated to isolates from semen.

Foetuses and placentas

Six species were isolated from foetal material, the most common being *M. bovigenitalium,* followed by *A. laidlawii.* Seven species were isolated from placentas, *A. laidlawii* being predominant, followed by a number of unidentified species (Table 6).

Of interest, was the isolation of *A. axanthum* from a bovine placenta, that no multiple infections were encountered, and that all untypable specimens were regarded as single infections.

DISCUSSION

The use of a variety of media in the cultivation of mycoplasmas enhances the frequency of primary isolations and laboratory maintenance (Al-Aubaidi & Fabricant, 1968). Four different media were therefore used in our experiment, and, isolations proved most satisfactory. More than one species was often isolated from a single specimen, and combinations varied significantly, with 2 cows producing 3 species each (Table 1).

An assessment of the pathogenicity of genital mycoplasmosis in bovines often depends on circumstantial evidence. It is generally accepted that some strains

TABLE 3 Mycoplasmas isolated from bull semen

	Origin				
	AI centres				Private herds
	A	B	C	D	112
Individual sampled	375	53	24	188	346
Mycoplasma species and combinations					
A. laidlawii	3		1	1	15
M. bovigenitalium	66	7	2	31	55
M. bovis	3	1		1	
M. species Group 7	1			1	1
A. modicum		1		3	
M. alkalescens	3	2		6	2
M. canadense	1			2	1
M. verecundum			1		
M. conjunctivae		1			
Ureaplasma sp.	36	3		22	34
Untypable *Mycoplasma* sp.	19	1	2	13	15
A. laidlawii *M. bovigenitalium*		2	1		
A. laidlawii *M. bovis*		1			
M. bovigenitalium *M.* species Group 7	23	8	1	15	19
Total positives	155	27	8	95	142
% positive	41	51	33	50	41
% single infections	35	30	25	42	36
% multiple infections	6	21	8	8	6

TABLE 4 Ratio between mycoplasmas recovered from sheath washes and semen expressed as a percentage

	No. of animals cultured		Percentage positive	
	Sheath wash	Semen	Sheath wash	Semen
AI stations A	85	102	26	51
B	20	40	85	57
C	4	8	75	25
D	15	23	40	39
Private herds	878	114	24	34
Totals	1 002	287	50	41

TABLE 5 Comparison of the frequency of *Mycoplasma* species isolated from preputial wash and semen of 100 bulls

Mycoplasma species	Preputial wash	Semen
A. laidlawii	2	2
M. bovirhinis	1	
M. bovigenitalium	14	42
M. bovis	1	7
M. species Group 7		7
M. alkalescens	5	7
M. conjunctivae		1
M. canadense		1
A. modicum		3
Ureaplasma	12	16
Untypable *Mycoplasma* sp.	4	3
% Negative	61	11
% positive	39	89

within a species are more pathogenic than others, (Al-Aubaidi *et al.*, 1972), that pathogenicity of local strains may vary and that the association with other organisms may complicate matters. Something which should be borne in mind is that certain species combinations may possibly enhance pathogenicity. Current literature does suggest, however, that mycoplasmas do play a role in bovine genital diseases. Admittedly, their effect is very often subclinical, but they are suspected of occasionally preventing conception or killing the fertilized ovum (Hirth *et al.*, 1966).

In the genital tracts of the cattle examined, the *Mycoplasma* flora was dominated by *M. bovigenitalium*, followed by *Ureaplasma* sp. The presence of *M. bovigenitalium* in cattle all over the world has been reported in several reviews (Gourlay, 1973; Tourtellotte & Lein, 1976). It is commonly isolated from the urogenital tract of cows with normal breeding cycles, from herds with infertility problems (Langford, 1975) and from cows with low fertility which could not be attributed to other causes. However, this syndrome could not be reproduced experimentally with this specific field strain.

TABLE 6 Mycoplasmas isolated from 722 foetal and 232 placental specimens

Mycoplasma species isolated	Foetuses		Placentas	
	Number pos.	% pos.	Number pos.	% pos.
A. laidlawii	6	(25)	14	(40)
M. bovirhinis	2	(8)		
M. bovigenitalium	7	(29)	4	(11)
M. arginini	1	(4)	4	(11)
M. species Group 7	3	(12)	2	(6)
M. axanthum			1	(3)
M. canadense			1	(3)
Ureaplasma	2	(8)	3	(8)
Untypable *Mycoplasma* sp.	3	(12)	6	(17)
Total	24	(3)	35	(15)

No *M. bovis* isolations were made out of cervico-vaginal mucus specimens (Table 1).

During the survey it was observed that cervico-vaginal isolations were certainly more frequent than uterine isolations.

Saed & Al-Aubaidi (1983) inseminated 12 heifers during oestrus with semen, derived from a highly fertile bull free of *Mycoplasma* infection, to which was added a pathogenic strain of *M. bovigenitalium*. All the inseminated heifers developed a granular vulvovaginitis, and some had a mucopurulent vaginal discharge. Six of the heifers were inseminated more than once, yet none conceived.

M. bovis which, next to *M. mycoides* ss. *mycoides*, is probably the most pathogenic bovine *Mycoplasma*, was responsible for 0,5 % of isolates for both sheath washes and semen samples. Hirth *et al.* (1964) added a *M. bovis* strain pathogenic to the bovine udder to bull semen, and inseminated 12 virgin heifers. Ten of the 12 heifers required multiple inseminations before conceiving. Four failed to conceive after 5 inseminations. At post-mortem, they showed varying degrees of chronic endometritis and salpingitis.

Kirchhoff (1982) stated that *M. bovigenitalium*, *M. bovis* and *Ureaplasma* play a role in genital disorders. *M. bovigenitalium* mostly produces vaginitis and vulvitis. *M. bovis* often causes severe forms of endometritis, salpingitis and salpingoperitonitis, often leading to the closure of oviducts and temporary or permanent sterility. Cervicitis, endometritis and salpingitis could be produced by ureaplasmas.

Ruhnke, Doig, Palmer & Mackay (1978) associated severe vulvular hyperaemia and granularity accompanied by a purulent discharge 5–10 days post AI with *Ureaplasma* infection. We obtained similar results (Trichard & Jacobsz, 1981, unpublished work).

The importance of ureaplasmas in repeat breeders and long term infertility is now recognized (Kuhn & Hopkins, 1983). Stalheim, Proctor & Gallagher (1976) in their study of the effects of ureaplasmas on bovine oviduct organ cultures, found that ciliary activity was stopped and epithelial lesions developed.

In conducting a survey of the prevalence of *Mycoplasma* infection in the oviducts of dairy cows, Hoare (1969), reported the isolation of mycoplasmas from the oviducts of 71 % of cows slaughtered on account of repeat breeding. It is significant to note that mycoplasmas were the only isolates.

The high percentage of *Mycoplasma* isolations made from AI bull semen is rather disturbing, and the probability of semen damage comprising spermadsorption and spermagglutination (Taylor-Robinson & Manchee, 1967; Barile, 1979) exists. A *M. bovigenitalium* has been incriminated as impairing spermatozoan motility (Jurmanova & Sterbova, 1977). Panangala, Winter, Wijesinha & Foote (1981) mixed *M. bovigenitalium* with bull semen and were able to demonstrate a highly significant time and dose dependent depression in sperm mobility.

Holzmann, Thiemann, Flatscher & Prilhofer (1982) noted that clinical and spermatological changes were more frequent when mycoplasmas were isolated from the ejaculate than from the prepuce alone. These changes included reduced motility and increased numbers of dead spermatozoa.

A hundred bulls taken at random from AI stations (Table 4) showed 39 % preputial wash and 89 % semen contamination. The low percentage of *Mycoplasma* isolations from the preputial cavity compared with that from semen is inconsistent with the usual concept that in the genital tract of bulls the most common habitat is the prepuce. A contributing factor to this phenomenon may have been the salt buffer solution used as a sheath wash. Admittedly, it is not the best transport medium available, but under prevailing conditions it was the most practical.

It must be remembered that the vast majority of sheath wash samples processed were collected for *Campylobacter* and *Trichomonas* isolations where buffered saline is the most satisfactory.

Additional data may still be required before the significance of mycoplasmas can be satisfactorily resolved. That they are very often regarded as commensals is true, however, considering their prevalence in the semen of bulls as found in this study, their potential significance cannot be ignored, and intensive study is warranted.

Most infected bulls do not show clinical symptoms, and the quality of their semen is not impaired, but they do constitute a permanent and important source of infection to cows. In this regard, we concur with the findings of Truscott & Abreo (1977).

While studies on trichomoniasis were being conducted in the Section of Reproduction, 2 bulls naturally infected with *M. bovigenitalium* were used to serve 17 heifers negative to genital *Mycoplasma* infection. After a single service, 14 of these heifers proved positive for *M. bovigenitalium* within 21 days. It would thus seem desirable that an antibiotic to which *Mycoplasma* is sensitive be added to AI semen be favourably considered and evaluated under practical conditions. Routine screening of semen for *Mycoplasma* contamination would be the ideal.

The isolation of mycoplasmas from only 3,3 % of aborted foetuses (Table 6) may be due partly to lysis of the foetuses, but whether these organisms made a substantial contribution to these abortions could not be verified. Considerably more isolations (15 %) were made from placental material. Soiling, which normally accompanies these specimens, and the dominance of *A. laidlawii* favours evidence of vaginal and external contamination.

These isolates can therefore not be regarded as of greater importance than the foetal isolations.

Available literature and our findings suggest that mycoplasmas cannot be regarded as an important cause of abortion but rather of low conception rates, caused by colonization of both male and female urogenital tissues. Embroyonic development may thus be hampered (Klein, Buckland & Finland, 1969).

The most interesting isolate, *A. axanthum*, came from a placenta that originated from a farm where cattle, geese and pigs are kept on the same premises.

Finally, the high frequency at which infection was detected stressed the fact that genital mycoplasmosis has become an important factor.

Although it is accepted with certain reservations that mycoplasmas often constitute part of the normal flora of the bovine genital tract, all circumstantial evidence considered, it is suggested that a high standard of breeding hygiene be maintained and that semen pollution should be limited to a minimum.

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