

MYCOPLASMAS OF THE UROGENITAL  
TRACT OF SHEEP

Alistair Gordon Rae

A thesis submitted for the degree  
Doctor of Philosophy  
University of Edinburgh  
1985



## CONTENTS

Acknowledgements	(i)
Declaration	(ii)
Dedication	(iii)
Abbreviations Used in Text	(iv)
Abstract	(vii)
CHAPTER 1 Review of the Literature	
Introduction	1
Mycoplasmas of the Urogenital Tract of Sheep	3
(1) Members of the Genus Mycoplasma	3
(2) Members of the Genus Acholeplasma	5
(3) Members of the Genus Ureaplasma (T-mycoplasmas)	6
Ureaplasmas of the Human Urogenital Tract	9
Ureaplasmas of the Bovine Urogenital Tract	11
CHAPTER 2 Materials and General Methods	
Media Components	16
Media Used for the Isolation of Mycoplasmas	20
Isolation and Cultivation of Mycoplasmas	23
Identification of Isolates	25
Bacteriological Techniques	30
Chlamydiological Techniques	30
Virological Techniques	31
Preparation of Antigens for Production of Hyperimmune Sera	31
Production of Hyperimmune Sera	32
CHAPTER 3 Assessment of Media for the Isolation and Cultivation of Ovine Ureaplasmas	
Section 1. The Ability of Different Ureaplasma Media to Support the Growth of Ovine Ureaplasmas	34
Section 2. Modifications to TA2 and TB2	38
Section 3. Modifications to TB2 Undertaken to Improve the Yield of Ovine Ureaplasmas	43

CHAPTER 3 cont'd

Section 4.	The Effect of Buffers on the Bulk Production of Ovine Ureaplasma	50
Section 5.	The Effect of Agitation on Broth Cultures of Ovine Ureaplasma	52
Section 6.	Dialysis Treatment of Bulk Cultures.	53
	Discussion	54
CHAPTER 4	Survey of the Mycoplasmas of the Ovine Urogenital Tract	
	Materials and Methods	62
	Results	
	Isolation of Mycoplasmas from the Urogenital Tract	64
	Isolation of Mycoplasmas from the Upper Urogenital Tract	68
	Discussion	71
CHAPTER 5	Investigations on the Pathogenicity of Ovine Ureaplasmas	
	<u>In vitro</u> Systems	81
	(i) Cell Cultures	81
	(ii) Organ Cultures	82
	(iii) Embryonated Hens' Eggs	83
	<u>In vivo</u> Systems	84
	(i) Mice	84
	(ii) Rats	85
	(iii) Guinea Pigs	86
	(iv) Hamsters	86
	(v) Mouse Mastitis Model	86
	(vi) Bovine Mastitis Model	88
	Results	
	<u>In vitro</u> Systems	89
	(i) Cell Culture Studies	89
	(ii) Organ Culture Studies	91
	(iii) Embryonated Hens' Eggs	92
	<u>In vivo</u> Systems	95
	(i) Rats, Hamsters and Guinea Pigs	95
	(ii) Mice	95
	(iii) Mouse Mastitis Model	97
	(iv) Bovine Mastitis Model	102
	Discussion	104

CHAPTER 6	Investigation on the <u>In vivo</u> Pathogenicity of Ovine Ureaplasmas	
	Materials and Methods	118
	Experimental Design	119
	Results	122
	Discussion	133
CHAPTER 7	Serological Studies with Ovine Ureaplasmas	
	Materials and Methods	144
	Results	
	(i) Growth Inhibition	148
	(ii) Mycoplasmacidal	148
	(iii) Metabolism Inhibition	149
	(iv) Indirect Immunofluorescence	149
	(v) Agar Gel Double Diffusion	151
	(vi) Polyacrylamide Gel Electrophoresis	154
	Discussion	156
CHAPTER 8	General Discussion	163
	Incidence of Mycoplasmas in the Ovine Urogenital Tract	167
	(i) Mycoplasmas	167
	(ii) Ureaplasmas	169
	Pathogenicity Factors of Ureaplasmas	174
	Serology of Ureaplasmas	179
References		184
Appendices		203
Publications		



ACKNOWLEDGEMENTS

I would like to thank all the people who have assisted me during the course of this thesis. I am particularly indebted to my two supervisors Drs. G.E. Jones and J.E. Phillips for their guidance, advice, criticism and careful reading of this manuscript. Most of the work was carried out in the Department of Microbiology and I am very grateful to my colleagues, Drs. W. Donachie and A. Herring, Messrs. P. Nettleton, I. Anderson, M. Quirie, N. Inglis and J. Redmond for performance of the bacteriology, virology, chlamydiology, protein estimations and polyacrylamide gel electrophoresis. I am particularly pleased to acknowledge the loyal support and excellent technical assistance provided by Miss A. Wood. My thanks also goes to Mr. G.W. Robinson and his staff for the unfailing supply of glassware, media and cell cultures, and to Messrs. B. Mitchell and D. Henderson and their staff for the supply and care of the experimental animals. I am also grateful to Messrs. J. Gilmour and E. Brown for advice and assistance with the pathological examinations, and to Messrs. B. Easter and A. Inglis for the excellent photographic work. My sincere thanks go to the librarian Mrs. J. Moon for her patience and assistance. I would also like to thank the staff at the Veterinary Investigation Centres in Scotland and England who supplied many of the survey samples.

Finally, I would like to add a special thanks to my wife Vivian, for her careful and speedy typing of the manuscript.

DECLARATION

I hereby declare that this thesis has been composed by myself and the work described has been carried out by myself or where jointly with others this fact has been acknowledged.

A. G. Rae

DEDICATION

I dedicate this thesis to my wife Vivian, for her invaluable support, and especially to my children, Caroline and Alan who have missed out on so much during the course of this study.

ABBREVIATIONS USED IN THE TEXT

AB	Broth used for cultivation of arginine-hydrolysing mycoplasmas
apd	Average pore diameter
BB	Bromothymol blue broth
BHK	Baby hamster kidney cells
BSS	Balanced salt solution
ccu	Colour changing units
cm	Centimetres
dai	Days after infection
D.P.X.	Mounting fluid, used in microscopical examination of cell cultures.
dw	Distilled water
FBS	Foetal bovine serum
FCA	Freund's complete adjuvant
FLB	Foetal lamb brain
FLK	Foetal lamb kidney
FLS	Foetal lamb skin
FLT	Foetal lamb Testes
g	Relative centrifugal force
GHL	Glycyl-L-histidyl-L-lysine acetate
GI	Growth inhibition
h	hours
HGM	Hog gastric mucin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonate
HIB	Heart infusion broth
IFA	Immunofluorescence antibody
Ig	Immunoglobulin
i/m	Intramuscular

i/n	Intranasal
i/p	Intraperitoneal
i/v	Intravenous
MC	Mycoplasmacidal
MES	(2-N-Morpholino) ethane sulphonate
min	Minutes
MI	Metabolism inhibition
ml	Millilitres
mm	Millimetres
MTM	Mycoplasma transport medium
MW	Molecular weight
NB2	Nutrient broth No. 2
NGU	Nongonococcal urethritis
OA,OB	Solid and broth media for cultivation of glycolytic mycoplasmas
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMN	Polymorphonuclear neutrophils
RT	Room temperature
SBA	Sheep blood agar
s/c	Subcutaneous
SPF	Specific pathogen-free
STh	Sheep thyroid
SUt	Sheep uterus
SV	Sheep vaginal epithelium
TA2,TB2	Media used initially for cultivation of ureaplasmas
TRIS	Hydroxymethylaminomethane
UA,UB	Media developed for cultivation of ureaplasmas
U.V.	Ultraviolet

U4	Bovine ureaplasma culture medium
U9C	Human ureaplasma culture medium
VTM	Virus transport medium
v/v	Volume by volume
w/v	Weight by volume

# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 7.9)

Name of Candidate ..... ALISTAIR G. RAE .....

Address ..... MOREDUN RESEARCH INSTITUTE, 408 GILMERTON ROAD, EDINBURGH .....

Degree ..... Doctor of Philosophy ..... Date ..... July, 1985 .....

Title of Thesis ..... Mycoplasmas of the Urogenital Tract of Sheep .....

No. of words in the main text of Thesis ..... 38,000 .....

### ABSTRACT

The role of mycoplasmas, especially ureaplasmas, in the urogenital tract of sheep was investigated both by survey and by pathogenicity experiments in vitro and in vivo.

The survey revealed that Ureaplasma are the most frequently isolated species from the ovine urogenital tract, and demonstrated an association between the occurrence of vulvovaginitis and the presence of ureaplasmas. M.capricolum and A.axanthum, two species not previously reported in the United Kingdom, were among the other seven species isolated.

Differences in virulence of the strains of ureaplasma isolated from sheep were investigated using both in vitro and in vivo systems. The in vitro systems used were cell and organ cultures as well as embryonated hens' eggs, but although none of these gave successful results, a cytopathic effect observed in ovine uterus cell cultures did suggest that these organisms are potentially pathogenic. The various in vivo systems investigated included inoculation of several animal species by a variety of routes. The production of mastitis in lactating mice after intramammary inoculation confirmed the pathogenicity seen in cell cultures but possible variations in virulence between strains were not identified by this model. However, the strains varied in their ability to induce mastitis in the lactating bovine udder, suggesting that variations in virulence do occur but are revealed only by in vivo systems.

Transmission experiments in sheep were carried out and demonstrated that inoculation of these organisms per vaginam resulted in vulvitis. They showed also the importance of venereal transmission in the spread of the organisms to previously unmated ewes, some of which developed vulvitis. Ureaplasmas could be consistently recovered from all animals which developed the disease. The vulvitis did not affect conception rates or the reproductive efficiency of the ewes.

Two serological tests (metabolism-inhibition and indirect immunofluorescence) demonstrated that at least three groups of ovine ureaplasmas occur in the United Kingdom. All 106 strains examined in this study could be assigned to one of the three groups, using antisera prepared in specific pathogen-free lambs against a representative of each group.

## CHAPTER ONE

### REVIEW OF THE LITERATURE

#### INTRODUCTION

The first isolation and description of a mycoplasma was by Nocard and Roux in 1898, when a minute and filterable organism, now known as Mycoplasma mycoides subsp.mycoides, was cultivated from a case of contagious bovine pleuropneumonia. Twenty-five years later Bridr  and Donatien (1923) isolated M.agalactiae, the causative agent of contagious agalactia of sheep and goats. The importance of M.agalactiae and the disease it produced in sheep and goats may have inhibited the search for mycoplasmas from other anatomical sites in sheep because reports of the isolation of mycoplasma from sheep were very few until the 1960's. However, the discovery in the early 1960's that Eaton's agent, which had been isolated from a case of primary atypical pneumonia in man and maintained in cotton rats and eggs for 20 years, was not a virus but a mycoplasma (Hayflick, 1965), stimulated greater interest in this group of organisms. This increasing interest can be measured by the greater numbers of isolations of mycoplasma made from sheep. Prior to 1968 only M.agalactiae had been positively identified as a recognised species. Just over a decade later, in 1979, the number of recognised mycoplasma species known to occur in sheep had grown to nine, namely M.arginini, (Barile, Del Giudice, Carski, Gibbs and Morris, 1968), M.capricolum (Swanepoel,



Efstratiou and Blackburn, 1977), M.conjunctivae (Barile, Del Giudice and Tully, 1972), M.mycoides subsp.mycoides (Al-Aubaidi, Dardiri and Fabricant, 1972 ; Ernø, Freundt, Krogsgaard-Jensen and Rosendal, 1972), M.ovipneumoniae (Carmichael, St. George, Sullivan and Horsfall, 1972), A.laidlawii (Krauss and Wandera, 1970), and Ureaplasma sp. (Livingston and Gauer, 1975) This list does not include those mycoplasmas which have been isolated once or rarely from sheep, or those which are normally associated with other hosts, namely M.bovis, (Cottew, 1979), M.bovirhinis (Cottew, 1970, 1974) and A.granularum (Cottew, 1979). Also not included are the strains, as yet unnamed but designated 2D, from the urogenital tract of sheep in Australia (Carmichael, et al., 1972).

The isolations of mycoplasmas from sheep and the conditions with which they have been associated have been fully reviewed by Jones (1978) and Cottew (1979).

The increase in the number of mycoplasma species isolated from sheep has been mirrored in other hosts, and this has necessitated changes in the taxonomy of the Mycoplasmatales. In 1967 the order Mycoplasmatales was placed in a new class, Mollicutes, containing one family, Mycoplasmataceae (Edward and Freundt, 1967). Twelve years later three families had been established, Mycoplasmataceae, containing two genera, Mycoplasma and Ureaplasma; Acholeplasmataceae, containing the single genus Acholeplasma; and Spiroplasmataceae, containing the genus Spiroplasma. (Subcommittee on the Taxonomy of Mycoplasmas, 1979). Table 1 gives the present classification according

to Freundt and Edward (1979) and Freundt (1983). The isolation of mycoplasmas from the ovine urogenital tract is less well documented and is reviewed here.

### Mycoplasmas of the Urogenital Tract of Sheep

Interest in mycoplasmas of the sheep urogenital tract increased during the period of this study, and several articles appeared in the literature. This section will therefore review only publications which appeared prior to December 1979.

#### (1) Members of the Genus Mycoplasma

The occurrence of classical, large-colony mycoplasmas in the ovine urogenital tract has been reported by several authors. The first reported isolations from this site were by McIlwain and Bolin (1967), who recovered mycoplasmas from the testes of three lambs with orchitis. Their isolates were non-glycolytic and non-haemolytic and stained positively in the immunofluorescence technique with M.gallisepticum antiserum. However, the identity of these isolates remains doubtful since M.gallisepticum is both glycolytic and haemolytic.

In Australia, Carmichael et al. (1972), isolated mycoplasmas from seven vaginal and three preputial swabs taken from a flock in which there was a high incidence of clinical pneumonia. These isolates were found to be of one biotype, designated 2D, which was not related serologically to any of 11 other serotypes of ovine or caprine

mycoplasmas. Biotype 2D was similar to M.agalactiae, differing only in its ability to reduce tetrazolium aerobically.

Cottew, Lloyd, Parsonson and Hore (1974), also in Australia, isolated similar mycoplasmas which they designated SH from 9 of 13 cases of vulvovaginitis in sheep. These isolates were found by growth-inhibition tests to belong to one serological type which was closely related to M.mycooides subsp. mycooides, but which differed from this species by not fermenting glucose, by producing a surface film on horse serum medium and by reducing phosphatase. The SH strains differed serologically from the biotype 2D strains of Carmichael et al. (1972). Experimental inoculation of these strains by subcutaneous, intraperitoneal, supraocular, intravenous and intravaginal routes into pregnant ewes failed to reproduce genital disease, although the mycoplasma proliferated in the vagina of some, and was recoverable from two sheep for as long as 122 days. One sheep infected intravenously, yielded the organism from the uterus at necropsy five weeks later. From this strong circumstantial evidence, namely easy isolation of the organism from the vagina of natural cases, and its persistence in experimentally infected sheep, the authors considered the mycoplasma was responsible for the vulvovaginitis observed in the outbreak.

Bar-Moshe, Goldberg-Weiss, Steiff and Israeli (1974) carried out a survey on the prevalence of mycoplasmas in sheep and cattle in Israel. Of the ovine

material examined, four isolates were obtained from 39 aborted fetuses, one from the placentae of eight aborted fetuses and one from a single preputial swab. Biochemical characterisation or serological identification of the isolates was not reported.

In Turkey, numerous cases of abortion were seen in sheep which had been vaccinated with a sheep-pox vaccine: mycoplasmas, which were not identified or characterised, were isolated from the vaccine and from the aborted fetuses of sheep inoculated with the vaccine (Aktan, Güley and Doguer, 1955).

M.ovipneumoniae has been isolated from the reproductive tract of normal sheep in England (Leach, Cottew, Andrews and Powell, 1976) and Texas, (Livingston and Gauer, 1978). M.ovipneumoniae is more usually isolated from the respiratory tract and is commonly found in sheep with pneumonia but may also occur in healthy sheep (Carmichael et al, 1972; Jones, Foggie, Mould and Livitt, 1976).

The only report of M.arginini from the ovine urogenital tract was by Jones and Rae (1979), who isolated the organism from two of 160 vaginal or preputial swabs taken from normal sheep.

## 2) Members of the Genus Acholeplasma

Acholeplasma laidlawii has been isolated from vaginal swabs taken from normal sheep in Australia (Cottew, 1974), from vaginal swabs and semen of sheep in England (Leach, et al., 1976) and from a preputial swab of a tup in Scotland (Jones and Rae, 1979). In India,

mucosal scrapings taken from the uterine mucosa of 40 apparently healthy sheep yielded 12 A.laidlawii isolates (Kumar and Pathak, 1979). Doig and Ruhnke (1977), in Canada, isolated A.laidlawii from one of 19 vaginal swabs taken from ewes with vulvitis. A.laidlawii is generally considered to be saprophytic (Whittlestone, 1972) but Olson, Seymour, Boothe and Dozsa (1960) associated A.laidlawii with pathological changes in the genital tract of cattle. Hoare (1969) isolated A.laidlawii from a high percentage of repeat breeder cows with lesions of the oviduct and/or ovaries. These findings led the authors to suggest that in some instances A.laidlawii may be an opportunist pathogen.

(3) Members of the Genus Ureaplasma (T-Mycoplasmas)

The first recorded isolation of ureaplasmas from the ovine urogenital tract was by Livingston and Gauer (1975) in Texas, who isolated ureaplasmas from the urine of 3 of 42 lambs, 18 of 34 aged ewes and one goat, and from the vagina of two goats. These observations were extended in a subsequent study (Livingston and Gauer, 1978), when ureaplasmas were isolated from 22 clinically normal sheep, 32 of 115 vaginal or cervical swabs, 11 of 17 semen samples and 8 of 75 preputial swabs from wether lambs. No abnormalities were reported in any of these animals.

In Northern Ireland, McCaughey, Ball and Irwin (1979) reported the isolation of ureaplasmas from progesterone impregnated vaginal sponges after their removal from 60

ewes in an experimental crossbred flock. The sponges had been in place for 12 days. Heavy bacterial contamination affected mycoplasmological examinations, but 30 samples gave a positive urease colour change, and ureaplasmas were confirmed in two. No clinical abnormalities of the vulva or vagina had been noted in this flock, in which the conception rate was 78%.

Ureaplasmas have been isolated from three normal sheep flocks in Scotland (Jones and Rae, 1979). Vaginal swabs collected from 79 ewes, ranging from two to nine years old, yielded 66 (85%) ureaplasma isolates and 32 (83%) of the preputial or vaginal swabs taken from 39 lambs born to these ewes were also positive for ureaplasmas.

An association between ureaplasma infection and granular vulvitis in sheep has also been reported. Doig and Ruhnke (1977) examined a group of 27 sheep, part of a flock of 40, 19 of which had a mild vulvitis. The disease was characterised by hyperaemia of the vulvar epithelium, associated with excess mucus production. A very fine granularity of the vulvar epithelium was seen in 7 of the 19 affected ewes. Ureaplasmas were isolated from 18 of the 19 ewes with vulvitis and from 4 of 8 apparently healthy flock mates. The ewes had been running with rams for four months, and although all were presumed to be pregnant, only 20 of the 40 were subsequently found to be so. Experimental transmission studies were performed on three, one month old lambs and one ewe. Vaginal swabs taken from these animals prior to infection did not yield mycoplasmas or ureaplasmas. Animals were infected by swabbing the

vulvar epithelium with a cotton wool swab which had been soaked in a ureaplasma broth culture. Hyperaemia and slight granularity were observed in the vulvar epithelium of the lambs 11 days after-infection (dai) and in the ewe at 18 dai. Ureaplasmas could be recovered from the vulva up to seven weeks after infection. It was suggested that the low conception rate in the natural outbreak could have been due to the vulvitis.

Livingston, Gauer and Shelton (1978) studied 19 ureaplasma strains isolated from the urogenital tract of sheep. Three of these isolates were obtained from the uterus of ewes which were known to have been mated and had been shown to be pregnant by laparotomy, but which were not pregnant at necropsy 45 days later. Two pregnant ewes inoculated with one of these strains aborted, but the route of inoculation, the stage of gestation at inoculation, or the time of abortion were not reported.

These reports indicate conflicting roles postulated for ureaplasmas in the ovine urogenital tract. The isolations from normal sheep (Livingston and Gauer, 1975, 1978; McCaughey et al., 1979; Jones and Rae, 1979), suggest that ureaplasmas may be commensal in the urogenital tract and that their association with granular vulvitis (Doig and Ruhnke, 1977) and infertility and abortion (Livingston, et al., 1978), is purely coincidental. However, ovine ureaplasmas are not homogeneous, as shown by Livingston et al. (1978), who examined 19 ureaplasma isolates from the ovine urogenital tract and identified nine major



serological groups. One serotype was associated with uterine infection which resulted in infertility. It is possible, therefore, that only certain serotypes are pathogenic.

The pathogenicity of ureaplasmas in the human and bovine urogenital tracts is also uncertain and controversial. As both these areas have been more extensively studied it is worthwhile reviewing the relevant literature to assess the problems involved in determining the role of ureaplasmas in urogenital tract disease.

#### Ureaplasmas of the Human Urogenital Tract

The literature relating to ureaplasmas in the human urogenital tract has been extensively reviewed by Taylor-Robinson(1979).

Ureaplasmas were first isolated from the urethral discharge of men with non-gonococcal urethritis (NGU) (Shepard, 1954). Since then they have been isolated from a variety of sources (Taylor-Robinson, 1979). The role of ureaplasmas in human urogenital tract disease has been controversial since their first isolation. The weight of evidence is enough to suggest that ureaplasmas may be a cause of NGU in a proportion of cases. The difficulty in obtaining appropriate controls has meant that epidemiological studies have not been particularly helpful in assessing the role of ureaplasmas in NGU, but human and animal inoculation experiments have proved more useful. Human ureaplasmas introduced into the urethra of



chimpanzees multiplied in the urethra and stimulated a polymorphonuclear (PMN) response (Taylor-Robinson, Purcell, London and Sly, 1978). Experimental infection of humans has been confined to two volunteers, both workers in the field, who inoculated themselves intraurethrally with a strain of ureaplasma which had been isolated from a case of NGU (Taylor-Robinson, Csonka and Prentice 1977). Both subjects developed urethritis and ureaplasmas and PMN leucocytes were present in the urine. Antibiotic therapy resulted in the elimination of the ureaplasmas in both subjects, although PMN leucocytes persisted in the urine of one subject for a further six months. These observations demonstrate that ureaplasmas are pathogenic in the male urogenital tract, but more work is required to determine the actual importance of ureaplasmas as a cause of NGU.

In women a strong association has been noted, between spontaneous abortion and the presence of ureaplasmas, either in the cervix of the mother or in the aborted foetus (Kundsinn and Driscoll, 1970), but conclusive evidence that ureaplasmas may cause abortion is lacking. Ureaplasmas have also been found to be associated with chorioamnionitis (Shurin, Alpert, Rosner, Driscoll, Lee, McCormack, Santamarina and Kass, 1975) and with low birthweight but not decreased gestational age (Braun, Lee Klein, Marcy, Klein, Charles, Levy and Kass, 1971). However, evidence is again lacking that ureaplasmas are directly responsible for these conditions.

In one study ureaplasmas were isolated from cases of nonspecific vaginitis (Romano and Romano, 1968), but a separate investigation (Mårdh and Weström, 1970) was unable to demonstrate any significant difference in the isolation rate of ureaplasmas from cases of urogenital tract infection, including cervicitis and vaginitis, compared with a control group of women with no signs of disease. Experimentally, human ureaplasmas have been shown to cause mastitis in cattle (Howard, Gourlay and Brownlie, 1973), goats (Gourlay, Brownlie and Howard, 1973) and mice (Howard, Anderson, Gourlay and Taylor-Robinson, 1975).

These various studies illustrate the highly uncertain state of knowledge concerning the role of ureaplasmas in conditions of the human urogenital tract.

#### Ureaplasmas of the Bovine Urogenital Tract

The first reported isolation of ureaplasmas from the bovine urogenital tract was in 1967 (Taylor-Robinson, Haig and Williams, 1967). In subsequent studies ureaplasmas were isolated from the vaginal mucus of 11-14% of fertile cows and heifers (e.g. Langford, 1974; Onoviran, Truscott, Fish, Barker and Ruhnke 1975). Slightly higher isolation rates (26%) were obtained from the vaginal mucus of repeat breeder cows, but this difference was not statistically significant (Panangala, Fish and Barnum, 1978). The first report which associated ureaplasma with bovine urogenital tract disease was by Anderson (1974), who isolated ureaplasmas from 9 of 11 vulvar samples from cows that developed a purulent vulvar discharge shortly after

service and from the frozen semen used to inseminate one of the cows. In 1978 Doig and Ruhnke reported on the association of ureaplasmas with bovine granular vulvitis (BGV). A subsequent study on this condition (Ruhnke, Doig, MacKay, Gagnon and Kierstead, 1978) revealed that 100% of vulvar swabs from acute cases of BGV, 74% from chronic cases and 23.5% from clinically normal herd-mates yielded ureaplasmas. Examination of breeding records suggested that the infection had a significant effect on the fertility of affected cows.

In contrast, Langford, Jurmanova and Stalheim (1978) did not observe such an association in cattle and found no significant evidence to implicate ureaplasmas with reduced conception rates. A report by Jurmanova and Sterbova (1977) stated that spermatozoa in bovine semen samples which contained ureaplasmas were less motile than those in samples which were free of ureaplasmas, but the extent to which the ureaplasmas were responsible was not determined. A separate study (Taylor-Robinson, Thomas and Dawson, 1969) demonstrated that the conception rate of cows inseminated with ureaplasma-containing semen was similar to that of cows given semen from which ureaplasmas were not recovered.

Ball, Neill, Ellis, O'Brien and Ferguson, (1978) isolated ureaplasmas more frequently from the vaginal mucus of cows which had aborted than from control animals. However, it was not possible to deduce from their evidence whether the organisms were directly involved in the abortions. In a follow up investigation Ball and McCaughey

(1979) studied the distribution of mycoplasmas within the urogenital tract of cows and found ureaplasma infection to be limited to the lower genital tract. It seems unlikely, therefore, that ureaplasmas can colonise the upper genital tract of normal cows. However, an in vitro study has shown that bovine ureaplasmas can damage bovine oviducts in organ culture (Stalheim, Procter and Gallagher, 1976), suggesting the possibility of infertility if infection of the oviduct occurs in vivo.

#### AIMS OF THE THESIS

Mycoplasmas, and ureaplasmas in particular, have frequently been found in the ovine urogenital tract, but their role, if any, in disease remains largely unknown.

The aim of this study was to assess the importance of mycoplasmas and ureaplasmas in diseases of the ovine urogenital tract, to study the occurrence and incidence of ureaplasma serotypes in various sheep populations, to investigate their association with naturally occurring disease and to determine pathogenicity by in vitro and in vivo experiments.

Table 1. Taxonomy of the Class Mollicutes.

Class: Mollicutes.

Order: Mycoplasmatales.

Family 1: Mycoplasmataceae.

1. Require sterol for growth.
2. Genome size  $5 \times 10^8$  daltons.
3. NADH oxidase in cytoplasm.

Genus 1: Mycoplasma

1. 54 species
2. Most species require either glucose or arginine as energy source.
3. Do not hydrolyze urea.
4. G+C content of DNA ranges from 22.8 - 40.8 moles%.

Genus 2: Ureaplasma

1. Two species, each with several serotypes.
2. Hydrolyze urea but do not utilise glucose or arginine
3. G+C content of DNA ranges from 27.7 - 28.5 moles%.

Family 2: Acholeplasmataceae

1. Do not require sterol for growth.
2. Genome size  $1.0 \times 10^9$  daltons.
3. NADH oxidase in membrane.

Genus 1: Acholeplasma

1. Seven species.
2. Ferment carbohydrates, do not utilise arginine or urea.
3. G+C content of DNA ranges from 29.3 - 35.7 moles%.

Family 3: Spiroplasmataceae

1. Helical morphology during some stage of growth.
2. Sterol required for growth.
3. Genome size  $1.0 \times 10^9$  daltons.
4. NADH oxidase in cytoplasm.

Genus 1: Spiroplasma

1. Three species.
2. Glucose and mannose fermented.

Genera of Uncertain Taxonomic Position.

I. Anaeroplasma

1. Two species.
2. Strict anaerobes.
3. Sterol dependent and sterol independent strains known.
4. G+C content of DNA ranges from 29.3 - 40.3 moles%

II. Thermoplasma

1. Only one species.
  2. Strict aerobes.
  3. Temperature range for growth 45-62°C.  
Optimum 59°C.
  4. pH range for growth 0.5-4.0. Optimum 2.0.
  5. Do not require sterol.
  6. Genome size  $1 \times 10^9$  daltons.
  7. G+C content of DNA 46 moles%.
-

## CHAPTER TWO

### MATERIALS AND GENERAL METHODS

Mycoplasmas are fastidious in their cultural requirements, yet because of the small size of their genome they have limited biosynthetic capabilities and therefore require to be supplied with a large number of precursors for the synthesis of macromolecules. Many complex media have been formulated for the isolation and cultivation of mycoplasmas and most contain serum as a source of lipoproteins, especially cholesterol, (Washburn and Somerson, 1979) yeast extract for growth factors, a bacteriological broth such as beef heart or brain-heart infusion broths, DNA to supply nucleic acid precursors and various salt solutions which are used to adjust the tonicity of the complete medium. As there is a large number of mycoplasma media in use, it is pertinent to list the source or preparation of the components which were included in the media employed in this study.

#### Media Components

All components were sterilised through membrane filters of 0.22  $\mu\text{m}$  average pore diameter (apd) (Millipore Corporation, Bedford, Mass. U.S.A.) unless otherwise stated.

1. Agar - two types were used:

- (i) Agarose For Electrophoresis  
(BDH Chemicals, Ltd. Poole, Dorset)
- (ii) Agar Noble (Difco Laboratories, Detroit, Michigan, U.S.A.)

Both were made up in sterile distilled water (dw) to give concentrations of 0.9% or 1% in the complete media.

2. L-arginine hydrochloride. (Sigma Chemical Company, St. Louis, Missouri, U.S.A.). A 20% (w/v) stock solution was prepared in dw and stored at 4°C.

3. Bacteriological broths - two types were used.

(i) Brain-heart infusion broth (Difco Labs.). The lyophilised powder, reconstituted by adding 37g to 1 litre dw, was sterilised by autoclaving at 121°C for 15 minutes.

(ii) Hartley's digest broth (Oxoid Ltd.). Lyophilised powder, reconstituted by adding 29g to 1 litre of dw, was sterilised by autoclaving at 121°C for 15 minutes.

4. Buffers - four types were used.

(i) N-2-hydroxyethylpiperazine  
N'-2-ethanesulphonate (HEPES) (BDH Chemical Co.)  
Stock solution of 0.05M.

(ii) Hydroxymethylaminomethane (TRIS) (BDH Chemical Co.) Stock solution of 0.05M.

(iii) (2-N-Morpholino) ethane sulphonate (MES) (BDH Chemical Co.) Stock solution of 0.05M.

(iv) Phosphate buffer, which comprised 2 components:

(a) Di-sodium hydrogen orthophosphate  
(Analar, BDH Chemical Co.)

(b) Potassium dihydrogen orthophosphate  
(Analar, BDH Chemical Co.)

Stock solutions of 1.5 M were used in a mixture of 7 parts (a) to 3 parts (b).



5. Cysteine hydrochloride (BDH Chemical Co.). Stock solution 0.2% (w/v) in dw.
6. Deoxyribonucleic acid (sodium salt). (BDH Chemical Co.) From calf thymus gland, stock solution 0.2% (w/v) in dw.
7. Digitonin (Sigma Chemical Co.) Stock solution 1.5% (w/v) in ethanol.
8. Dithiothreitol (Sigma Chemical Co.) Stock solution 10% (w/v) in dw.
9. Manganous sulphate (BDH Chemical Co.) Stock solution 1.5% (w/v) in dw.
10. Medium 199 (Wellcome Diagnostics, Dartford, Kent). Supplied as a 10X solution without sodium bicarbonate or glutamine.
11. Phenol red (BDH Chemical Co.). Stock solution 0.4% in 0.0125M sodium hydroxide. Sterilised by steaming overnight.
12. Sera
  - (i) Foetal Calf ) (Gibco Biocult.)
  - (ii) Newborn Calf) Mycoplasma &
  - (iii) Lamb ) virus
  - (iv) Horse ) screened
  - (v) Swine - prepared from blood collected at local abattoir.
  - (vi) Rabbit - prepared from Moredun stock.
  - (vii) Guinea pig - prepared from Moredun stock.
13. Urea (Aristar, BDH Chemical Co.). Stock solution 10% (w/v) in dw.

14. Yeast extract. Prepared at Moredun following the method of Hers, cited by Marmion (1967). One kg. of fresh bakers yeast, was added to one litre of warm (40°C) dw. The yeast and water were well mixed until a homogenous suspension was obtained. The mixture was heated to 80°C, 6.5ml of concentrated HCl was added, and the temperature was maintained at 80°C for 30 minutes with continuous stirring. After cooling the suspension was centrifuged at 1000g for 20 mins then filtered through Whatman's No.2 filter paper. The filtrate was adjusted to pH 4.5 with HCl and sterilised by filtration. Aliquots of 100 ml were stored at -20°C. Fresh batches of yeast extract were prepared every three months, any unused extract being discarded after this period.

15. Bacterial and Fungal Inhibitors.

(i) Ampicillin (Penbritin, Beecham Animal Health, Brentford Middlesex.). Stock solution of 100mg per ml in dw.

(ii) Amphotericin B (Fungizone, E. J. Squibb, Princeton, New Jersey, U.S.A.) Stock solution of 0.5mg per ml in dw.

(iii) Thallous acetate (BDH Chemical Co.). Stock solution 10% (w/v) in dw.

Other reagents.

1. Nutrient Broth No.2(NB2) (Oxoid Ltd.). Lyophilised powder reconstituted by adding 25g to 1 litre of dw. Dispensed in 10ml amounts and sterilised at 121°C for 15 mins.

2. Phosphate Buffered Saline (PBS) Prepared at Moredun Research Institute as a 0.15M solution pH 7.2.

Media used for the isolation of mycoplasmas.

The formulae of these media are given in Table 2.

OA and OB

These media, described by Jones, Foggie, Mould and Livitt (1976), were originally formulated for the isolation and cultivation of M.ovipneumoniae but have subsequently been shown to be suitable for the growth of most glycolytic mycoplasmas. Both media were adjusted to pH 7.6 to 7.8.

AB

This broth medium devised for the isolation of arginine-hydrolysing mycoplasmas, was similar to OB but included arginine hydrochloride at a final concentration of 1% (w/v). The medium was adjusted to pH 6.6 to 6.8. The solid medium OA was found to be satisfactory for the cultivation of arginine-hydrolysing mycoplasmas.

TA2 and TB2

These media, which were used for the isolation of ureaplasmas at the start of this study, were similar to OB and OA but contained 20% swine serum and 50% medium 199 and were supplemented with 0.1% final concentration of urea, 0.1% dithiothreitol, 0.002% calf thymus DNA and 0.02% thallos acetate. These media had some drawbacks both in preparation and in use, and early on in the study it was decided that they should be compared with other widely-used

ureaplasma media to determine the best medium for further use. This media assessment is detailed in Chapter Three.

#### Mycoplasma transport medium (MTM)

MTM was used to maintain the viability of mycoplasmas during transport of swabs and tissue samples. The initial formulation of Jones, Gilmour and Rae (1978) was modified, initially by the omission of thallos acetate since this substance has been shown to suppress the growth of human ureaplasmas (Black, 1973) and might have affected ovine ureaplasmas in a similar manner. However, this step resulted in heavy contamination by fungi: this was largely overcome by the addition of amphotericin B at a final concentration of 5µg per ml (w/v) to the transport medium. MTM was dispensed in 4ml amounts and stored at -20°C until required.

Culture media were prepared according to the formulae in Table 2. For routine use 500 ml or 1 litre batches were prepared and dispensed in 1.8 ml volumes and stored at -20°C until required. All broths and plates in daily use were stored at 4°C. Plates were discarded after three weeks storage, freshness of preparation being particularly important for plates used for the isolation of ureaplasmas. Media used to grow antigens for the preparation of hyperimmune sera, or for use in serological tests, were prepared in 5 or 10 litre volumes, which were sterilised by filtration through 0.22µm apd membrane filters, and stored at -20°C while sterility checks were performed. Neither ampicillin, to avoid loss of activity during storage, nor

TABLE 2

## FORMULAE OF GROWTH AND TRANSPORT MEDIA

	VOLUME IN 100 ml.						MTM
	OB	AB	TB2	OA	TA2		
Distilled Water	54	49.5	43.2	45	43.2	-	
10X Medium 199	6.0	5.5	4.8	5.0	4.8	-	
Brain Heart Infusion Broth	20	20	20	20	20	90	
Yeast Extract	10	10	10	10	10	5	
Swine Serum	10	10	20	20	20	5	
Arginine (20%)	-	5	-	-	-	-	
Urea (20%)	-	-	0.5	-	0.5	-	
Dithiothreitol (10%)	-	-	0.5	-	0.5	-	
Calf Thymus DNA (0.2%)	-	-	1.0	-	1.0	-	
Phenol Red (0.4%)	1.5	1.5	1.5	1.5	1.5	-	
Thallos Acetate (10%)	0.25	0.25	0.25	0.25	0.25	0.25	
Ampicillin (100 mg/ml)	1.0	1.0	1.0	1.0	1.0	1.0	
Agarose (w/v)	-	-	-	0.9	0.9	-	
pH	7.6-7.8	6.6-6.8	6.0-6.2	7.6-7.8	6.0-6.2	7.0-7.2	

Final concentrations of supplements: Ampicillin: 1mg/ml, Arginine: 1%, Calf Thymus DNA 0.002%,

Dithiothreitol: 0.05%, Thallos acetate: 1 in 4000,

Urea: 0.1%, Phenol red: 0.006%

amphotericin B, as it is a suspension and is removed by filtration, were added at this time.

#### Isolation and cultivation of mycoplasmas

All samples for mycoplasma isolation were submitted in MTM. Samples submitted by Veterinary Investigation Centres which had been in transport for 24 hours or more were generally processed without further treatment. Freshly taken tissues and swabs were thoroughly shaken in MTM and incubated at 37°C for 60 mins to allow the microbial inhibitors to act before processing began. Kaklamanis, Thomas, Stavropoulous, Borman and Boshwitz (1969) demonstrated that ground tissue releases substances which are inhibitory to mycoplasmas. To avoid this, tissues were coarsely chopped and approximately 1g was added to nine ml NB2 and processed by a Colworth Stomacher (A.J. Seward, London) to give a 10% suspension. Swabs in MTM were thoroughly mixed using an automatic mixer (Autovortex, Stuart Scientific Co., England). Prepared samples were inoculated into the three broths OB, AB and TB2 and streaked onto the two solid media OA and TA2. Semen, vaginal mucus and preputial washings were titrated in ten-fold steps to determine the numbers of organisms present. The titre was the highest dilution of broth which gave a colour change, and was expressed as colour-changing units (ccu) per 0.2ml. For these particular samples the initial dilution in MTM was regarded as 1 in 10. Broth cultures were incubated aerobically and solid media in a humidified atmosphere of 95% air, 5% CO<sub>2</sub> at 37°C. TA2

plates were initially incubated under various gaseous conditions to determine the best environment for optimum growth. The findings are discussed in Chapter Three.

All broth cultures were examined daily for signs of growth as indicated by pH change or slight turbidity, and subcultured if considered necessary to fresh broth and solid medium. Cultures showing heavy turbidity indicative of bacterial contamination were filtered through membrane filters of 0.45 $\mu$ m apd before subculture. Because some mycoplasma species do not produce any colour change in the growth media, blind subcultures to fresh OB and OA were performed with all OB cultures which showed no colour change after seven days incubation. Broths were kept for at least three weeks before being discarded as negative.

Plates were examined three times a week for growth of colonies. Where necessary, for example if the corresponding broth culture of a positive plate gave no colour change or if the broth was contaminated, subculture was performed by excision and transfer of a block of agar-bearing colonies to fresh broth or a fresh plate.

#### Cloning of mycoplasmas

Isolates were cloned from plates with well separated colonies. A capillary Pasteur pipette was used to remove and transfer a plug of agar containing a single colony to broth, which was then incubated until growth was observed. The culture was passed through a membrane filter of 0.45 $\mu$ m apd and the filtrate streaked onto a fresh plate. This procedure was repeated at least three times as recommended by the Subcommittee on The Taxonomy of Mollicutes (1979).

All ureaplasma isolates and some large colony isolates were stored as broth cultures at  $-70^{\circ}\text{C}$ .

#### Identification of isolates

All large colony mycoplasmas were identified by the growth inhibition and, or, indirect immunofluorescence tests.

Ureaplasma isolates were identified by their ability to metabolise urea, indicated by the development of an alkaline pH in the ureaplasma broth, and by their colony morphology. At an early stage of the study manganous sulphate was added to the solid medium (Shepard and Lunceford, 1976). This facilitated identification of ureaplasmas, especially in mixed cultures, since ureaplasma colonies stain dark brown - black while other mycoplasmas do not. (Fig 2.1).

#### Growth inhibition test (GI)

The GI test was performed according to the method of Clyde (1964) as modified by Dighero, Bradstreet and Andrews (1970). Blank paper discs (Mast Laboratories Liverpool) saturated with  $12\ \mu\text{l}$  of specific antiserum, were lyophilised and stored at  $4^{\circ}\text{C}$  until required. Digitonin, used for the differentiation of non-sterol-requiring acholeplasmas from sterol-requiring mycoplasmas and ureaplasmas, was also used in the GI test. Discs were prepared by the method of Freundt, Andrews, Ernø, Kunze and Black (1973) by adding  $25\ \mu\text{l}$  of a 1.5% (w/v) ethanolic solution of digitonin to each disc. Discs were dried

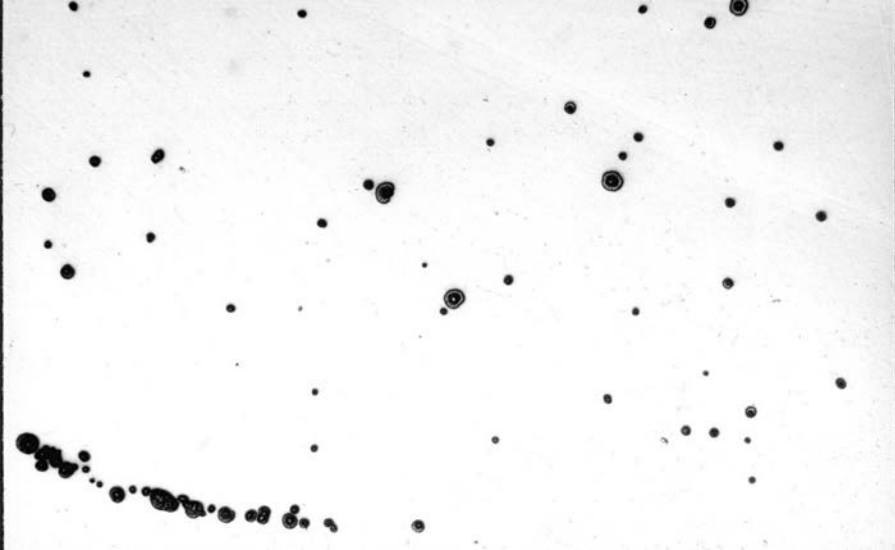


Fig 2.1

Colony morphology of strain 7860/1 on solid medium containing  $\text{MnSO}_4$ .

Ureaplasma colonies stain black due to production of manganese dioxide.

Four days incubation at 37°C



overnight at 37°C and stored at 4°C until ready to use. Isolates were subcultured in broth until they were growing well and producing a colour change in 24 - 48 hours. OA plates which had been dried at 37°C for 20 mins were flooded with 10-, 100-, or in the case of rapidly growing mycoplasmas, 1000- fold dilutions of culture, excess fluid was removed and the plates allowed to dry at room temperature for 15 - 20 min.

Appropriate antiserum discs, determined by cultural characteristics and morphology of the isolate, and a digitonin disc were placed on the dried plates, which were incubated until growth was sufficient for a result to be obtained, usually within 48 - 72 hours. Isolates were identified by a zone of inhibition around a specific antiserum disc. Failure to identify isolates in the initial GI test was often encountered due to 3 factors:

- (i) growth of the isolate being too heavy or rapid and therefore obscuring any inhibition.
- (ii) the presence of two or more mycoplasmas in the culture with one species growing in the zone of inhibition of the other
- (iii) poor growth of colonies due to insufficient adaptation to artificial media.

The first two problems were usually overcome by repeating the test with higher dilutions of culture or by incubating the test plates at a lower temperature (25-30°C) to slow down growth. If identification was still not achieved the test was repeated using a further range of

antisera against mycoplasmas or acholeplasmas as indicated by the reaction to the digitonin disc. If the presence of a mixed culture was suspected and a result was not obtained by the above, pure cultures of each organism were prepared by the filter cloning technique and retested. The third problem was overcome by further passage of the organism in broth.

In practice most large-colony isolates could be identified using antisera to M.arginini, M.ovipneumoniae and A.laidlawii.

#### Indirect immunofluorescence antibody test (IFA)

The method of Rosendal and Black (1972) was followed. This uses fluorescein-conjugated antiserum to the primary anti-mycoplasma immunoglobulin (IgG). The test was carried out as follows: Plates were seeded with a dilution of broth culture and incubated at 37°C until small discrete colonies were visible, usually in 24 - 48 hours. Blocks of agar-bearing colonies were cut from the plate and placed colony side up on glass slides. Rabbit anti-mycoplasma sera diluted to predetermined optional concentrations in PBS (1 in 20 or 1 in 40) were placed in 25µl volumes on each block, which were then incubated for 30 min. at room temperature (RT) in a moist chamber. The blocks were washed twice in PBS, 10 min. each wash, excess fluid was removed then the colonies were flooded with 25 µl of fluorescein conjugated sheep anti-rabbit IgG (Wellcome Diagnostics, Dartford, Kent) diluted in PBS to 1 in 20 or 1 in 40. The blocks were incubated for a further 30 min. at RT, washed twice as before and placed colony-side up on

slides. The microscope used was equipped with an attachment for incident illumination, with U.V. light provided by a mercury vapour lamp (Leitz Orthoplan).

Sheep anti-rabbit IgG was used because of the unavailability of anti-rabbit IgG prepared in other hosts. Negative controls using normal rabbit serum were included in each test to avoid any confusion which may have arisen due to the possible presence of mycoplasmal antibodies in the conjugated antiserum.

The IFA test was used in two ways:

(i) Identification of isolates. The test was frequently used to confirm results obtained by the GI test, and mixed cultures could be identified without the need for cloning. A distinct advantage of this test was that isolates could be identified without the need for subculture. This was found to be very useful for organisms which were difficult to subculture from primary isolation plates.

(ii) Identification of different serotypes of ureaplasma. The test was modified according to Howard and Gourlay (1981), treatment times with the specific antisera and conjugated antiserum being extended to 60 min. each and carried out at 4°C. Blocks were washed for 30 min. with three changes of PBS after each treatment. When lamb anti-mycoplasma hyperimmune serum was used the conjugate was fluorescein conjugated rabbit anti-sheep IgG (Wellcome Diagnostics).

### Metabolism inhibition (MI)

The MI test is basically a growth inhibition test carried out in liquid medium. The method of Taylor-Robinson, Purcell, Wong and Chanock (1966) was used for the identification of glycolytic mycoplasmas. Although the test is relatively easy to perform difficulties have been encountered in reproducibility and the interpretation of results (Taylor-Robinson, 1983): thus the test was only used to identify isolates when the GI or IFA tests had failed.

Before isolates could be tested they had to be in pure culture, growing well and producing an adequate colour change in the test medium (OB). Freshly growing broth cultures were usually used as antigen, and these were diluted 100- or 1000- fold in OB for the test. Rabbit hyperimmune sera were heat inactivated at 56°C for 30 min. before adding to the test plates. Guinea pig serum as a source of complement was added to the test medium ( 5% v/v). Tests were performed in U-bottom microtitration plates sealed with individual non-toxic plate sealers. Plates were incubated at 37°C, or 30°C for rapidly growing species such as acholeplasmas, until the pH of the medium in the antigen control wells had fallen by approximately 0.5 pH unit.

Isolates were identified by the inhibition of metabolism by a specific antiserum, with resultant lack of colour change in the test medium.

The test, in modified form, was also used in the serotyping of ureaplasma isolates. These modifications and results are discussed in Chapter Seven.

### Agar gel double diffusion (AGDD)

This was carried out according to the method of Ouchterlony (1948). Agarose (BDH 2% w/v) was dissolved in PBS containing 1% sodium azide and poured in 15 ml volumes onto 8 x 8 cm glass plates. The plates were held in a humidified chamber for 24-48 hours and examined over a light box with diffused light for presence of precipitin lines.

### Bacteriological techniques

Samples were examined for bacteria by inoculating aliquots of homogenised tissues on to 7% sheep blood agar (SBA) and into nutrient broth No.2 (NB2). Swabs were smeared over an SBA plate then broken off into 10 ml of NB2. Plates and broths were incubated aerobically at 37°C. Identification of isolates was carried out by routine bacteriological procedures.

### Chlamydiological techniques

Samples were collected into chlamydia transport medium and stored at -70°C until examined. A 1 in 4 dilution of the original sample in Medium 199 was centrifuged onto BHK cell cultures grown on circular coverslips pretreated for three days with 5-iodo-2-deoxyuridine. Inoculated cultures were incubated at 37°C for three days, the coverslips were removed, fixed in methanol and stained with 5% Giemsa stain, before being mounted in D.P.X and examined for the presence of chlamydial inclusion bodies.

### Virological techniques

Samples for virological examination were collected in virus transport medium (VTM). Selected samples only were examined, due to the lengthy procedures involved in culturing material for viruses; these included vaginal swabs from ewes with vulvovaginitis and swabs or tissue from tups with penile lesions. Samples in VTM were treated in an ultrasonic bath, filtered through membrane filters of 0.45 $\mu$ m and then inoculated in 0.2 ml volumes onto foetal lamb brain (FLB) or foetal lamb kidney (FLK) cell cultures. These were allowed to adsorb at 37°C for 60 mins, after which one ml of maintenance medium (Medium 199 with 2% foetal bovine serum) was added. Cultures were examined daily for the development of a cytopathic effect. Samples were passaged three times before being discarded as negative. A haemadsorption test was performed on the final passage to check for the presence of non-cytopathic haemadsorbing viruses.

### Preparation of antigens for the production of hyperimmune sera

Selected, cloned ureaplasma strains were grown in ten-fold increasing volumes of ureaplasma broth to a maximum final broth volume of five or ten litres. The final broth was incubated for 20 hours at 37°C and the organisms harvested by centrifugation at 15,000 g for 45 min. at 4°C.



Pellets were washed three times in Hanks BSS or in PBS before finally being resuspended in dw to give a 500-fold concentration. Ureaplasma suspensions were treated in an ultrasonic bath before protein determinations were obtained by a semi-micro Kjeldahl method. The ureaplasma suspensions were then diluted if necessary to 2mg of protein per ml.

#### Production of hyperimmune sera

(i) In rabbits; Hyperimmune sera were produced in rabbits according to the method of Morton and Roberts (1967). Aliquots of mycoplasma suspension\* were emulsified with an equal volume of Freund's complete adjuvant (FCA, Difco Laboratories). Primary vaccination of rabbits with antigen in adjuvant was by the inoculation of 0.1 ml into each hind footpad, 0.1 ml intradermally into eight sites along the flanks and 0.5 ml intramuscularly (i/m) into two sites on each shoulder. Three weeks later one ml was inoculated i/m into each hind leg. Four weeks after the second injection, 0.5 ml of antigen in dw was inoculated intravenously (i/v). A second i/v inoculation of one ml of antigen in dw was given a week later. Animals were bled out 5 - 7 days after the final i/v inoculation. Sera were filtered through 0.45µm apd membrane filters and stored at -20°C.

(ii) In lambs; Specific pathogen-free (SPF) lambs (Brotherston, 1968; Hart, MacKay, McVittie and Mellor, 1971) were inoculated by a modification of the method described by Howard and Gourlay (1981). The inoculum comprised two ml of ureaplasma suspension and two ml of

\* see Appendix A for mycoplasma species used.

FCA: lambs were injected subcutaneously (s/c) at two sites on the neck on two occasions four weeks apart. Two and four weeks after the final s/c inoculation, two ml of ureaplasma suspension in dw were injected i/v. Serum collected two weeks after the final i/v inoculation was filtered through 0.45 $\mu$ m apd membrane filters and stored at -20°C.

### CHAPTER THREE

#### ASSESSMENT OF MEDIA FOR THE ISOLATION AND CULTIVATION OF OVINE UREAPLASMAS

The special growth requirements of Ureaplasma spp. (i.e. lower optimum pH and a requirement for urea) dictate that media different to those generally employed for the growth of large colony mycoplasmas are required for their isolation. Several media have been formulated for the cultivation of human and bovine ureaplasmas. This chapter deals with the comparison of the four most widely used of these media, with the one in use at this laboratory for the isolation of ovine and bovine ureaplasmas. Several aspects affecting the cultivation of ovine ureaplasmas were also examined.

##### Section 1

##### The Ability of Different Ureaplasma Media to Support the Growth of Ovine Ureaplasmas

Two media used for the isolation of U.urealyticum, U9C and Bromothymol blue Broth (BB) (Shepard and Lunceford, 1970, 1976; Robertson, 1978), U4 used for the isolation of U.diversum (Howard, Gourlay and Collins, 1978) and Heart Infusion Broth (HIB) (Livingston, 1972) used for the isolation of ovine and caprine ureaplasmas were compared with TA2 and TB2 (Jones, 1978). Broths and solid media were prepared as closely as possible to their published formulae, the unavailability of Tryptic digest broth necessitated its replacement in U9C with Trypticase soy broth (Difco, Laboratories). Where ingredients were

common to more than one broth the same batch was used to avoid any inter-batch variation in quality. Broths were dispensed in 1.8ml volumes, and solid media in 8 ml volumes.

### Culture Techniques

To test the efficiency of these media, freshly isolated ureaplasma strains were obtained from a flock of Finnish-Landrace ewes, a high percentage of which had previously been shown to have a urogenital ureaplasma infection (Jones and Rae, 1979). Vaginal swabs from 15 ewes were collected into MTM, vigorously shaken and 0.2ml aliquots inoculated into each of the five broths, ten-fold dilutions to  $10^{-4}$  were made in each of the broths, plates being inoculated directly from the MTM. Broths were incubated aerobically, plates in 5%  $\text{CO}_2$  in air, at  $37^\circ\text{C}$  for five days; the titres produced in each broth and the number and size of colonies on plates was determined.

### Results

Isolation rates are shown in Table 3.1: TB2 detected the greatest number of positive samples with 10 of the 15 swabs yielding, ureaplasmas followed by U9C (7), U4 (5) and HIB (2), ureaplasmas were not isolated with BB. Titres ranged from  $10^2$ - $10^4$  ccu/0.2ml with the highest titres being achieved in TB2. A single isolation was made on solid media, one strain producing a few colonies on TA2.

To test the reproducibility of these results the 10 positive ewes were swabbed a second time and the swabs

titrated as before but this time ten-fold dilutions to  $10^{-8}$  were made in each broth. Again TB2 gave the highest isolation rate although on this occasion only eight ewes yielded ureaplasmas, U9 detecting five isolates and U4 four isolates, BB and HIB failed to detect any of the eight isolates. The titres obtained in each broth were similar to those obtained on the first sampling, only two giving titres greater than  $10^4$  ccu/0.2ml in TB2. (Table 3.1). Ureaplasma colonies were not seen on any solid media.

A third trial utilising only TB2, U9C, U4 broths and their solid media was carried out by collecting vaginal swabs from a further 30 ewes, from three different flocks, and processing them as before. Eight isolates were detected in TB2, nine gave an alkaline colour change in U9C, but four of these were contaminated by fungi which may have been responsible for the increase in pH, and four were positive in U4. Titres of ureaplasma on this occasion were lower than in the first two trials, none being greater than  $10^3$  ccu/0.2ml. Growth of ureaplasma colonies was not obtained on solid media. Throughout these three trials TB2 had given the best results detecting 26 isolates, U9C and U4 detected 17 and 12 isolates respectively. Although TB2 was the best broth for isolating ovine ureaplasmas the titres obtained were low. This may have been a true reflection of the numbers of organisms present or there may have been a deficiency in some growth requirement in the broth. None of the solid media gave satisfactory results, only 1 of the 26 isolated strains growing on solid media (TA2). Nonetheless, because of the relative success of TB2

TABLE 3.1

ISOLATION RATES OF OVINE UREAPLASMAS IN VARIOUS  
CULTURE MEDIA.

		UREAPLASMA TITRE*				
		TB2	U4	IN U9C	BB	HIB
<u>Trial 1.</u>						
Ewe No.	784	$10^4$		$10^2$		$10^2$
	880	$10^2$	-	$10^2$	-	-
	882	$10^3$	$10^2$	$10^2$	-	-
	886	$10^4$	$10^2$	$10^2$	-	$10^2$
	896	$10^2$	$10^2$	-	-	-
	903	$10^4$	$10^2$	$10^2$	-	-
	917	$10^2$	$10^4$	$10^2$	-	-
	919	$10^2$	-	-	-	-
	933	$10^2$	-	$10^2$	-	-
	999	$10^3$	-	-	-	-
<u>Trial 2.</u>						
Ewe No.	784	$10^5$	$10^3$	$10^3$	-	-
	880	$10^3$	-	-	-	-
	882	$10^4$	-	$10^2$	-	-
	886	$10^4$	-	$10^2$	-	-
	896	$10^2$	$10^2$	-	-	-
	903	$10^4$	$10^3$	$10^2$	-	-
	917	$10^5$	$10^4$	$10^2$	-	-
	919	$10^3$	-	-	-	-
	933	-	-	-	-	-
	999	-	-	-	-	-

\* ccu/o.2 ml

it was chosen for further study and various modifications to it and to TA2 were undertaken in an effort to improve the titres obtained in broth, and the growth on solid media.

## Section 2

### Modifications to TA2 and TB2

One problem frequently encountered in the production of TA2 was the denaturation of the protein content of the medium by dithiothreitol. This invariably happened during warming of the broth to 56°C prior to addition of the agarose, if the broth was held at this temperature for more than 15 minutes a cloudy, white precipitate formed. Although care was taken to avoid any visual change in the broth the possibility that dithiothreitol was responsible for unseen chemical changes which had an adverse effect on the ability of the media to support growth was suspected. The replacement of dithiothreitol by another reducing agent, cysteine hydrochloride at a final concentration of 0.02%, averted this problem of denaturation. Batches of solid media containing each of these reagents were compared for their ability to grow ureaplasmas on primary isolation. Vaginal swabs were obtained from the same Finnish-Landraces used earlier and of eight samples positive in TB2, five were detected on TA2 containing cysteine but only two on TA2 with dithiothreitol, thus confirming earlier suspicions. However, the size of the colonies produced on these solid media was very small and because there is often debris derived from the sample, e.g. mucus, dead cells, present on primary isolation media the possibility that

such small colonies could be overlooked was considered, and modifications to the media which could increase the size of colonies were investigated.

The inclusion of various buffers into ureaplasma media has been reported to enhance colony growth. Manchee and Taylor-Robinson (1969) achieved increased colony size of human, simian and feline ureaplasmas by the inclusion of N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate (HEPES) buffer. Similarly Romano, Ajello, Massenti and Scarlatta (1975) incorporated (2-N-morpholino)-ethanesulphonate (MES) or HEPES buffers in their media and obtained improved growth of a human ureaplasma. Windsor, Edward and Trigwell (1974) showed that inclusion of a mixture of di-sodium hydrogen orthophosphate and potassium dihydrogen orthophosphate buffers increased the sensitivity of their medium for growth of human ureaplasmas, in addition to increasing the size of the colonies, a white precipitate was also formed, which made detection of ureaplasmas colonies easier. Similarly, Shepard and Lunceford (1976) have demonstrated that the inclusion of manganous sulphate into primary isolation media aids in the detection of ureaplasma colonies, by the formation of manganese dioxide within and on the surface of the colonies which gives them a dark-brown to black appearance. The initial problem in the present work was the confident detection of ureaplasmas on primary plates and therefore the supplements described by Windsor et al. (1974) and Shepard and Lunceford (1976) were incorporated into TA2.



The gaseous environment in which ureaplasma cultures are incubated also influences the isolation rate of ureaplasmas. Razin, Masover, Palant and Hayflick (1977) recommended that incubation in 100% CO<sub>2</sub> increased the size of U.urealyticum colonies. However, Kundsinn, Parreno and Poulin (1978) demonstrated that different strains of U.urealyticum preferred different gaseous conditions for optimum growth.

Thus, varying atmospheric conditions, plus the modifications outlined above, were investigated to determine their effect on the size of ovine ureaplasma colonies.

#### Materials and Methods

TA2 was made up according to the formula given in (Table 2) with the following modifications: dithiothreitol was replaced by cysteine hydrochloride (0.02% final concentration) and the medium was supplemented with either 1.0% manganous sulphate or 2.5% phosphate buffer (the composition of these reagents is given in Chapter Two). Six, low-passage isolates, which had been stored at -70°C, were subcultured into TB2 and incubated overnight, 1 in 10 dilutions of these were used to inoculate the plates which were incubated at 37°C under a variety of conditions, namely, 5% CO<sub>2</sub> in air, 100% CO<sub>2</sub>, aerobically and anaerobically (Gaspak System, Becton Dickinson). After five days incubation the size of the colonies was measured.

#### Results

All the strains grew on both media and in all

atmospheres and the colonies were readily detectable due to either, the production of a white precipitate in plates containing phosphate buffer, or to pigmentation of the colonies by manganese dioxide. Ten to twenty colonies were measured on each plate, the inoculation titre of each strain and the colony size attained is shown in Table 3.2. There was a wide variation in the size of colonies produced under the various conditions but four strains A801/1, A801/2, A801/3 and A801/6 produced largest colonies in 100% CO<sub>2</sub>, two strains (A801/5 and 7860/1), preferred aerobic conditions. The largest colonies were produced on media supplemented with phosphate buffer, under all gaseous environments, except for strains A801/1 and A801/5 which in 5% CO<sub>2</sub> and anaerobically produced bigger colonies on media containing manganous sulphate. The strains used in this test had all been passaged at least twice in TB2 and the possibility that adaption to growth in artificial media was more important in determining the growth and size of colonies rather than the addition of either ingredient was considered. However, subsequent use demonstrated that inclusion of both ingredients, either singly or in combination, increased the detection of ureaplasmas on primary agar plates. The routine procedure adopted therefore, was to include phosphate buffer and manganous sulphate in primary isolation plates and phosphate buffer only, in plates used for subsequent subcultures. As different strains apparently preferred different gaseous environments, primary isolation plates were incubated in both 5% and 100% CO<sub>2</sub>. Although differences in colony

TABLE 3.2

AVERAGE COLONY SIZE OF OVINE UREAPLASMAS GROWN UNDER DIFFERENT CONDITIONS OF INCUBATION.

Strain	Titre <sup>b</sup> of inoculum	TA2 supplement <sup>c</sup>	Average <sup>a</sup> colony size (µm)			
			Aerobic	5% CO <sub>2</sub>	100% CO <sub>2</sub>	Anaerobic
A801/1	10 <sup>5</sup>	PB	37	18	39	30
		MnSO <sub>4</sub>	27	33	32	65
A801/2	10 <sup>5</sup>	PB	28	25	47	16
		MnSO <sub>4</sub>	20	17	20	18
A801/3	10 <sup>6</sup>	PB	37	47	52	38
		MnSO <sub>4</sub>	18	36	32	20
A801/5	10 <sup>4</sup>	PB	43	22	38	24
		MnSO <sub>4</sub>	28	37	24	26
A801/6	10 <sup>5</sup>	PB	66	69	87	81
		MnSO <sub>4</sub>	34	43	55	73
7860/1	10 <sup>6</sup>	PB	42	38	23	26
		MnSO <sub>4</sub>	21	21	19	19

a Average of 10 to 20 colonies

b ccu/0.2 ml

c PB = phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>) 7:3 mixture incorporated in media at 2.5% (v/v).

MnSO<sub>4</sub> = manganous sulphate (1.5% solution) incorporated in media at 1% (v/v).

size were observed on several occasions all the strains isolated in the survey (Chapter Four) grew in both atmospheres; therefore, once an isolate had been obtained on primary agar plates, subsequent subcultures were incubated in 5% CO<sub>2</sub> only.

### Section 3

#### Modifications to TB2 Undertaken to

#### Improve the Yield of Ovine Ureaplasmas

The use of the modified TA2 along with TB2 permitted successful isolation of ovine ureaplasmas. Some strains however, failed to grow after a few passages in TB2, and when attempts were later made to produce antigenic material by culturing ureaplasmas in large volumes, for immunisation purposes and serological studies, the titres obtained never exceeded 10<sup>6</sup> ccu/ml. Various modifications were made to TB2 in an attempt to overcome these problems. These included the incorporation of HEPES, MES, TRIS (hydroxymethylaminomethane) and phosphate buffers, as well as alterations to the serum, yeast extract and urea components of TB2.

#### Materials and Methods

Fifty ml volumes of TB2 were prepared as in Table 2 except that dithiothreitol was replaced by 0.02% cysteine hydrochloride. Four batches of media were supplemented with either 0.05 M HEPES, MES TRIS buffers or 2.5% phosphate buffer, a fifth batch had no additives. Two, strains (A801/1 and 7860/1) at a low passage level, were inoculated into each batch of medium so that initially the

cultures contained  $10^1$  to  $10^2$  ccu/0.2ml, and incubated at  $37^\circ\text{C}$ . The number of viable organisms was determined at various periods during incubation by titrating samples from each batch in the appropriate media. The pH of the media was also monitored throughout the incubation period.

Sera from a variety of hosts, namely, lamb, horse, foetal calf, newborn calf and rabbit were tested at concentrations of 5, 10, 20 and 30% in TB2 to assess their effect on ureaplasma growth. The effect of varying the concentrations of urea and yeast extract in TB2 were also examined.

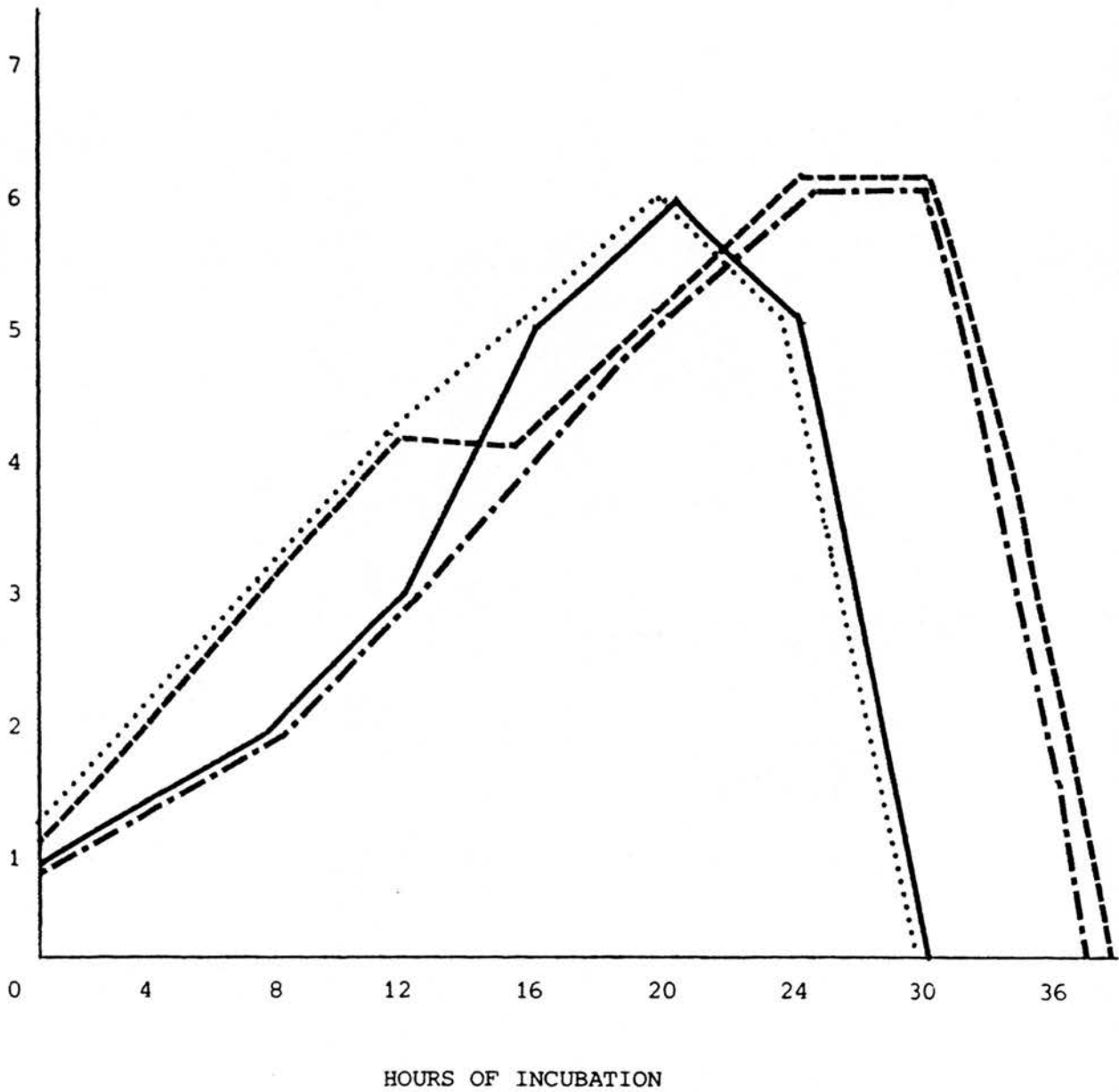
### Results

Figure 3.1 demonstrates the effect the various buffers had on the growth of the strain 7860/1, Table 3.3 shows the change in pH which occurred during growth. In unbuffered medium maximum titres ( $10^6$  ccu/0.2ml) were achieved by both strains by 20 h of incubation. Titres fell rapidly after this time and by 30h incubation, viable organisms were not detected. Initially the pH of the broth was 6.2, the final pH being 8.3- 8.5. The strains grown in MES and TRIS buffered broth gave similar results, maximum titres being achieved by 20h incubation however, the final pH was 7.2.-7.4 in MES buffered broth and 8.5 in the TRIS buffered broth. Although there was no increase in the maximum titres attained by the two strains in HEPES or phosphate buffered media, the time taken to achieve maximum titres was extended to 24h and viable organisms could still be recovered at 30h but not at 36 hours. The final pH was 7.6-7.8 for the HEPES buffered broth and 8.0-8.1 for the

Fig 3.1

EFFECT OF VARIOUS BUFFERS ON THE GROWTH OF OVINE UREAPLASMA  
STRAIN 7860/1.

ccu/0.2 ml  
(log<sub>10</sub>)



- = UB
- · - · = UB + 0.05 M HEPES
- · · · = UB + 0.05 M MES
- - - - = UB + 2.5% phosphate buffer

The growth curve produced in UB + 0.05 M TRIS was very similar to that in UB and is omitted from the figure for clarity.

The growth curves produced by strain A801/1 were similar to those produced by 7860/1.

TABLE 3.3

EFFECT OF VARIOUS BUFFERS ON THE pH OF BROTH CULTURES OF OVINE UREAPLASMAS.

MEDIUM	FINAL pH OF CULTURES AFTER 36 H INCUBATION	
	A801/1	7860/1
TB2	8.5	8.3
TB2 + 0.05 M HEPES	7.8	7.6
TB2 + 0.05 M MES	7.4	7.2
TB2 + 0.05 M TRIS	8.5	8.5
TB2 + PB*	8.0	8.1

The initial pH of all broths was 6.0-6.2 except for the TRIS buffered broth in which the pH was 7.0.

\* = phosphate buffer ( $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ ) 7:3 mixture incorporated in media at 2.5% (v/v).

phosphate buffered broth.

Increased titres of the two strains were not achieved by altering the concentration of the swine serum. Similarly incorporation of horse, foetal calf or newborn calf sera did not affect titres. Both strains however, had reduced titres ( $10^4$  ccu/0.2ml) when rabbit serum was incorporated at all concentrations tested. Inclusion of lamb serum at concentrations of 20 and 30% led to growth of the organism being delayed for up to three days, but no reduction in the final titre was noted. The effect of urea on the growth of ureaplasmas was examined by including the substrate at final concentrations of 0.1, 0.05 and 0.01% in 50 ml volumes of TB2. Strains A801/1 and 7860/1 were incubated and titrated in the appropriate media, as before, for estimation of viable organisms. The titres obtained were dependent on the concentration of urea used, maximum titres being obtained when urea was incorporated at 0.05%, strain A801/1 giving a 10-fold increase and 7860/1 a 100-fold increase compared with titres in TB2. The titres obtained with 0.01% urea were the same as those achieved with 0.1% urea but maximum titres were not attained until 24h incubation. Similarly, the time taken to achieve maximum titres with 0.05% urea was also delayed until 24h. (Table 3.4). There was no difference however in the rate of decline in viability of the organisms, none being recovered after 30h incubation.

Yeast extract was incorporated at final concentrations of 10%, 5% and 1% in 50 ml volumes of TB2 and the effect on the titres of the two strains determined



TABLE 3.4

EFFECT OF VARYING CONCENTRATIONS OF UREA ON GROWTH  
OF OVINE UREAPLASMAS.

CONCENTRATION OF UREA IN BROTH	INCUBATION TIME AT WHICH MAXIMUM TITRES* WERE OBTAINED			
	A801/1		7860/1	
0.1%	20h	$10^6$	20h	$10^6$
0.05%	24h	$10^7$	24h	$10^8$
0.01%	24h	$10^6$	24h	$10^6$

\* ccu/0.2 ml

h = hours

as above. No difference in the maximum titres attained, or the incubation time required to achieve them were noted between media containing 5 or 10% extract. The titre of strain 7860/1 was ten-fold less in TB2 containing 1% extract.

The above results led to the following modifications being made to TB2;

- (i) the replacement of dithiothreitol by cysteine,
- (ii) the urea concentration being reduced from 0.1% to 0.05% final volume,
- (iii) Yeast extract incorporated at a final concentration of 5%.

Additionally, as thallos acetate had already been replaced in MTM by amphotericin B, for reasons already discussed (Chapter Two), it was also replaced in TB2. The efficiency of this modified ureaplasma broth (UB) was tested against that of TB2 by titrating a further 20, low-passage isolates in both broths and comparing the titres obtained. Seven of the strains tested attained the same titres in both broths, the titres of 13 strains however, increased by 10- or 100-fold in UB. These results indicated that UB was superior to TB2 and was subsequently used for all isolation and cultivation procedures.

These modifications were also made to TA2, in addition to the incorporation of the buffers described earlier. The formulae of these two modified media, UB and UA, are given in Table 3.5.

Although the use of UB allowed increased isolation

TABLE 3.5

FORMULAE OF MEDIA USED FOR THE ISOLATION AND CULTIVATION OF OVINE UREAPLASMAS.

	VOLUME IN 100 ml	
	UB	UA
Distilled Water	45	24
10X Medium 199	4.5	4.5
Brain Heart Infusion Broth	20	30
Yeast Extract	5	5
Swine Serum	20	30
Urea (10%)	0.5	0.5
Cysteine (2%)	1.0	1.0
Calf Thymus DNA (0.2%)	1.0	1.0
Phenol red (0.4%)	1.5	-
Amphotericin B (0.2 mg/ml)	1.0	-
Ampicillin (100mg/ml)	0.5	0.5
Phosphate buffer	-	2.5
KH <sub>2</sub> PO <sub>4</sub> /Na <sub>2</sub> HPO <sub>4</sub> (1.5M solns.) 7:3 mixture		
Agarose (w/v)	-	0.9
pH	6.0-6.2	6.5

Final concentrations of supplements: Ampicillin: 0.5 mg/ml  
 Calf thymus DNA: 0.002%, cysteine: 0.02%,  
 amphotericin B: 0.002 mg/ml, phosphate buffer: 0.025%,  
 phenol red: 0.006%, urea: 0.05%

The addition of Manganous sulphate (1.5% in dw), 1 ml to 100 ml of solid media, allows easier identification of ureaplasmas on primary isolation plates. The ureaplasma colonies stain dark-brown to black due to the formation of manganese dioxide within and on the surface of the colonies.

titres of ovine ureaplasmas, the yield from large volumes of UB was still poor and several cultural aspects were examined in an attempt to improve yields.

#### Section 4

##### The Effect of Buffers on the Bulk Production of Ovine Ureaplasmas

Previous work had demonstrated that the inclusion of HEPES or phosphate buffer in TB2 prolonged the viability of ovine ureaplasmas and that inclusion of phosphate buffer in solid medium resulted in increased colony size. These buffers were therefore included in UB to determine if the yield of ureaplasmas obtained from large volumes of broth could be increased.

##### Materials and Methods

Three ureaplasma strains (US1 7860/1 and A801/1) were each inoculated into five litres of basic UB or UB supplemented with either 0.05m HEPES or 2.5% phosphate buffer. The broths were incubated for 30h at 37°C, ureaplasmas were harvested by centrifugation at 15,000 g for 45 minutes and the pellets obtained washed twice in PBS, resuspended in 5 ml of dw and the protein content determined. The final pH of the broths was also measured.

##### Results

There was no significant difference in the protein yields from each strain (Table 3.6). The final pH of the three broths ranged from 7.9-8.0 for UB + HEPES, 8.0-8.3 for phosphate buffered UB, and 8.3-8.5 for basic UB. Thus, despite the earlier demonstration that the ureaplasmas

TABLE 3.6

EFFECT OF BUFFERS ON THE YIELD OF OVINE UREAPLASMAS  
FROM LARGE-VOLUME CULTURES.

MEDIUM	PROTEIN YIELD (mg/ml)*			FINAL pH OF BROTH
	US1	7860/1	A801/1	
UB	0.95	0.9	1.0	8.3-8.5
UB + 0.05 M HEPES	0.92	1.0	1.1	7.9-8.0
UB + 2.5% PB <sup>+</sup>	0.88	0.99	0.95	8.0-8.3

\* 1,000 fold concentration from original volume.

+ phosphate buffer ( $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ ) 7:3 mixture.



remained viable for longer periods by buffering the media, this did not lead to increased yields of organisms from bulk cultures.

## Section 5

### The Effect of Agitation on Broth Cultures of Ovine Ureaplasmas

Growth of some mycoplasmas is improved by gentle agitation of the cultures (Kenny, 1979). The effects of agitation on two strains of ovine ureaplasma was examined.

#### Materials and Methods

Two strains (US1 and A801/1) were each inoculated into duplicate five litre volumes of UB and incubated at 37°C, one set of cultures being agitated the other remaining static. After 30h incubation ureaplasmas were harvested and protein estimations determined as before.

#### Results

Both agitated cultures were very turbid by 16h incubation. At 20h, titres of both strains under each cultural condition, were  $10^6$  ccu/0.2 ml. The protein content of US1 and A801/1 from static cultures was 1.0 mg/ml and 1.2 mg/ml respectively, from agitated cultures the yields were 3.5 mg/ml and 2.8 mg/ml. The turbidity of the agitated cultures and the increased protein yield initially suggested that there may have been bacterial or fungal contamination of the broth, but neither was isolated. As no increase in the titre of ureaplasmas had been obtained,

it was assumed therefore, that the increased protein yield was due to denaturation of the medium proteins by agitation at 37°C.

To test this hypothesis, a further two uninoculated five litre volumes of UB were agitated for 16h at 37°C. The broth became very turbid and after centrifugation yielded pellets which contained 2.9 mg/ml and 3.5 mg/ml of protein. As a precaution against the broth having become contaminated during centrifugation, it was resterilised by filtration and inoculated with the same two ureaplasma strains and agitated as before. The broth again became turbid and titres of  $10^6$  ccu/0.2 ml were obtained for each strain: the protein yield for US1 and A801/1 was 3.2 mg/ml and 3.4 mg/ml respectively. These results indicate that agitation of UB at 37°C readily denatures the protein content of the broth and although agitation and centrifugation of the broth prior to inoculation removed a large proportion of this denatured protein, more was produced by subsequent agitation of the broth. The prior removal of some this protein did not affect the subsequent growth of the two strains.

## Section 6

### Dialysis Treatment Of Bulk Cultures

The production of a toxic product by ureaplasmas during growth has been suggested as a possible reason for the poor yields obtained in bulk cultures (Furness, 1973). Attempts were made to remove this toxic product(s) by culturing the ureaplasmas in a dialysis system.

### Materials and Methods

Strains A801/1 and US1 were each grown in 500 ml of UB in sterilised dialysis tubing, suspended in five litres of UB. Cultures were incubated at 37°C for 48h, samples being removed after 24h, 36h and 48h for determination of viable units. After 48h incubation the yield of ureaplasma protein, produced in each dialysis tube, was determined as before. The ability of each dialysate to support growth was also tested by taking samples at 24h and 48h incubation, re-adjusting the pH to 6.0-6.2 and making ten-fold dilutions of strains US1 and A801/1 in each dialysate.

### Results

The titres of both strains after 24h dialysis were  $10^7$  ccu/0.2ml, but viable organisms were not detected at 36h or 48h. The dialysate supported growth of both strains; samples taken at 24h allowing growth of both to  $10^6$  ccu/0.2ml, and titres of  $10^6$  ccu/0.2 ml for A801/1 and  $10^7$  ccu/0.2ml for US1 were obtained in the 48h dialysate. The protein yields obtained for US1 and A801/1 were 0.34 mg/ml and 0.23 mg/ml respectively. These results indicate that yields of ovine ureaplasma protein can not be increased by dialysis of the growth medium.

### DISCUSSION

The differences observed in the isolation rates of ovine ureaplasmas in the five media were not entirely unexpected. None of these media are chemically defined and there are major differences in the complexity of the media



formulations, Table 3.7 summarises the ingredients in each. The simplest media are BB and HIB which use commercially prepared infusion broths for their base and relatively few supplements; U4 uses Hanks balanced salt solution as a base and is supplemented with Hartley's digest broth, foetal calf serum, yeast extract, urea and magnesium sulphate. Shepard's U9C is more complex using trypticase soy broth as a base supplemented with horse serum, yeast extract, urea, cysteine, GHL-tripeptide, and magnesium chloride. The most complex of the five media tested was TB2 which has Medium 199, a chemically defined cell culture medium (Morgan, Morton and Parker, 1950) as its base further supplemented with brain heart infusion broth, swine serum, fresh yeast extract, urea, dithiothreitol and DNA.

The increasing complexity of the media was reflected in the number of isolations obtained; the two most complex media, U9C and TB2, giving the highest isolation rates; additionally the highest titres were obtained with TB2. It is unlikely that any one component was responsible for the improved isolation rate achieved by TB2 but doubtless, the many vitamins, amino acids, co-factors and other constituents in Medium 199 provided a more suitable medium for growth of ovine ureaplasmas than was provided by ingredients in the media used for growth of U.urealyticum and U.diversum. These other media however, have been shown to be suitable for growing the Ureaplasma spp. for which they were devised, and their inability to grow freshly isolated strains of ovine ureaplasmas suggests that these

TABLE 3.7

SUMMARY OF INGREDIENTS IN THE FIVE MEDIA TESTED FOR THEIR ABILITY TO GROW OVINE UREAPLASMAS.

	BROTH				
	HIB	BB	U4	U9C	TB2
Base	HIB	PPLO	HBSS	TSB	Medium 199
Supplements	HS	HS	HDB	HS	BHIB
	YE	YE	FCS	YE	SS
	Urea	Urea	YE	Urea	YE
	NaCl	GHL	Urea	GHL	Urea
			MgSO <sub>4</sub>	Cysteine MgCl	Dithiothreitol Calf Thymus DNA

- BHIB - Brain heart infusion broth  
 FCS - Foetal calf serum  
 GHL - Glycyl-L-histidyl-L-lysine acetate  
 HBSS - Hanks balanced salt solution  
 HDB - Hartley's digest broth  
 HIB - Heart infusion broth  
 HS - Horse serum  
 PPLO - Pleuropneumonia-like organism broth  
 SS - Swine serum  
 TSB - Trypticase soy broth  
 YE - Yeast extract

organisms are more fastidious in their growth requirements than human or bovine strains. A recent report by Stemke, Stemler and Robertson (1984) partially supports this observation. The growth characteristics of ureaplasmas isolated from several hosts were compared, using BB broth, and the results demonstrated that the broth was adequate for growth of all ureaplasmas except certain strains of ovine and bovine origin, and these authors suggested that ovine and bovine ureaplasmas had more demanding nutritional requirements than those isolated from other species. The failure to isolate any ovine ureaplasma using BB broth in this study confirms the inadequacy of this broth for use with ovine ureaplasmas.

However, the successful isolation of ovine ureaplasmas by the relatively simple HIB (Livingston, 1972) argues against the suggestion that all ovine ureaplasmas are more fastidious, and the poor performance of HIB in this study is difficult to explain. It may be possible that strains of ovine ureaplasmas vary in their nutritional requirements and it is the less demanding ones which are detected by HIB. Variations in the fastidiousness of strains of U.urealyticum have been shown by Kundsinn et al. (1978), who demonstrated that the different media they examined varied in their ability to support the growth of all ureaplasma strains, and they suggested that several media should be used to ensure confident isolation of human ureaplasmas.

TB2 was adequate for isolation purposes and only minor modifications were required to allow its use for

routine cultivation of ovine ureaplasmas. The only modification which increased titres of ureaplasmas was the reduction of the urea concentration of the broth. The role of urea in the metabolism of ureaplasmas has not been fully elicited. Ford, McCandlish and Gronlund (1970), using carbon<sup>14</sup> labelled urea, demonstrated that carbon is not incorporated in the ureaplasma cell but 95% is evolved as CO<sub>2</sub>. The reports of Masover and Hayflick (1973) and Masover, Benson and Hayflick (1974), that ureaplasmas can grow in media without added urea, or where urea has been replaced by putrescine or allantoin suggest that urea may not be an essential metabolite for these organisms. However, the difficulties involved in ensuring that no contaminating urea is present in any of the medium components make confident interpretation of these results difficult. Conversely, Kenny and Cartwright (1977) demonstrated that urea is a growth-limiting factor for U.urealyticum and the titres obtained in their study were dependent on the concentration of urea in the growth medium, maximum titres being obtained at a concentration of 32mM urea (approximately 0.2 mg/ml). The concentration of urea in TB2 was 1mg/ml, the reduction of this level to 0.5mg/ml resulted in 10-fold and 100-fold increases in the titres of the two strains examined.

From the report of Kenny and Cartwright (1977) and the limited work in the present study, it would appear that urea is required for optimum growth of ureaplasmas and that the maximum titres achieved are dependent on the urea

concentration in the media.

Yeast extract, at the usual 10% incorporation level in media, has been shown to be partially inhibitory to many strains of U.urealyticum in primary culture (Shepard and Howard, 1970; Shepard and Lunceford, 1975). No such inhibition was seen in this study. However, yeast extract incorporated at 5% supported growth of ovine ureaplasmas to the same titres as the 10% concentration and subsequently a level of 5% was routinely incorporated in the medium.

Apart from the differences noted when the medium was supplemented with rabbit or lamb sera, the source or concentration of the sera did not affect the growth of ureaplasmas. Many mycoplasma species fail to grow well in media containing rabbit serum (Senterfit, 1983), the poor growth obtained with ovine ureaplasmas therefore, is not surprising. The nature of the inhibitor present in lamb serum was partially investigated and it was shown to be thermostable at 56°C and non-dialyzable. Gel immuno-electrophoresis, using pig anti-IgG<sub>1</sub> and IgG<sub>2</sub> serum demonstrated a high proportion of IgG in the lamb serum. Enquiries to the supplier revealed that although the product was called "lamb" serum, it was actually collected from animals up to 12 months old and therefore, it is possible that the serum from such animals may contain mycoplasmal antibodies. The presence of inhibitory substances, some of which have been shown to be antibody, has previously been demonstrated in batches of animal serum used for growth of other mycoplasma species. (Tully and Rose, 1983).

Attempts to increase the ureaplasma protein yield obtained from large-volume cultures was not successful. Of the buffers tested, HEPES and phosphate buffer maintained a lower pH in the broth and allowed longer survival of the ureaplasmas but no increase in yield was obtained. Although several buffers have been shown to increase the size of ureaplasma colonies on solid media (Manchee and Taylor-Robinson, 1969, Romano, et al., 1975; Windsor et al., 1974) they do not appear to significantly increase yields of ureaplasmas from broth cultures. The studies carried out in this work with HEPES and phosphate buffer confirm these findings.

Several studies have investigated other methods of maintaining a low pH in ureaplasma broth cultures, such as growing cultures under a flow of air to reduce the ammonia concentration (Hendley and Alfred, 1972), by adding ion-exchange resins to remove ammonium ions from the broth (Windsor and Trigwell, 1976) or by buffering cultures with CO<sub>2</sub> (Masover, Razin and Hayflick, 1977). Although the viability of the organisms was prolonged in some of these systems no significant increase in the protein yield was obtained. These studies indicate that an increase in pH alone is not the sole factor preventing higher yields of ureaplasmas. The production of a toxic product(s) has also been suggested to be involved (Furness 1973). In this study attempts to remove this product by dialysis during growth did not result in increased yields and the dialysate did not inhibit growth of either strain tested, suggesting that the toxic product was retained within the dialysis

tubing. Furness (1973) reported only a slight increase in the numbers of ureaplasmas grown in dialysed medium compared with normal cultures, but the organisms remained viable for longer periods which suggested that the toxic product was removed by dialysis. The dialysate supported growth of two strains of human ureaplasmas after 24 h incubation, but was inhibitory at 41h suggesting that the toxic product was only slowly dialyzable. The discrepancies between these two studies may perhaps be explained by an increased sensitivity of ovine ureaplasmas to this toxic product resulting in a more rapid decline in viability of the organisms within the dialysis tubing, and therefore, the production of the toxic metabolite ceasing before inhibitory levels are reached in the dialysate.

The modifications to TA2 and TB2 described in this chapter provided media (UA and UB) suitable for the isolation and cultivation of ovine ureaplasmas. However, the yield of protein obtained from large-volume cultures of these organisms was poor. Attempts to increase this yield by further modifying UB or altering the cultural conditions under which the ureaplasmas were grown were unsuccessful.



## CHAPTER FOUR

### SURVEY OF THE MYCOPLASMAS OF THE OVINE UROGENITAL TRACT

It was apparent from the review of the literature that the ovine urogenital tract may be colonised by several species of mycoplasma and that some of these may be associated with vulvovaginitis (Cottew et al., 1974), granular vulvitis (Doig and Ruhnke, 1977) or infertility and abortion (Livingston et al., 1978). At the outset of this work the only mycoplasmological studies on the urogenital tract of sheep reported in the United Kingdom concerned normal animals: the species which had been isolated were M.ovipneumoniae (Leach et al., 1976) M.arginini and A.laidlawii (Jones and Rae, 1979) and Ureaplasma sp. (McCaughey, Ball and Irwin, 1979; Jones and Rae, 1979).

This survey was undertaken to amplify knowledge of the incidence, occurrence and distribution of mycoplasmas in the normal ovine urogenital tract, and to compare the findings with those obtained from sheep which had experienced several different forms of reproductive tract failure or disease

#### MATERIALS AND METHODS

The methods used in the isolation of mycoplasmas, bacteria, viruses and chlamydia have already been described (Chapter Two).



Material obtained from the following sources were examined for the presence of mycoplasmas:

- (i) Normal sheep of various ages
- (ii) Sheep slaughtered at a local abattoir
- (iii) Specific pathogen-free lambs
- (iv) Sheep which had aborted
- (v) Sheep with vulvovaginitis
- (vi) Barren ewes
- (vii) Fertile and infertile rams

Most of the samples from animals in groups (iv), (v) and (vii) were submitted from Veterinary Investigation Centres in Scotland and England in response to a request for material from sheep with urogenital tract disorders. Animals were identified as diseased by veterinary staff at these centres, and it is their descriptions which are used when reference is made to the various conditions encountered in the survey. Samples from the other groups were collected by the author. Swabs of the vagina or prepuce were the only samples examined from sheep in groups (i), (iii), (v) and (vi). The material cultured from group (ii) animals included vaginal swabs and uterine mucosa from non-gravid uteri. Intact gravid uteri were collected at a local abattoir and transported to the Institute for sampling. Amniotic fluid, obtained by sterilising the exterior uterine wall with alcohol then aspirating 20 mls of fluid by syringe, was stored at  $-70^{\circ}\text{C}$  until examined. The uterus was then opened, the foetus transferred to a sterile tray and samples of brain, spleen, liver, kidney, lung, stomach contents and cotyledons

removed for culture. In group (iv), vaginal swabs from the ewes, stomach contents from the aborted fetuses and samples of placenta, if available, were cultured. Semen and preputial swabs were cultured from animals in group (vii). All samples were examined for mycoplasmas by inoculation into the appropriate media as described in Chapter Two. Samples from groups (i), (iv), (v), (vi) and (vii) were additionally cultured for bacteria and in some cases, viruses and chlamydia.

### RESULTS

A total of 664 samples were examined for mycoplasmas from which 135 isolations were made. The samples examined and the mycoplasmas isolated are detailed in Table 4.1. Ureaplasma sp. were the most frequently isolated organisms, comprising 77 (57%) of the isolates. The other mycoplasma species isolated were M.arginini, M.capricolum, M.ovipneumoniae, A.axanthum, A.laidlawii and A.oculi. Ureaplasmas were isolated exclusively from semen, and preputial and vaginal swabs; foetal tissues, amniotic fluid, uteri and placentae all being negative. Ten of the 14 ewes from which intact non-gravid uteri were obtained yielded ureaplasmas from vaginal swabs taken immediately before removal of the uterus complete with Fallopian tubes.

#### Isolation Of Mycoplasmas From The Lower Urogenital Tract

Six species of mycoplasma were isolated from the lower urogenital tract. The most commonly isolated was Ureaplasma sp. with an overall isolation rate from vaginal

TABLE 4.1ISOLATION OF MYCOPLASMAS FROM UROGENITAL TRACT SAMPLES

SPECIMEN	NUMBER CULTURED	NUMBER POSITIVE FOR:			
		Ureaplasma sp.	A.laid.	Other spp.	
Vaginal swab (all groups except (iii))	174	43	17	6	(M.arginini)
				4	(M.capricolum)
				3	(A.axanthum)
				3	(A.oculi)
Semen/ Preputial swab	217	34	8	-	
Aborted foetus/ Placenta	149	-	4	1	(M.ovipneumoniae)
				1	(Unidentified)
Normal foetus/ Placenta	72	-	1	-	
Non-Gravid Uterus	14	-	4	2	(M.arginini)
				2	(M.ovipneumoniae)
Neonatal Lambs	38	-	2	-	
<b>TOTAL</b>	<b>664</b>	<b>77 (11.6%)</b>	<b>36 (5.4%)</b>	<b>22 (3.3%)</b>	

swabs of 25%. Grouping of results according to source (Table 4.2) reveals that normal, barren and aborted ewes in all of which the vulva appeared normal, yielded only five ureaplasma isolates from 98 samples (5.6%). In comparison, ewes with vulvovaginitis yielded 38 isolates of ureaplasma from 86 (44%) samples examined. Isolation titres of ureaplasma from vaginal swabs ranged from  $10^2$  to  $10^5$ ccu/0.2ml of sample.

A.laidlawii, the next most commonly isolated mycoplasma from vaginal swabs, was cultured from 17 (9.8%) samples. No significant differences were apparent in the isolation rates of A.laidlawii from the different groups.

M.arginini was isolated from four of six vaginal swabs collected from one outbreak of vulvovaginitis and from two of four sampled in a separate outbreak. Both outbreaks occurred just after tupping.

Two other species of mycoplasmas isolated from vaginal swabs were M.capricolum and A.axanthum. Both these mycoplasmas were isolated from a group of 120 housed ewes which were part of a flock of 2250 (Jones, Rae, Holmes, Lister, Jones, Grater and Richards, 1983). An outbreak of vulvovaginitis occurred in this group two to four weeks prior to lambing. Lesions which developed initially just below the tail became haemorrhagic and bled when the scabs which formed on the vulva were removed. Vulvar scabs from four of six affected animals yielded M.capricolum. A.axanthum was also identified in three of these samples.

A.oculi was the only other mycoplasma isolated from vaginal swabs. It was isolated from one normal and two

TABLE 4.2ISOLATION OF MYCOPLASMAS FROM VAGINAL SWABS

CONDITION	NUMBER CULTURED	NUMBER POSITIVE FOR:		
		Ureaplasma sp.	A.laid.	Other spp.
Normal	37	1 (2.7%)	3 (8.1%)	1 (A.oculi)
Post-Abortion	16	1 (6.3%)	3 (18.7%)	-
Barren	35	3 (8.6%)	-	1 (A.oculi)
Vulvovaginitis	86	38 (44%)	11 (12.8%)	6 (M.arginini) 4 (M.capricolum) 3 (A.axanthum)
TOTAL	174	43 (25%)	17 (9.8%)	16 (9.2%)

barren ewes; the latter had been culled, and at the time of sampling, exhibited a creamy, mucopurulent vaginal discharge.

With the exception of Ureaplasma sp. and A.laidlawii the incidence of the other mycoplasmas isolated from the ovine urogenital tract was very low. The individual isolation rates from vaginal swabs were; M.arginini (3.6%), M.capricolum (2.3%), A.axanthum (1.7%) and A.oculi (1.7%).

#### Isolation Of Mycoplasmas From The Upper Urogenital Tract

From the upper urogenital tract three species were isolated. A.laidlawii was isolated nine times, M.arginini twice and M.ovipneumoniae on three occasions. Four of the isolations of A.laidlawii, the two of M.arginini, and two of M.ovipneumoniae were from non-gravid uteri obtained at the local abattoir. One isolate of A.laidlawii was made from a normal placenta, but the remaining four A.laidlawii isolates and one M.ovipneumoniae isolate were obtained from aborted placentae (Table 4.1).

Table 4.3 analyses the isolations from semen and preputial samples according to whether the source animal was "diseased" (suffering from balanoposthitis, granular posthitis, poor semen quality, or implicated as the cause of repeated service or vulvovaginitis) or apparently normal. Ureaplasma sp. and A.laidlawii were the only organisms isolated from these animals. No significant differences were observed in the isolation rate of ureaplasmas from normal or "diseased" rams, 21 isolates being obtained from 149 "normal" ram samples (14%), and 13

from "diseased" ram samples (17%). A.laidlawii, undetected in "normal" animals, was isolated from eight (11%) "diseased" ram samples.

Mycoplasmas have been isolated from the urogenital tract of unmated lambs (Livingston and Gauer, 1978; Jones and Rae, 1979) and it was suggested that these lambs became infected per vaginam at parturition. In an attempt to confirm this supposition two flocks of pregnant ewes were examined. Flock A consisted of 29 Scottish Blackface ewes and flock B consisted of 24 Greyface ewes intended for the supply of SPF lambs. Vaginal swabs were taken from the dams and vaginal or preputial swabs from the lambs in Flock A at parturition and again when the lambs were two weeks old. A.laidlawii, the only mycoplasma isolated, was obtained from three ewes, and from two preputial swabs taken from the 38 lambs at the second sampling. Neither of the positive lambs were the progeny of the ewes which had yielded A.laidlawii.

Mycoplasmas were not isolated from any ewe in Flock B, nor from any of their 39 lambs at one week of age.

The virtual absence of mycoplasmas and total absence of ureaplasmas thwarted the aim of this study. Thus no conclusions can be made concerning the possible infection of lambs during passage through the lower urogenital tract at parturition.

The samples examined bacteriologically yielded bacteria of many groups: Micrococcus, Coliforms, Streptococcus, Neisseria, Staphylococcus, Bacillus, Corynebacterium, Escherichia and Proteus. Most of these

TABLE 4.3ISOLATION OF MYCOPLASMAS FROM SEMEN AND PREPUTIAL SWABS

CONDITION	NUMBER CULTURED	NUMBER POSITIVE FOR:	
		Ureaplasma sp.	A.laidlawii
Normal	149	21 (14%)	-
Diseased*	68	13 (19%)	8 (11.1%)
TOTAL	217	34 (15.7%)	8 (3.7%)

\*Diseased -- tups with balanoposthitis, granular posthitis, orchitis, poor semen quality or implicated as the cause of repeated service or vulvovaginitis.



are regarded as part of the normal flora of the urogenital tract of many animals including sheep (Buxton and Fraser, 1977). No one group was consistently isolated or associated with any condition, and no differences were apparent between diseased or normal animals regarding the groups isolated.

Because of the time-consuming procedures involved in examining for viruses and chlamydia selected samples only were examined. Material from aborted and barren ewes, ewes with vulvovaginitis and semen from infertile rams were examined. No isolates were obtained from any sample.

#### DISCUSSION

Seven species of mycoplasma have been shown to occur in the ovine urogenital tract in this survey. To the four species known at the start of this survey, namely M.arginini, M.ovipneumoniae, A.laidlawii and Ureaplasma sp. can be added M.capricolum, A.axanthum and A.oculi. Most of the 135 isolates obtained (118, or 87%) were from the lower urogenital tract (vaginal and preputial swabs or semen).

The most frequently isolated was Ureaplasma sp. which comprised 57% of all isolations. Significantly higher isolation rates of ureaplasmas were obtained from ewes with vulvovaginitis compared with that from ewes with normal vulvas. An association of ureaplasmas with granular vulvitis of sheep was similarly noted in Canada by Doig and Ruhnke (1977) in a single outbreak of vulvitis which affected 19 sheep in a flock of 40. The more extensive sampling of this present survey, which involved many

flocks, provides considerably more evidence to link ureaplasmas with vulvovaginitis.

The presence of ureaplasmas in vaginal swabs and the failure to isolate the organism from any upper urogenital tract samples, suggests that ureaplasmas are unable to ascend from the lower urogenital tract to colonise the uterus or the Fallopian tubes.

A similar situation appears to exist with bovine urogenital ureaplasmas. Langford (1975) examined uterine exudates and endometrial scrapings from 80 infertile and 65 pregnant cows but failed to recover ureaplasmas. Ball, et al. (1978) isolated ureaplasmas from the stomach contents of 5 of 237 aborted bovine fetuses and from 4 of 93 aborted placentae, but not from the placentae or foetal tissues from 80 cases of normal parturition. They suggested that ureaplasmas may have a role in diseases of the bovine placenta which result in abortion: however, vaginal mucus samples taken on the day of abortion from 225 cows yielded 19 ureaplasma isolates. The possibility exists therefore that the aborted fetuses became contaminated with ureaplasmas from the vagina during expulsion. In a follow-up study, Ball and McCaughey (1979) studied the distribution of mycoplasmas within the urogenital tract of 118 normal cows. Five regions were examined in each tract; ureaplasmas were isolated from 20% of the vulva/vestibule samples, but from only 1.5% of the cervical samples. These findings support those of this survey which indicate that, despite vaginal colonisation by ureaplasmas, there is little evidence to suggest that

colonisation of the upper urogenital tract occurs. However, it is possible that ureaplasmas may be introduced into the cervix or uterus by mechanical transmission of a vaginal infection or by deposition of ureaplasma-infected semen during mating. Establishment in the uterus might lead to deleterious effects on ovum fertilisation or on the developing conceptus.

No significant differences were observed in the isolation rates of ureaplasmas from normal and diseased rams. The overall isolation rate of ureaplasmas of 15.7% is similar to that reported by Livingston and Gauer (1978) who isolated ureaplasmas from 19 of 92 (23%) semen samples or preputial swabs taken from normal tups.

It is apparent therefore that a proportion of normal and healthy rams harbour ureaplasmas. The only indication from this study that ureaplasmas may be involved in disease was that isolation titres from "diseased" rams were slightly higher (mean  $10^{4.2}$ ccu/0.2 ml) compared with normal animals (mean  $10^{2.5}$ ccu/0.2ml). This difference is not significant.

The more frequent isolation of ureaplasmas from the semen of fertile tups than from tups whose semen was of poor quality or which were implicated as the cause of ewes returning to service, was surprising. Ureaplasmas have been shown in vitro to adsorb to and agglutinate human and bovine spermatozoa (Taylor-Robinson and Manchee, 1967; Gnarpe and Friberg, 1973), and the presence of ureaplasmas and other mycoplasma species in bull semen has been suggested as a cause of reduced sperm counts and impaired

motility (Jurmanova and Sterbova, 1977), and therefore possible causes of infertility.

The quality of semen collected from the fertile rams was good, being creamy in colour and with strong spermatozoal motility. The presence of ureaplasmas in these semen samples suggests that they do not affect either the motility of ovine spermatozoa or the fertility of the tup.

The isolations of M.capricolum and A.axanthum were of interest as this was the first time they had been isolated from sheep in the United Kingdom.

M.capricolum has been isolated only from sheep and goats, and early reports suggest that it is a pathogenic mycoplasma. It has been associated with several conditions including polyarthrititis in sheep and kids (Swanepoel, et al., 1977; Cordy, Adler and Yamamoto, 1955), and a contagious agalactia syndrome in goats (Perreau and Breard 1979; Talavera Boto, 1980). Experimentally, it has been shown to produce pneumonia and death in kids when given orally (Da Massa, Brooks, Adler and Watt, 1983).

M.capricolum has not previously been isolated from the urogenital tract and there are no other reports of its association with vulvovaginitis. In an intensive follow-up study on the flock from which these isolates were obtained, samples were examined from a further 203 ewes and 35 rams but no further isolations of M.capricolum were made (Boughton and Jones, 1983). No significant serology was obtained on examining 645 sera from the flock. There is no supportive evidence therefore, to indicate whether the

M.capricolum was responsible for the cases of vulvovaginitis seen in this flock.

The other mycoplasma species isolated from this flock of ewes, A.axanthum has previously been isolated from several hosts including cattle, pigs, horses and birds (Tully, 1979). It has not been reported from sheep nor has the organism been associated previously with vulvovaginitis. Experimentally, the intranasal inoculation of A.axanthum into four to six month old specific pathogen-free pigs produced only slight lesions in the lungs (Stipkovits, Romvary, Nagy, Boden and Varga, 1974). Gourlay, Howard and Brownlie (1979) inoculated a strain of A.axanthum, reported by others to induce mastitis, into the bovine mammary gland. The organism did not become established or multiply in the gland, indicating that the strain was not pathogenic.

M.arginini was isolated on six occasions from ewes with vulvovaginitis. In the only isolation of M.arginini reported previously from the ovine urogenital tract 2 of 160 swabs from clinically normal sheep yielded the organism (Jones and Rae, 1979).

M.arginini occurs in many hosts, but has been associated principally with the respiratory tract of sheep and goats (Cottew, 1979). The organism has been isolated on a few occasions from the bovine urogenital tract, in particular, semen samples from a bull with vesiculitis (Leach, 1970) from clinically healthy bulls (Jurmanova and Krejci, 1971) and from the uterus of an infertile cow (Langford, 1975). It has also been found in cattle with

mastitis (Leach, 1970). Attempts to examine the pathogenicity of M.arginini in the hosts from which it has been isolated have consistently failed to show a pathogenic role for this species. Foggie and Angus (1972) inoculated two specific pathogen-free lambs, one intratracheally and one intranasally. The organism was not re-isolated from the lungs of either lamb, suggesting that the minimal lesions seen in the lamb inoculated intratracheally resulted from the broth in which the mycoplasma was diluted. Watson, Cottew, Erdag and Arisoy (1968) inoculated goats subcutaneously with Type N mycoplasmas, subsequently identified as M.arginini, but failed to bring about infection. Endobronchial inoculation of M.arginini into three calves failed to produce lesions in two of the lungs (Jurmanova, Cerna, Sisak and Hajkova, 1975). Evidence presently available therefore indicates that a pathogenic role for M.arginini is unlikely.

In addition to its isolation from the upper genital tract A.laidlawii was isolated from the vaginal swabs of 6.8% of ewes with normal vulvas and from 12.8% of ewes with vulvovaginitis.

A.laidlawii has been isolated from the urogenital tract of normal sheep on several occasions (Cottew, 1974; Leach, et al., 1976; Kumar and Pathak, 1979; Jones and Rae, 1979). Doig and Ruhnke, (1977) also isolated the organism from 1 of 19 sheep with granular vulvitis. A.laidlawii is usually considered to be saprophytic (Whittlestone, 1972) although it has been associated with lesions in the bovine genital tract (Olson et al., 1960; Hoare, 1969).

Furthermore, Ball et al. (1978) isolated A.laidlawii more often from aborted than from non-aborted cattle placentae, but since the organism was also isolated more frequently from the vaginal mucus of cows on the day of abortion than from non-aborting cows, the possibility of contamination of the aborted placentae by A.laidlawii from the vagina cannot be excluded.

The only other mycoplasma isolated from vaginal swabs was A.oculi which was isolated on three occasions. A.oculi was first isolated from the eyes of goats with keratoconjunctivitis (Al-Aubaidi, Dardiri, Muscoplatt and McCauley, 1973). A.oculi has subsequently been reported from several other hosts, namely pigs (Kuksa and Gois, 1975), horses (Moorthy, Kirchoff, Heitmann, Plumer and Spradbrow, 1977) camels, (Al-Aubaidi, Ernø and Al-Shammery, 1978), sheep (Arbuckle and Bonson, 1980) and cattle (Kelly, Jones and Hunter, 1983). There have been two reports of its isolation from the ovine urogenital tract, both from India. Kumar and Pathak (1979) isolated A.oculi from one of 47 samples of uterine mucosa examined at slaughter, and Tiwana and Singh (1982) isolated the organism from the vaginas of 6 of 50 ewes with vulvovaginitis.

As yet the only experimental evidence of a pathogenic role for A.oculi comes from the work of its original isolators Al-Aubaidi et al., (1973) who produced a pneumonic condition and generalised septicaemia in goats following intravenous inoculation. No further supportive evidence indicating A.oculi to be a pathogen has been produced, suggesting that its role in diseases of animals,



particularly goats, remains doubtful.

The isolations of M.arginini, M.ovipneumoniae and A.laidawii from the upper genital tract were obtained from uterine material in which the possibility of cross-contamination from other sites could not be excluded. Due to the system of killing employed at the abattoir, uteri had generally to be collected from the floor of the killing hall. M.arginini and M.ovipneumoniae are commonly found in the respiratory tract of abattoir slaughtered sheep (Alley, Quinlan and Clark, 1975; Jones, Gilmour, Donachie, Rae, Anderson and Nettleton, unpublished observations). Thus, despite attempts to avoid contamination of the uterine mucosa by organisms on the exterior surface, it is possible that the isolations of M.arginini and M.ovipneumoniae were due to contamination of the uterus by respiratory tract material during evisceration of the animal.

M.ovipneumoniae has not been recorded from the upper urogenital tract of sheep but it was isolated from one of 167 vaginal swabs taken from a flock of housed sheep in England (Leach et al., 1976). No urogenital tract disease was reported in these sheep. The doubt concerning the source of the M.arginini and M.ovipneumoniae isolates, and the infrequency of their occurrence in the ovine urogenital tract, suggests that neither organism is likely to be a significant pathogen at this site.

The finding that the lower urogenital tract is often colonised by mycoplasmas, in high numbers in some instances, but that the upper tract is rarely infected,



suggests that either the cervix is an efficient barrier to the entry of these organisms or that the uterine environment is unsuitable for mycoplasmal colonisation.

With the exception of A.laidlawii and Ureaplasma sp. the incidence of the other five mycoplasmas in the ovine urogenital tract was very low, suggesting that this site is not their normal habitat, and that none is likely to have a significant pathogenic role in urogenital tract diseases. Similarly, although A.laidlawii was isolated from 10% of vaginal swabs and from 5.4% of all samples, it is generally considered to be non-pathogenic.

The overall isolation rate of ureaplasmas from vaginal swabs was 24.7% and from semen or preputial swabs 15.7%. The higher incidence of ureaplasmas in the urogenital tract would suggest that this site is more readily colonised by ureaplasmas than by other species of mycoplasmas and that ureaplasmas may be merely commensals in the urogenital tract. However, the difference in isolation rates between those ewes with normal vulvas (5.6%) and those with vulvovaginitis (44%) is significant. An association of ureaplasmas with ovine vulvitis has been noted previously in Canada by Doig and Ruhnke (1977), who isolated ureaplasmas from 18 of 19 ewes with vulvitis; the authors suggested that the 50% conception rate in the flock may have been due to the vulvitis. In Texas, one strain of ureaplasma isolated from the urogenital tract of sheep was associated with a uterine infection resulting in infertility (Livingston and Gauer, 1978).

The evidence from these two reports suggesting a

pathogenic role for ureaplasmas in the ovine urogenital tract, the parallel observations with human and bovine ureaplasmas, (reviewed in Chapter One) and the high incidence of ureaplasmas in cases of ovine vulvovaginitis found in this survey, all indicate a need for a more thorough investigation into the role ureaplasmas may have in the ovine urogenital tract.

CHAPTER FIVEINVESTIGATIONS ON THE PATHOGENICITY OF  
OVINE UREAPLASMAS

The survey yielded 77 ureaplasmas isolated from normal and diseased animals. In order to determine which isolates should be used for pathogenicity studies in sheep, selected strains were initially screened in several model systems. Strains were selected on the basis of their serological reactions in the metabolism inhibition test or on their association with vulvovaginitis. Eight strains were tested in most of the systems used.

IN VITRO SYSTEMS(i) Cell Cultures

The cell cultures used were foetal lamb kidney (FLK), foetal lamb testes (FLT), foetal lamb skin (FLS), sheep thyroid (STh), sheep vaginal epithelium (SV) and sheep uterus (SUt). All cell suspensions were dispensed in tubes with flying coverslips at a concentration of  $1 \times 10^5$  cells/ml. The growth medium for all cells was Medium 199 supplemented with 10% foetal bovine serum (FBS) and ampicillin (0.012mg/ml).

When the cell monolayer was 50-70% confluent the medium was removed and the monolayers washed three times with PBS. Sixteen hour ureaplasma broth cultures were used as inocula. The pH of these were generally alkaline and therefore the inocula were diluted 1 in 100 with PBS to

avoid pH-related cellular damage, 200 $\mu$ l of this dilution was added to each of 20 tubes and the ureaplasmas allowed to adsorb to the cells at 37°C for 60 min. Control tubes were inoculated with 200 $\mu$ l of PBS. After this adsorption period the inoculum was removed and 1.5ml of Medium 199, supplemented with 2% FBS and ampicillin, was added to each tube and these were incubated at 37°C. Cell monolayers were examined daily, coverslips were removed and stained using the DNA-specific stain bisbenzamide (Chen, 1977). Aliquots of media from the infected monolayers were also assayed for ureaplasma titre.

#### (ii) Organ Cultures

Sheep uteri, complete with Fallopian tubes, were generally collected at a time when ewes would be in their oestrus cycle, from mid-November until the end of the year. The complete oviduct was placed in Hanks BSS containing ampicillin (1mg/ml) and amphotericin B (0.2mg/ml) to inhibit bacterial and fungal growth respectively, and incubated at 37°C. The tubes were excised from the body of the uterus, opened along their length and laid flat. Pieces of tissue, approximately three mm<sup>2</sup>, were cut from the opened ends and placed on prescored grids in 50 mm, vented plastic petri dishes (Sterilin Ltd.). Four pieces of tissue were placed in each dish and sufficient Medium 199, supplemented with 10% FBS, ampicillin and amphotericin B, was added to each dish to give a depth of two mm. Dishes were placed in a plastic box and incubated overnight at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Cultures were examined for

viability by examining ciliary activity using a stereo microscope fitted with oblique illumination. It was not always possible to see ciliary activity over the whole surface of the organ culture but activity at the edges was usually evident. Initially attempts were made to quantify the quality of ciliary activity but this very subjective measurement was subsequently abandoned and only the presence or absence of ciliary activity was noted.

Organ cultures which were viable the day after they were initiated were inoculated after removal of the medium, with 50-100 $\mu$ l of an 18 hour ureaplasma culture diluted 1 in 100 in PBS. After inoculation, the medium was replaced with Medium 199 supplemented with 2% FBS, ampicillin and amphotericin B. Cultures were examined daily for the presence of ciliary activity, and pieces of tissue and samples of medium were titrated for the presence of ureaplasmas. The medium of all cultures, except those detailed later, was changed daily.

(iii) Embryonated Hens' Eggs.

Studies using all eight strains were carried out in this system. Yolk sac inoculation was performed on 7 - 10 days old embryos by drilling a hole in the shell over the airspace and inoculating one ml of ureaplasma culture into the yolk sac. The inoculation site was swabbed and sealed

with "Sellotape". Eggs were incubated at 37°C and candled daily, embryonic deaths being recorded and pieces of yolk sac titrated for the presence of ureaplasmas. Yolk sacs were harvested and stored at -70° C until the results of the ureaplasma titrations were known: positive yolk sacs were coarsely chopped and resuspended in PBS to give a 1 in 10 dilution and passaged through eggs on a further two occasions.

#### IN VIVO SYSTEMS.

The pathogenicity of ovine ureaplasmas for small laboratory rodents was investigated principally in mice, but rats, guinea pigs and hamsters were also used. The intraperitoneal (i/p) or intranasal (i/n) routes of inoculation were used in all animal species; other routes of inoculation which were occasionally employed are detailed below. Animals were examined for clinical signs of disease during each experiment and necropsied at the end to check for any evidence of macroscopic abnormalities.

##### (i) Mice

Inbred Balb/C mice, four to six weeks old, of both sexes were used throughout, except where inoculation was per vaginam.

Cultures of each isolate were inoculated i/n (0.05ml) into 10 mice, and i/p (0.5ml) into a further 10 mice. Pairs of mice were killed daily from day one to day five, and the lungs from the i/n inoculated animals and, the

lungs, spleen, kidney, liver and brain from the i/p inoculated mice were examined for abnormalities and the presence of ureaplasmas. Ten mice per isolate were inoculated intravenously (i/v) with 0.1 ml of culture. Initially, pairs of mice were killed at daily intervals between days 0 and 5, but times of killing were later reduced to 15 min, 1h, 2h, 4h, and 24h after infection. At necropsy liver, lungs, spleen, kidney and whole blood were examined for ureaplasma presence.

Five strains were each inoculated per vaginam into 10 mice. Inoculation was performed using a polyethylene sheath over a 26G needle; the tip of the sheath was just introduced into the vagina and approximately 0.1 ml of culture deposited in the vulvovaginal vestibule. The mice were examined daily for the development of vulvitis. Pairs of mice were killed each day, the urogenital tract being dissected out and examined for the presence of ureaplasmas. Urine was also collected when possible and examined for ureaplasmas.

Serum pools from all groups of mice were prepared and tested in the metabolism inhibition test.

#### (ii) Rats

Each strain was inoculated separately by the i/n, i/p and per vaginam routes into groups of five rats. Animals were killed daily between days one and five, at necropsy the same tissues as from mice (except brain) were removed and examined for the presence of ureaplasmas.

(iii) Guinea Pigs

Guinea pigs were infected by the i/n and i/p routes only, five guinea pigs being used per isolate and inoculation route. One animal was killed daily over a period of five days and the lungs of the i/n infected animals as well as the lungs, liver and spleen from the i/p infected animals were sampled for the presence of ureaplasmas.

(iv) Hamsters

The numbers of hamsters used, the routes of inoculation and the tissues examined were as described for guinea pigs.

(v) Mouse Mastitis Model

This model was originally developed by Chandler (1970) to study the bacteria associated with bovine mastitis; it has since been adapted to study bovine and human ureaplasmas (Howard, Anderson, Gourlay and Taylor-Robinson, 1975).

Mice were inoculated via the teat canal on the 4th or 5th day of lactation, offspring having been removed at least one hour before inoculation. Initially, mice were anaesthetised with ether, but this was found not to be totally satisfactory as the level of anaesthesia needed to be monitored constantly during preparation and inoculation of the mice. It was found more convenient to anaesthetise the mice with sodium pentobarbitone (Sagatal, May & Baker),



diluted 1 in 10 in distilled water to provide 6 mg/ml. Each mouse was injected i/p with 0.1 ml of this solution, anaesthesia reached an effective level in less than five minutes and lasted for 15 to 40 minutes.

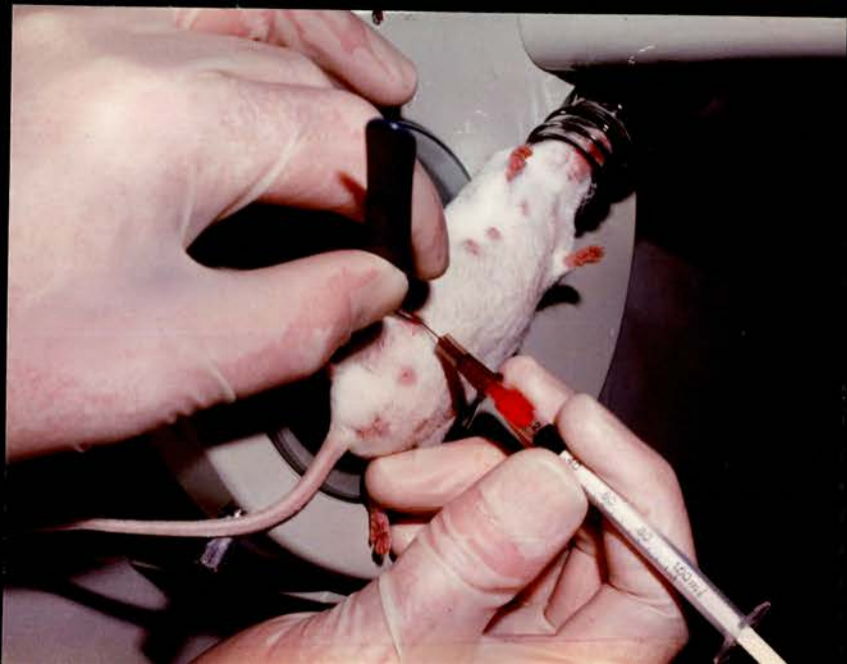
The mice were laid on their backs on a cork board and the mammary glands were swabbed with alcohol to lay down the hair around the teats and make observation easier. Inoculation via the teat canal was aided by the use of a stereo microscope and was carried out using a one ml syringe fitted with a 26 G, 1 cm needle (Fig. 5.1). An overnight broth culture of ureaplasmas, diluted 1 in 10 in PBS, was inoculated in 100 $\mu$ l volumes into one of the four abdominal mammary glands; the controls comprised 100 $\mu$ l of PBS inoculated into a second gland. Successful inoculation into the gland was marked by an absence of subcutaneous swelling at the base of the teat. Later, it was found that removal of the tip of the teat and the use of needles from which the bevelled point had been removed facilitated easier entry into the teat canal and gave more consistent inoculation of the mammary gland.

Each isolate was inoculated into a single mammary gland of 25 mice. Five mice were killed by cervical dislocation on days 1, 2, 3, 4 and 6 after inoculation. Mammary glands were examined macroscopically for any abnormalities before being dissected out. The glands were cut in two, one half being placed in 10% formol saline for histological examination and the other half homogenised in 2.5 ml of peptone water in a Colworth Stomacher. This

Fig 5.1

Inoculation procedure used in mouse mastitis model. Inoculation of the gland via the teat canal was performed with a 26G, 1cm needle.

Observation of the procedure was aided by the use of a stereo microscope. Latterly, the mice were anaesthetised by i/p injection of sodium barbitone.



homogenate was used to determine the titre of ureaplasma in the gland and also to monitor for the possible presence of contaminating bacteria.

### Bovine Mastitis Model

Inoculation into the bovine udder has been used as a means of assessing the virulence of human and bovine ureaplasmas (Gourlay, Howard and Brownlie, 1972; Howard, Gourlay and Brownlie, 1973).

Seven strains of ovine ureaplasma were tested in this system in an attempt to detect differences between strains.

Three Friesian cows in early lactation were used. Inoculation of the udder via the teat canal was performed using a 16 G polyethylene cannula (Portex Ltd.) immediately following the afternoon milking. The catheter was introduced 3 - 4 inches into the teat canal and 10 ml of an overnight broth culture diluted 1 in 10 in PBS inoculated. Each quarter received a different ureaplasma isolate, control culture or control broth. The cultures used as controls were strain A417C of Ureaplasma diversum (kindly supplied by Dr. C.J. Howard, Compton) which is known to be pathogenic in this system, (Gourlay et al., 1972), and A. laidlawii which is non-pathogenic for the bovine udder system (Jain, Jasper and Dellinger, 1969; Gourlay, et al., 1979).

At 17 hours post inoculation and daily thereafter for a period of 21 days, the udder was examined for signs of mastitis, the rectal temperature noted and the daily milk yield recorded. The appearance of the milk drawn from each quarter was noted and samples were taken to determine the

presence of bacteria and the numbers of ureaplasma and cells in the milk. Whey, prepared from each quarter of all cows on the day prior to inoculation and at 7, 14 and 21 days following inoculation was diluted 1 in 10 in PBS and tested in the MI test against its homologous strain.

## RESULTS

### IN VITRO SYSTEMS

#### (i) Cell Culture Studies

All eight strains tested, namely US1, US33, US50, US55, A801/1, 7790/2, 7860/1 and 6CT, were between the seventh and eleventh passage level in UB. Inoculum titres ranged from  $10^3$  ccu/0.2ml to  $10^5$  ccu/0.2ml.

Infected and control cells were stained at 2 - 3 day intervals and the medium titrated for the presence of ureaplasmas. Of the cell types used the most consistent results were obtained with the ovine uterus cells. All strains tested were able to infect these cell monolayers and multiply. Variable results were obtained with the other cell types used. STh and FLK cells showed no evidence of infection with any strain either on stained coverslips or by cultivation of the supernatant medium when first sampled three days after infection. Strains US1, US33, US50 and 6CT were detectable, by staining, in a few cells in the FLS, FLT and SV monolayers but they did not spread to infect other cells and could not be detected in the culture medium at any time.

After these initial studies which involved all cell types, further work concentrated on using the ovine uterus

cell cultures. The eight strains were re-tested in these cells and it was confirmed that all were able to infect the cells and multiply. Ureaplasmas were detectable in a few cells by bisbenzamide staining two days after infection (Fig. 5.2) and the organisms could also be recovered from the medium at this time. The numbers of infected cells increased and six days after infection nearly all cells were heavily infected (Fig 5.3). Maximum titres were reached five to six days following infection and remained at this level until day 10 after infection, when the monolayers became overgrown and began to detach from the glass. The cytopathic effect (CPE) produced was slight; cell cytoplasm became granular three to four days after infection in a few cells, and was evident in most cells by day seven (Fig 5.4). The overall appearance of the monolayer had become vacuolated by the tenth day (Fig 5.5), but rounding or detachment of the cells was not observed except when monolayers became overgrown and large areas of cells became detached. The inoculation of medium containing infected cells into another batch of cells induced a CPE similar to the above.

Failure to change the culture medium every second day led to the appearance of a non-specific CPE, similar to that produced by the ureaplasmas. However, under these circumstances the pH of the medium became very alkaline, the cell cytoplasm became granular and cells detached from the glass.

Fig 5.2

a. Ovine uterus cell culture.

Bisbenzamide stain      x1200

b. Ovine uterus cell culture infected with strain  
A801/1. Two days after infection.

Bisbenzamide stain      x1200

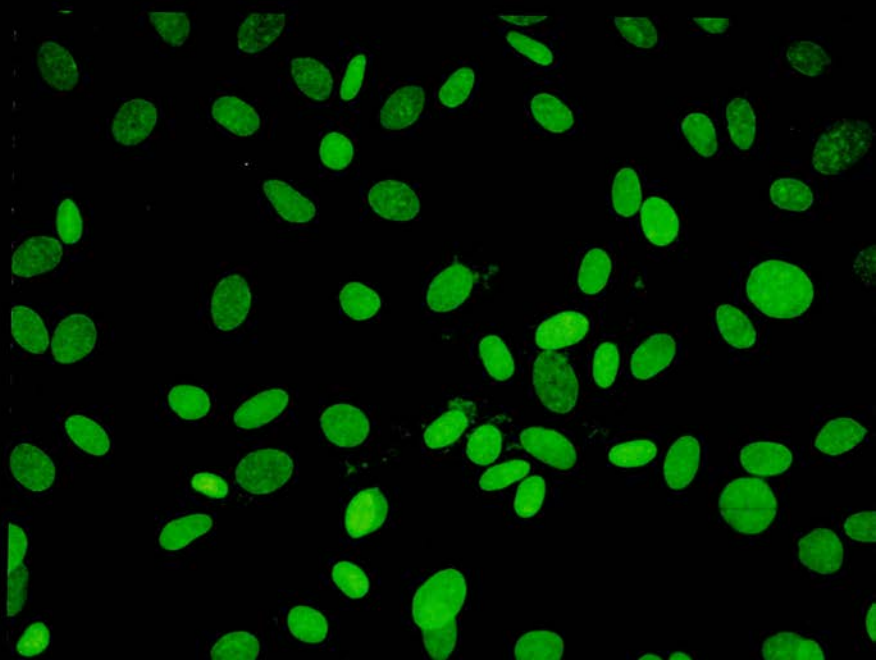
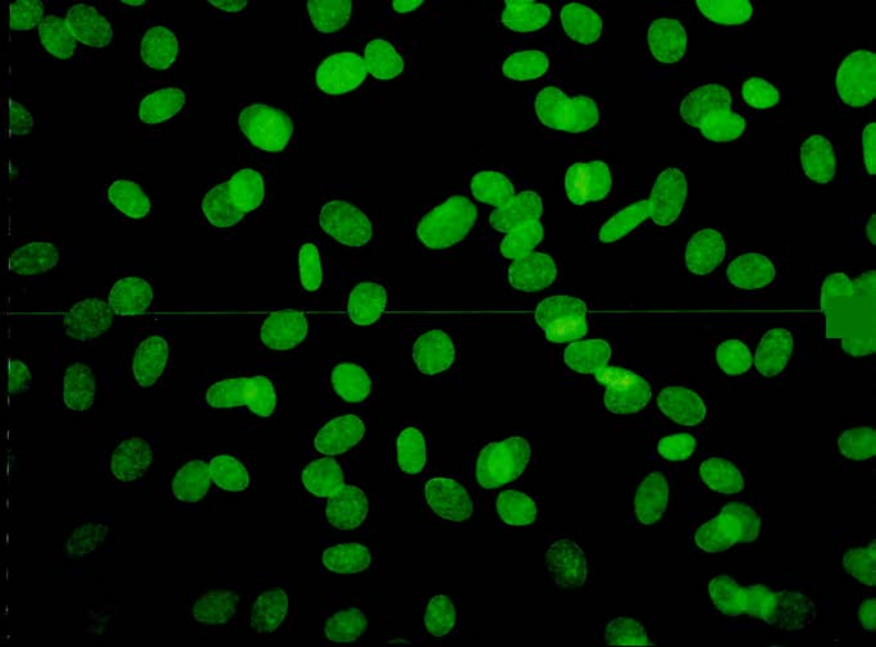




Fig 5.3

- a. Ovine uterus cell culture infected with strain A801/1. Six days after infection.

Bisbenzamide stain            x1200

- b. Ovine uterus cell culture infected with strain A801/1. Six days after infection.

Bisbenzamide stain            x3000

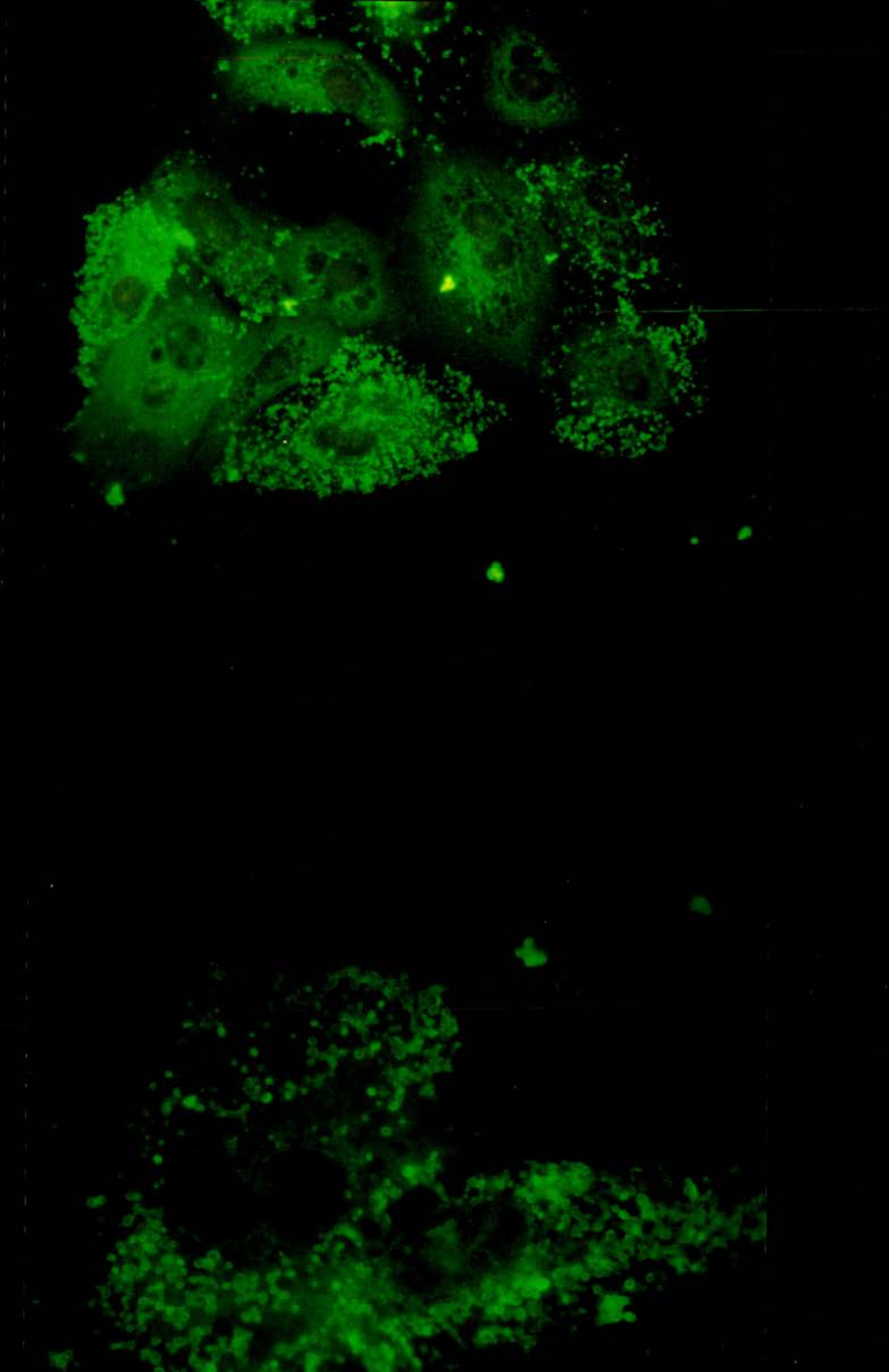


Fig 5.4

a. Ovine uterus cell culture.

Phase contrast            x96

b. Ovine uterus cell culture infected with strain  
A801/1. Seven days after infection.  
Early CPE is evident.

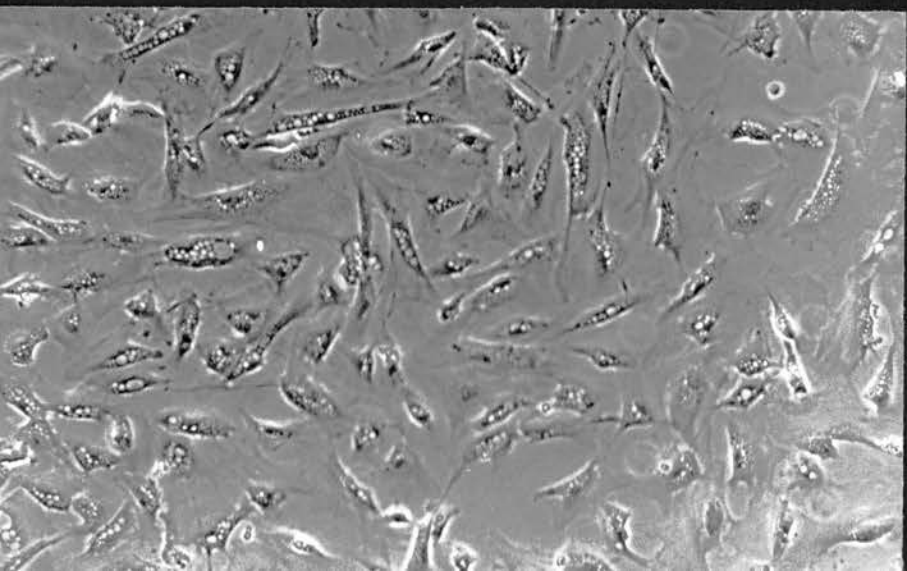
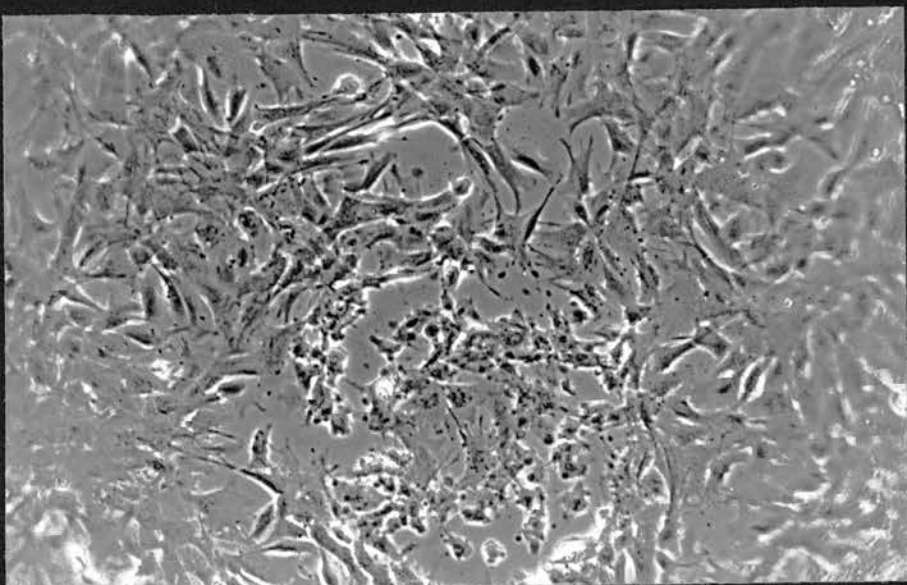
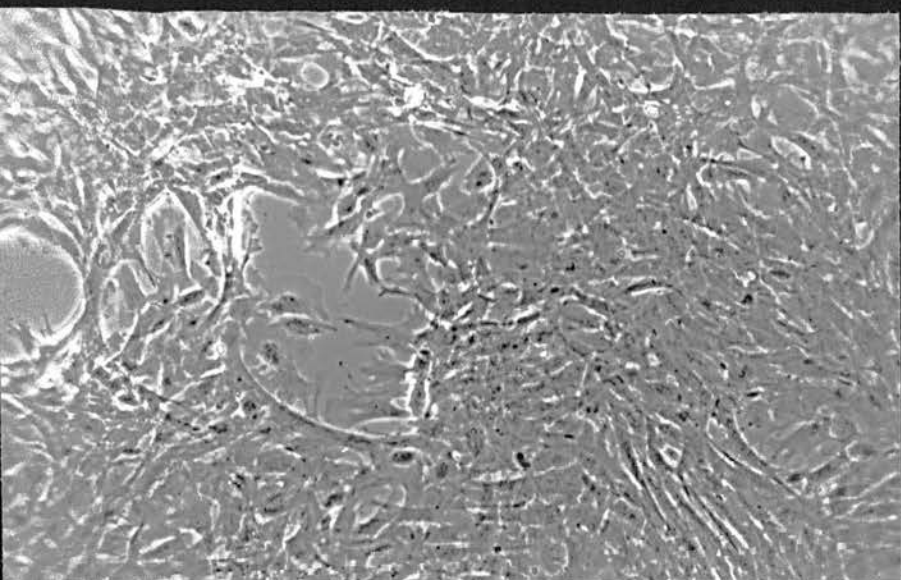
Phase contrast            x96

Fig 5.5

Ovine uterus cell culture infected with strain  
A801/1. Ten days after infection.

The cytoplasm of most cells is vacuolated.

Phase contrast            x96



Although ovine uterus cells could be infected with all eight ureaplasma strains tested no differences could be detected between strains in the effects produced on the cell monolayer.

Studies to determine whether three of the ureaplasma strains (6CT, US1 and 7860/1) could grow in the cell maintenance medium were also carried out. Tubes containing plain glass coverslips were seeded with two mls of culture medium and infected in the same manner as above. Samples of medium were examined for the presence of ureaplasmas at the same intervals as medium from infected cells.

The titre of the inoculum of all three strains was  $10^5$  ccu/0.2ml. Only strain US1 was recovered on a single occasion two days after infection, at a titre of  $10^3$  ccu/0.2ml, indicating that the medium alone was not capable of supporting growth of ureaplasmas.

(ii) Organ Culture Studies

Bacterial contamination was a frequent problem with organ cultures and studies were therefore limited to three strains namely 6CT, US1 and 7860/1. In experiments where the medium was changed daily all three strains could be recovered from the explant medium throughout the duration of the experiments. The inoculum titre of 6CT and 7860/1 was  $10^3$ ccu/0.2ml and that of US1  $10^5$  ccu/0.2ml; from twenty-four hours after inoculation until the experiment was terminated the titre of all three strains reached  $10^6$  ccu/0.2 ml and remained at this level (Fig 5.6). Ciliary activity was strong at 24 hours after infection and remained

so until days nine or ten, when activity began to decline. Ciliary activity in control cultures also declined at this time. No differences were noted in the appearance of the cilia between infected and control cultures.

In experiments where the medium was not changed, ureaplasmas were recoverable at 24h and 48h, but not at 72h after infection, and ciliary activity had ceased by this time. (Fig. 5.7). The pH of the medium became alkaline (pH 8.5 - 9.0) by 24h after infection and remained high throughout.

The medium used to maintain the organ cultures also appeared capable of maintaining viability of the ureaplasmas. However, the titres attained ( $10^2$  -  $10^3$  ccu/0.2 ml) were not as high as those achieved when tissue was also present.

All three strains tested gave comparable growth curves in organ cultures but none of the strains appeared to have any effect on the activity or appearance of the cilia.

### (iii) Embryonated Hens' Eggs

The eight strains were each inoculated in one ml. volumes into the yolk sac of five eggs. Inoculum titres are shown in table 5.1. Three days after inoculation with strain 6CT one embryo died, the yolk sac was harvested and titrated and ureaplasmas at a titre of  $\geq 10^7$  ccu/0.2 ml were recovered. No other deaths occurred and the embryos were killed by chilling ten days after inoculation. Aliquots of the individual yolk sacs inoculated with each strain were pooled and titrated. Ureaplasmas were not

recovered from yolk sacs inoculated with five of the strains but the remaining three strains, 6CT, US1 and A801/1, were recovered at titres of  $10^4$  to  $10^5$  ccu/0.2 ml, (Table 5.1)

The reisolation titre of 6CT from one egg three days (dai) after infection was 100-fold greater than its inoculum titre, indicating that it had multiplied in the yolk sac. However, the recovery titres of the three strains at 10 dai were similar to those of the inocula.

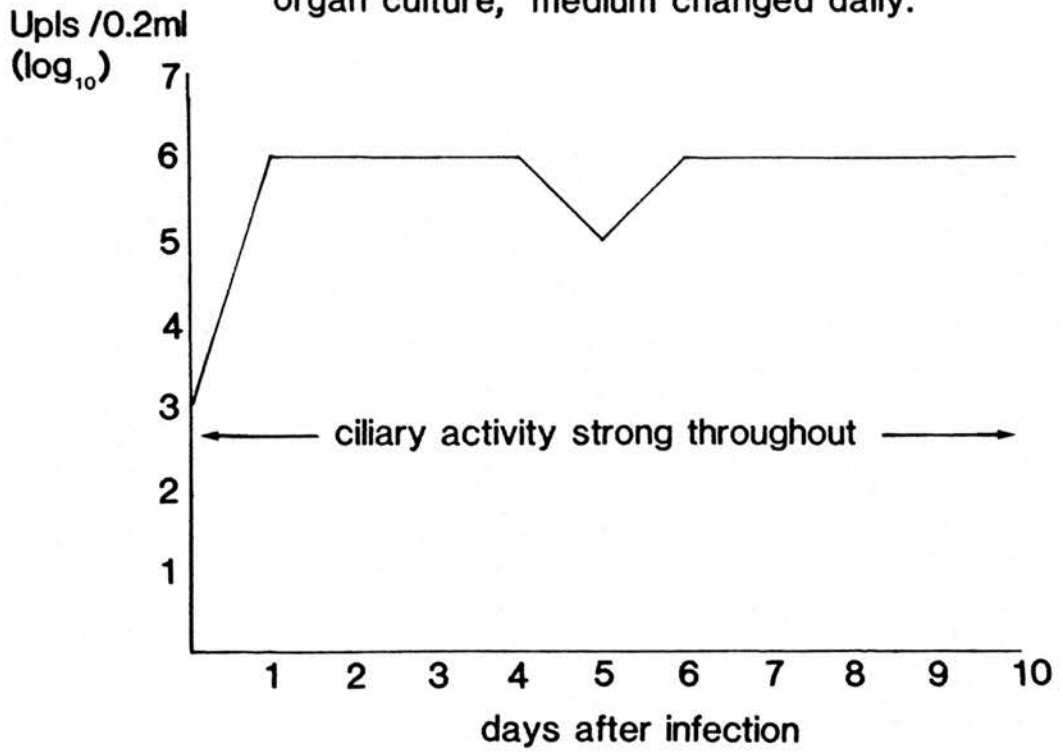
All yolk sacs, whether or not culturally positive for ureaplasmas, were pooled according to strain and stored at  $-70^{\circ}\text{C}$  until more embryonated eggs became available. Each of the eight pools was inoculated into a further five eggs. The titres of the positive yolk sacs from the first passage had fallen slightly during storage at  $-70^{\circ}\text{C}$ . No embryonic deaths were recorded in any of this second batch of eggs; embryos were killed as before, at 10 dai. Ureaplasmas were not recovered from any yolk sac inoculated with the five strains which had been negative in the first passage and low titres only were obtained with the three positive strains (Table 5.1).

The positive yolk sacs were inoculated, without being stored, into a third batch of eggs, five per strain. Again no deaths were recorded, embryos being killed and processed as before. Ureaplasmas were not recovered from any of the infected yolk sacs.

There was no evidence, during passage, of any infection in the embryo, nor were macroscopic lesions observed in any of the embryonic membranes.

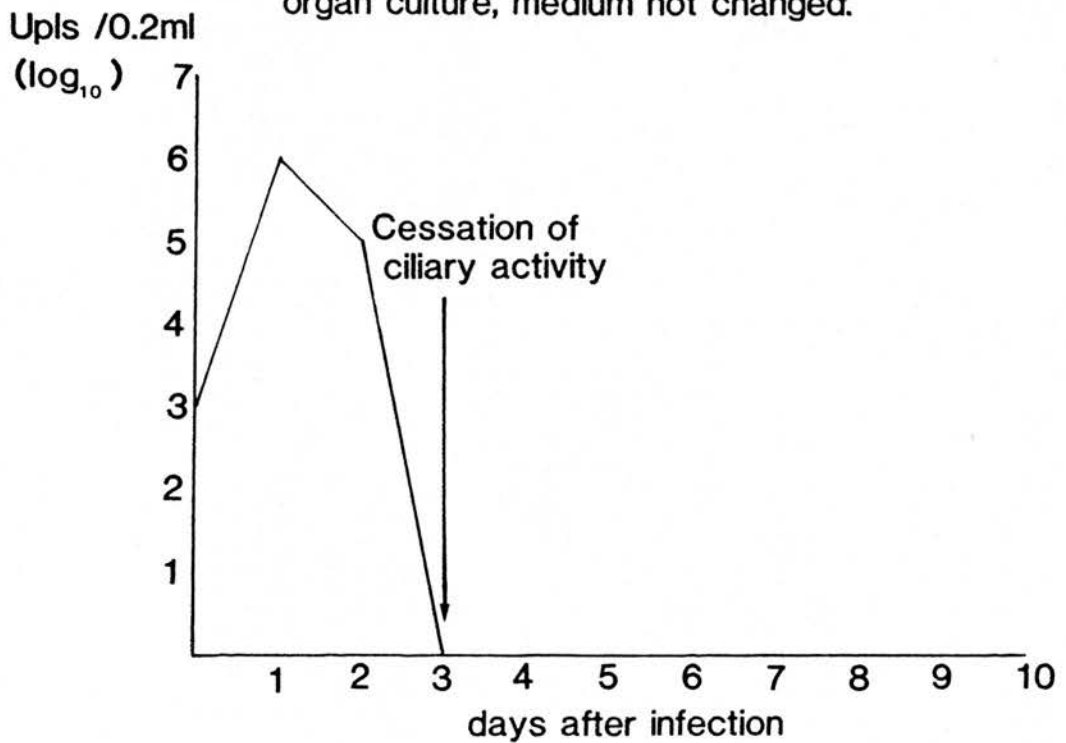
**Fig. 5.6**

Growth of 7860/1 in ovine Fallopian tube organ culture, medium changed daily.



**Fig. 5.7**

Growth of 7860/1 in ovine Fallopian tube organ culture, medium not changed.





**TABLE 5.1**

DETAILS OF UREAPLASMA STRAINS INOCULATED INTO EMBRYONATED HENS' EGGS.

UREAPLASMA STRAIN	TITRE OF INOCULUM ccu/0.2ml	RE-ISOLATION TITRE		
		1st pass	2nd pass	3rd pass
6CT	$10^4$	$10^5$	$10^3$	-
US1	$10^5$	$10^4$	$10^3$	-
7860/1	$10^4$	-	-	ND
US50	$10^3$	-	-	ND
US55	$10^5$	-	-	ND
US33	$10^4$	-	-	ND
A801/1	$10^4$	$10^4$	$10^2$	-
7790/2	$10^3$	-	-	ND

ND = not done

- = negative in culture

## IN VIVO SYSTEMS

### (i) Rats, Hamsters And Guinea Pigs

Neither clinical disease nor macroscopic lesions developed in rats, guinea pigs or hamsters. Ureaplasmas were not recovered from any of the tissues (Table 5.2).

### (ii) Mice

Clinical disease or macroscopic lesions were not observed in mice inoculated i/n, i/v or per vaginam, nor were ureaplasmas recovered from any tissues or urine sampled at necropsy (Table 5.2).

Ureaplasmas, at low titre, were recovered from the spleen of one mouse inoculated i/p with strain A801/1 one hour after inoculation but not at four, twenty-four or forty-eight hours. Hog gastric mucin (HGM) has been reported to increase the susceptibility of mice to infection with Pasteurella haemolytica following i/p inoculation. (Smith 1958; Evans, 1983). The possibility that HGM may enable ureaplasmas to become established in the mouse model was investigated. A mixture comprising four parts HGM and one part A801/1 culture was inoculated i/p into a group of twenty mice, another control group of twenty mice being inoculated with culture only. Five mice from each group were killed at the same intervals as before and tissues were examined for the presence of ureaplasmas. One of the mice inoculated with culture alone again yielded ureaplasmas from the spleen one hour after inoculation.

TABLE 5.2

## LACK OF PATHOGENICITY OF OVINE UREAPLASMAS FOR LABORATORY ANIMALS

SPECIES	TYPE	NUMBER IN GROUP	ROUTE OF INOCULATION	TITRE OF INOCULUM* ccu/0.2ml	RESULT DEATH/DISEASE	UREAPLASMA RECOVERED
Wistar rat	Newly Weaned	5	0.1 ml i/n	$10^4 - 10^6$	-	-
			0.1 ml p/v	$10^3 - 10^6$	-	-
			0.5 ml i/p	$10^4 - 10^6$	-	-
Guinea pig	Newly Weaned	5	0.1 ml i/n	$10^4 - 10^6$	-	-
			0.5 ml i/p	$10^4 - 10^6$	-	-
Hamster	Newly Weaned	5	0.1 ml i/n	$10^3 - 10^6$	-	-
			0.5 ml i/p	$10^4 - 10^6$	-	-
Mice	4 - 6 weeks of age	10	0.05 ml i/n	$10^4 - 10^6$	-	-
			0.1 ml i/v	$10^4 - 10^5$	-	-
			0.1 ml p/v	$10^3 - 10^6$	-	-

\* titre varied between strains; strains were at 7<sup>th</sup> - 10<sup>th</sup> passage in culture

Mice given both A801/1 and HGM and killed one hour after inoculation all yielded ureaplasmas from the spleen and uterus; the kidneys of three and the liver and peritoneal fluid of one were also positive. (Table 5.3). Ureaplasmas were not recovered from either group at 4, 24 or 48 hours after inoculation.

This experiment was repeated with two other strains of ureaplasmas, US1 and US50, and similar results were obtained.

No antibody was detected by the MI test in the serum from any of these animals.

(iii) Mouse Mastitis Model

All ureaplasma strains multiplied in the gland over the first few days until day four when titres in most glands began to decline, by day six ureaplasmas could be recovered from only one gland of one mouse inoculated with strain 7790/2, (Table 5.4).

The growth curves of all eight strains in the mammary gland were similar, maximum titres being achieved by day three and with the single exception noted above, becoming undetectable by day six. The growth curve of strain 7860/1 is shown in Fig 5.8.

At necropsy the glands in all mice were enlarged and contained milk. Some of the infected glands appeared hyperaemic in contrast with the creamy-white appearance of the control glands (inoculated with PBS or not inoculated, Fig. 5.9). This appearance was observed in most infected

TABLE 5.3

RECOVERY OF UREAPLASMAS FROM MOUSE TISSUES AFTER INTRAPERITONEAL  
 INOCULATION OF CULTURE ALONE AND CULTURE WITH HOG GASTRIC  
 MUCIN (HGM) ADDED.

INOCULUM	MOUSE NO.	RECOVERY OF UREAPLASMAS 1 HOUR AFTER INOCULATION				
		SPLEEN	LIVER	KIDNEY	UTERUS	PERITONEAL FLUID
A801/1	1	-	-	-	-	-
	2	-	-	-	-	-
	3	10 <sup>2*</sup>	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
A801/1 + HGM	1	10 <sup>3</sup>	-	+	+	+
	2	10 <sup>3</sup>	-	+	+	-
	3	10 <sup>2</sup>	10 <sup>2</sup>	-	+	-
	4	10 <sup>2</sup>	-	+	+	-
	5	10 <sup>3</sup>	-	-	+	-

\* ccu/ml of tissue suspension (10%)

+ Tissue not titrated, only checked for presence

- no ureaplasmas recovered

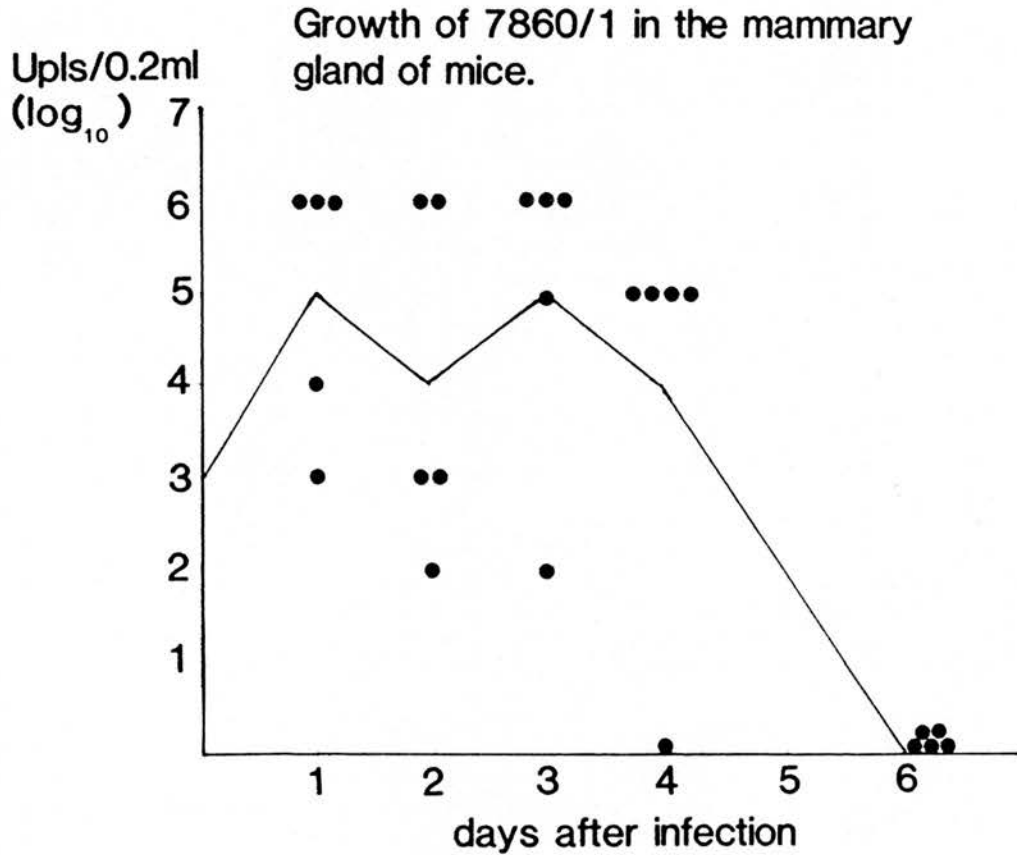
TABLE 5.4

RECOVERY OF OVINE UREAPLASMAS FROM THE MAMMARY GLAND FOLLOWING  
INTRAMAMMARY INOCULATION OF CULTURE INTO MICE

STRAIN	INOCULUM TITRE ccu/0.2ml	AV. TITRE RECOVERED ON DAY:				
		1	2	3	4	6
A801/1	$10^4$	$10^5$	$10^6$	$10^6$	$10^3$	0
US1	$10^6$	$10^5$	$10^6$	$10^6$	$10^2$	0
6CT	$\geq 10^7$	$10^5$	$10^6$	$10^7$	$10^4$	0
7860/1	$10^3$	$10^5$	$10^4$	$10^5$	$10^4$	0
7790/2	$10^4$	$10^4$	$10^5$	$10^5$	$10^5$	$10^2$
US33	$10^3$	$10^5$	$10^4$	$10^5$	$10^3$	0
US50	$10^5$	$10^5$	$10^5$	$10^6$	$10^2$	0
US55	$10^4$	$10^4$	$10^6$	$10^6$	$10^4$	0

25 lactating mice were used for each strain

Fig 5.8



Each point indicates the titre of ureaplasmas recovered from an individual gland . The line is drawn through the mean titre of ureaplasmas isolated on each day.

glands until day three, but declined thereafter and was absent by day six. All glands gradually reduced in size over the six days presumably in response to the lack of stimulation of milk production following the removal of offspring.

A summary of the histological findings produced by strain 6CT is given in Table 5.5. All glands were still lactating one day after infection and continued to do so until day four, when lactation in some glands began to diminish. Very few glands, infected or control, were still lactating on day six. The major histological change observed in infected glands was an inflammatory exudate, which was noted first in a few glands on day one and affected all infected glands by day three. The exudate was present in the milk sinuses and interstitium of the glands and consisted mainly of neutrophils. Pyknotic cells were seen in some glands on day two and by day three all infected glands had neutrophils and cellular debris present in the alveoli and milk sinuses (Fig. 5.10). Contraction of alveoli began to occur in both infected and control glands on day four and dead or dying cells were present in large numbers in the alveoli of all infected glands at this stage. Calcified cell debris was also noted at this time in some glands inoculated with PBS. By day six no inflammatory exudate could be seen in any infected gland but calcified cell debris was present in all infected glands (Fig. 5.11) and some PBS inoculated glands. All non-inoculated control glands remained normal throughout the six days. (Fig. 5.12).



TABLE 5.5

SUMMARY OF HISTOLOGICAL CHANGES IN THE MAMMARY GLAND IN MICE  
INOCULATED WITH STRAIN 6CT.

D.P.I.	MOUSE NO.	GLAND LACTATING			INFLAMMATORY EXUDATE IN						PYKNOTIC CELL IN ALVEOLI		
		L4	R5	R4	ALVEOLI			INTERSTITIUM			L4	R5	R4
					L4	R5	R4	L4	R5	R4			
1	1	+	+	+	-	-	-	+	-	-	-	-	-
	2	+	+	+	-	-	-	-	-	-	-	-	-
	3	+	+	+	+	ND	-	+	ND	-	-	ND	-
	4	+	+	+	-	-	-	-	-	-	-	-	-
	5	+	ND	+	+	ND	-	+	ND	-	-	ND	-
2	1	+	+	+	+	-	-	-	-	-	+	-	-
	2	+	+	+	+	-	-	+	-	-	-	-	-
	3	+	+	+	+	-	-	+	-	-	+	-	-
	4	+	ND	+	-	ND	-	-	ND	-	-	ND	-
	5	+	+	+	+	-	-	+	-	-	+	-	-
3	1	+	+	+	+	-	-	+	-	+	+	-	+
	2	+	+	+	+	-	-	+	-	-	+	-	-
	3	+	+	+	+	-	+	+	-	-	+	-	-
	4	+	+	+	+	-	-	+	-	+	+	+	-
	5	+	+	+	+	-	-	+	-	-	-	-	-
4	1	+	+	+	-	-	-	-	-	-	-	-	-
	2	+	+	+	-	-	-	-	-	-	+	+	+
	3	+	+	+	-	-	-	-	-	-	+	+	+
	4	±	+	-	+	-	-	+	-	-	+	-	+
	5	±	+	±	-	-	-	-	-	-	-	-	-
5	1	-	-	-	-	-	-	-	-	-	+	+	+
	2	-	-	-	-	-	-	-	-	-	+	+	+
	3	-	-	-	-	-	-	-	-	-	+	-	+
	4	±	±	±	-	-	-	-	-	-	+	-	+
	5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		L4	R5	R4	L4	R5	R4	L4	R5	R4	L4	R5	R4
		GLAND AFFECTED											

L4 = ureaplasma infected gland  
R4 = PBS inoculated gland  
R5 = uninoculated control gland

+ = strong positive reaction  
- = negative reaction  
± = weak reaction  
ND = tissue not examined

Fig 5.9

Mouse mastitis model. Appearance of mammary glands three days after inoculation.

Hyperaemia produced by inoculation of strain 6CT is seen in gland L4. The control gland (R5) and PBS inoculated gland (R4) are normal in appearance.

L4



R4

R5

Fig 5.10

- a. Mouse mammary gland three days after inoculation with strain 6CT, showing inflammatory exudate in milk sinuses and pyknotic cells in interstitium.

H & E      x120

- b. Alveoli of mouse mammary gland three days after infection with strain 6CT, demonstrating the presence of neutrophils and cellular debris.

H & E      x300

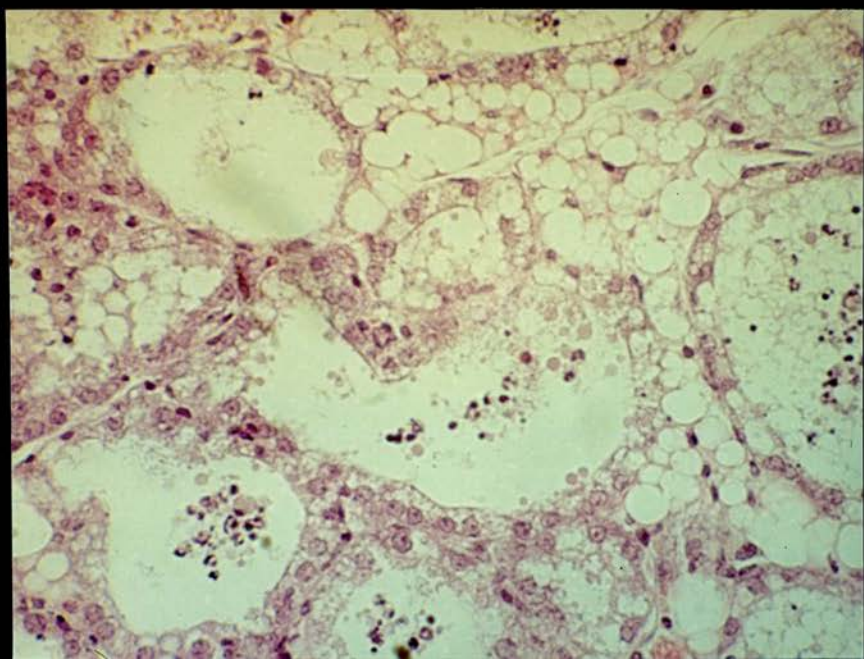
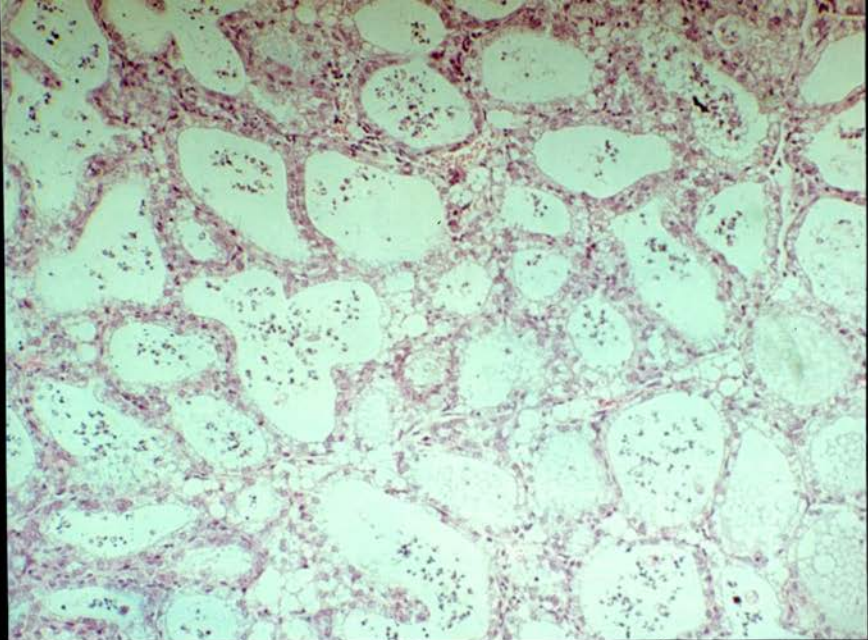


Fig 5.11

Mouse mammary gland demonstrating contraction of alveoli, reduction of the inflammatory exudate and calcification of cell debris (arrowed). Six days after infection with strain 6CT.

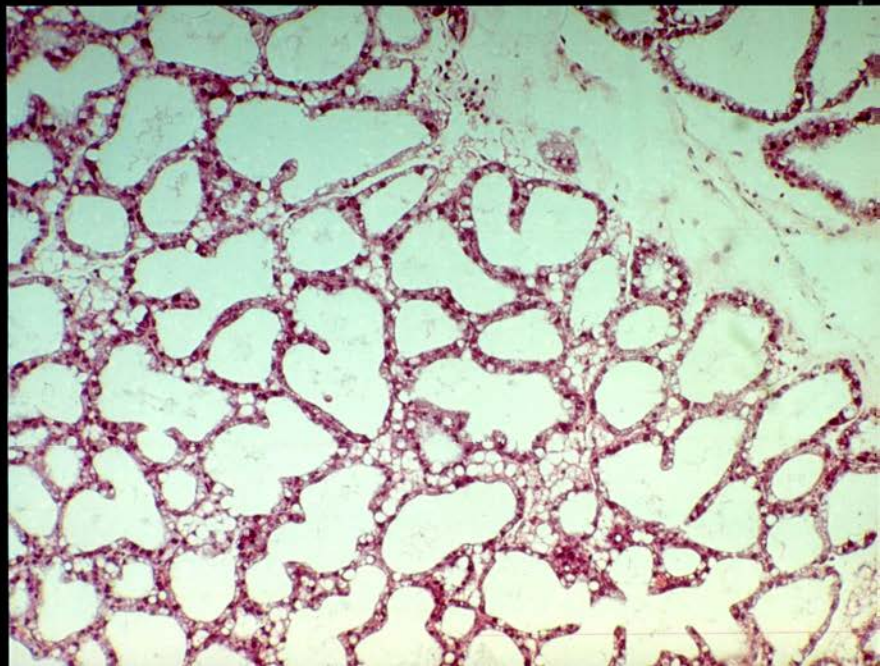
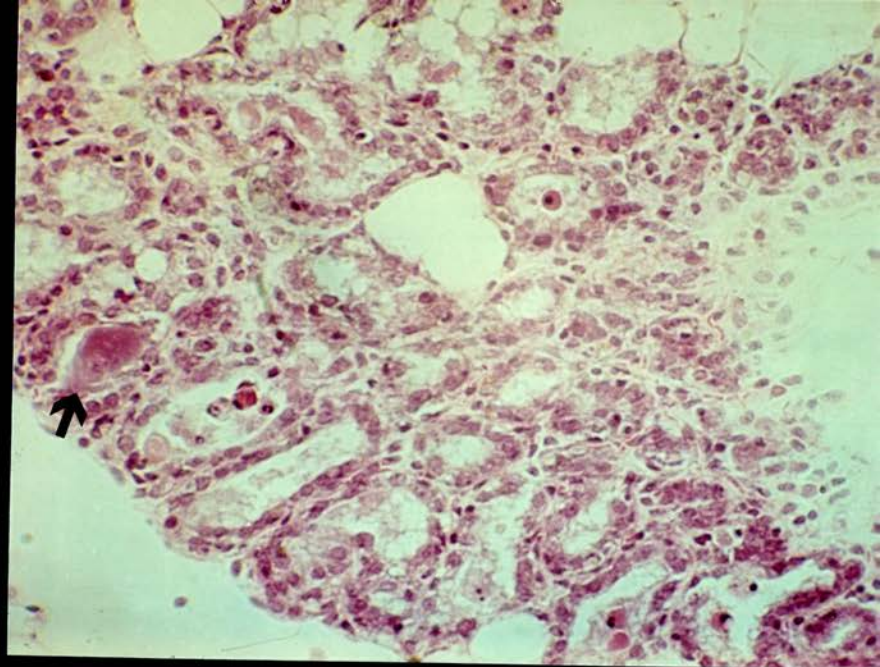
H & E            x150

Fig 5.12

Appearance of normal mouse mammary gland on day three. Note lack of debris in ducts.

H & E            x120





The gross appearance of the infected glands and the histological changes produced were similar for all the eight strains tested in this system.

Low numbers of Streptococcus, Staphylococcus and Bacillus were isolated from some infected and control glands. No histological changes were present in the control glands from which these bacteria were isolated, indicating that they were unlikely to have been responsible for the inflammatory exudate seen in ureaplasma infected glands.

#### Bovine Mastitis Model

The milk yield of each of the three cows was approximately 15 litres a day in the week prior to inoculation. Cell counts performed on the milk from each quarter were approximately  $10^5$  cells/ml or less. No mycoplasmas or ureaplasmas were cultured from the milk during this period. Details of the strains used are given in Table 5.6.

None of the cows showed a rise in rectal temperature, there were no obvious signs of abnormalities in the udder and milk yields were unaffected. All ureaplasma strains became established in the udder and, with one exception (US55) caused milk cell counts to rise to greater than  $10^6$  cells/ml. Four strains (A801/1, 7790/2, 7860/1 and US1) persisted in the milk at titres of  $10^4$ - $10^6$ ccu/ml throughout the period of observation, and cell counts in the quarters infected with these strains also remained high, the



TABLE 5.6

Details of strains inoculated into the bovine mammary gland.

COW NO.	QUARTER	STRAIN	ORIGIN	TITRE CCU/0.2ml
82	LF	A801/1	Vulvovaginitis	$10^4$
	LH	A.laid. <sup>†</sup>	Normal Vagina	$10^7$
	RF	7790/2	Vulvovaginitis	$10^4$
	RH	A417/C *	Bovine Lung	$10^5$
88	LF	7860/1	Vulvovaginitis	$10^5$
	LH	A.laid. <sup>†</sup>	Normal Vagina	$10^7$
	RF	US50	Normal Vagina	$10^5$
	RH	A417/C*	Bovine Lung	$10^5$
131	LF	US33	Normal Vagina	$10^6$
	LH	US1	Semen-Infertile Tup	$10^4$
	RF	US55	Normal Vagina	$10^5$
	RH	A417/C*	Bovine Lung	$10^4$

\* Strain of U.diversum. Kindly supplied by Dr. C.J. Howard, Compton.

+ Strain of A.Laidlawii. Isolated from ovine vaginal swab by author.

mean for all four strains over the 21 day period being  $10^{6.4}$  cells/ml. Cell counts with strain US1 exceeded  $10^7$  cells/ml from day 9 to day 15, and clots were present in the milk from this quarter on days one, two and again on days 12 and 13. (Fig. 5.13). Two strains (US33 and US50) were recovered from the milk only until 11 dpi; cell counts in the milk from these quarters fell to pre-inoculation levels by days 12 - 14. (Fig. 5.14).

The bovine strain A417C, which was included as a positive control, caused the cell count to increase to  $10^7$  cells/ml within 24 hours and although the number of cells in the milk from this quarter declined there were still around  $10^6$  cells/ml present at the end of the 21 day observation period. The organism was also isolated in high titres throughout this time (Fig. 5.15).

There was no response to the A.laidlawii infection other than a transient increase in cell counts on the two days following inoculation, and the organism was not re-isolated at any time. (Fig. 5.16). No other mycoplasmas or bacteria were isolated from the milk.

Only low levels of inhibitory activity were detected in the whey samples by the MI test, all had titres of 1 in 20 or less.

#### DISCUSSION

The large number of ureaplasmas isolated from the ovine urogenital tract made it desirable that a model system be found by which the infectivity and virulence of the different isolates could be compared. Such a system should

Fig. 5.13

Effect of strain US1 in the bovine mammary gland.

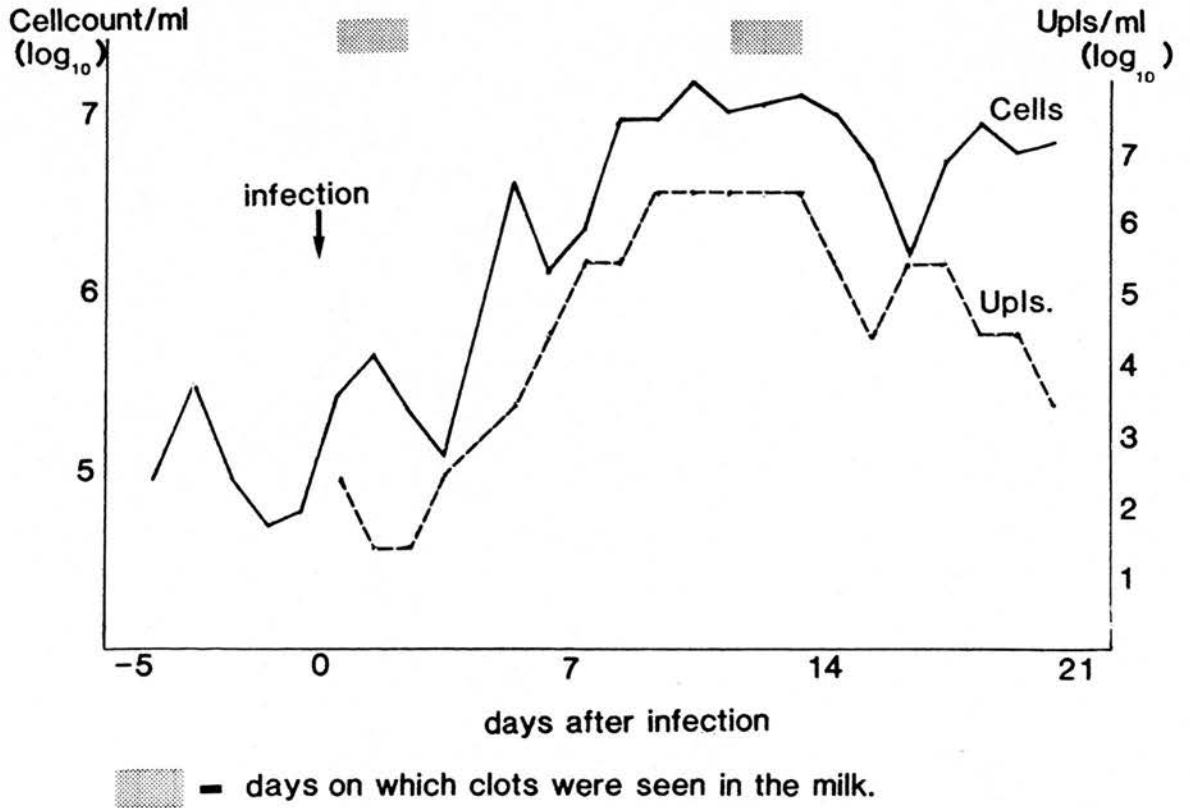
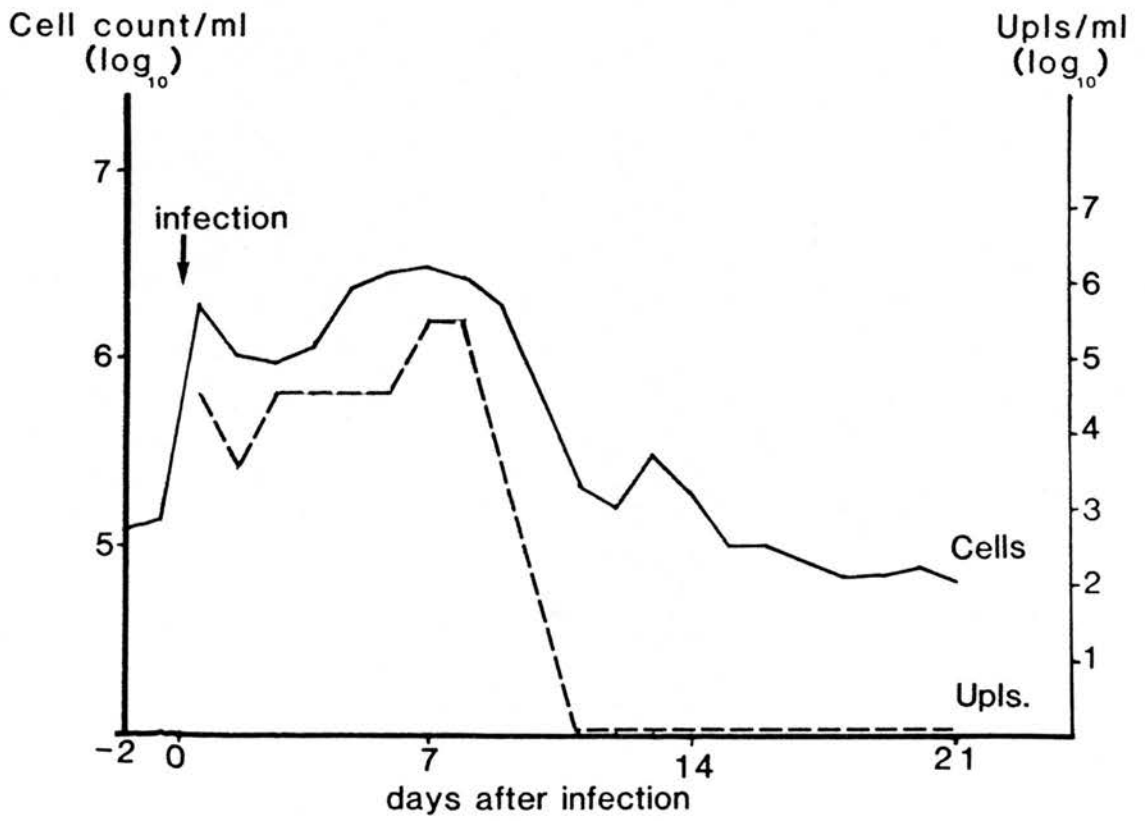


Fig. 5.14

Effect of strain US 50 in the bovine mammary gland.



**Fig. 5.15**

**Effect of bovine strain A417C in the bovine mammary gland.**

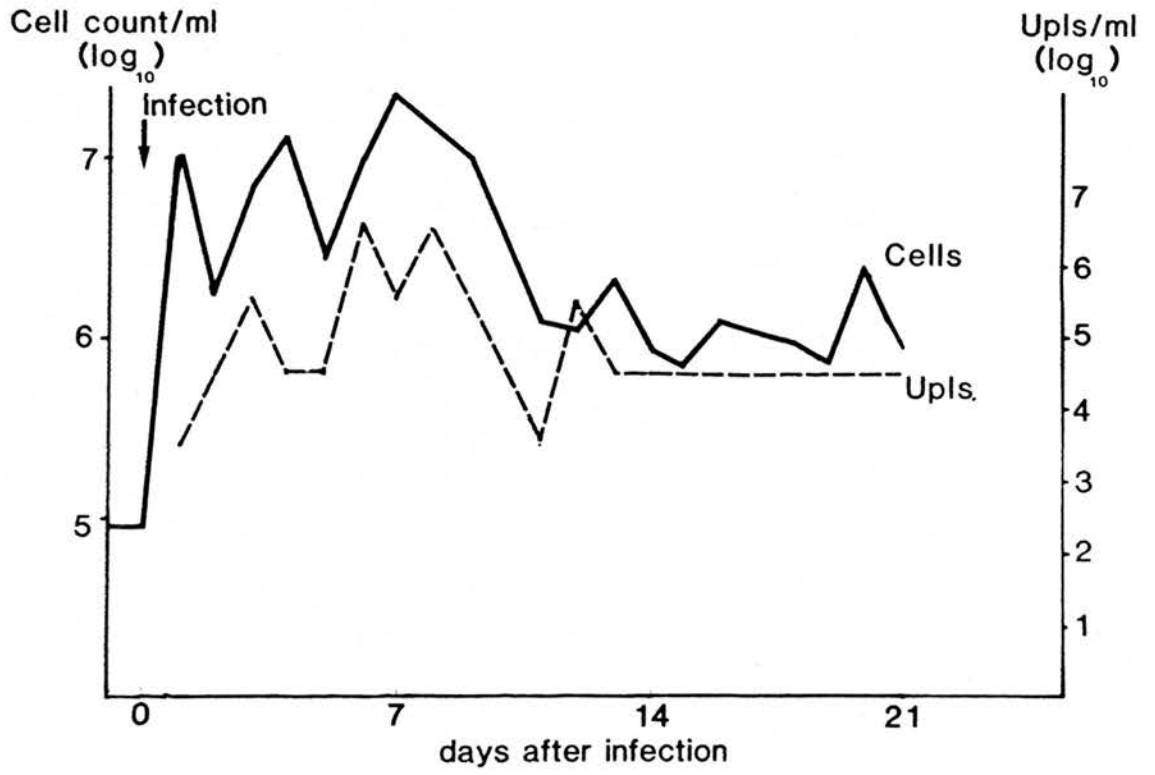
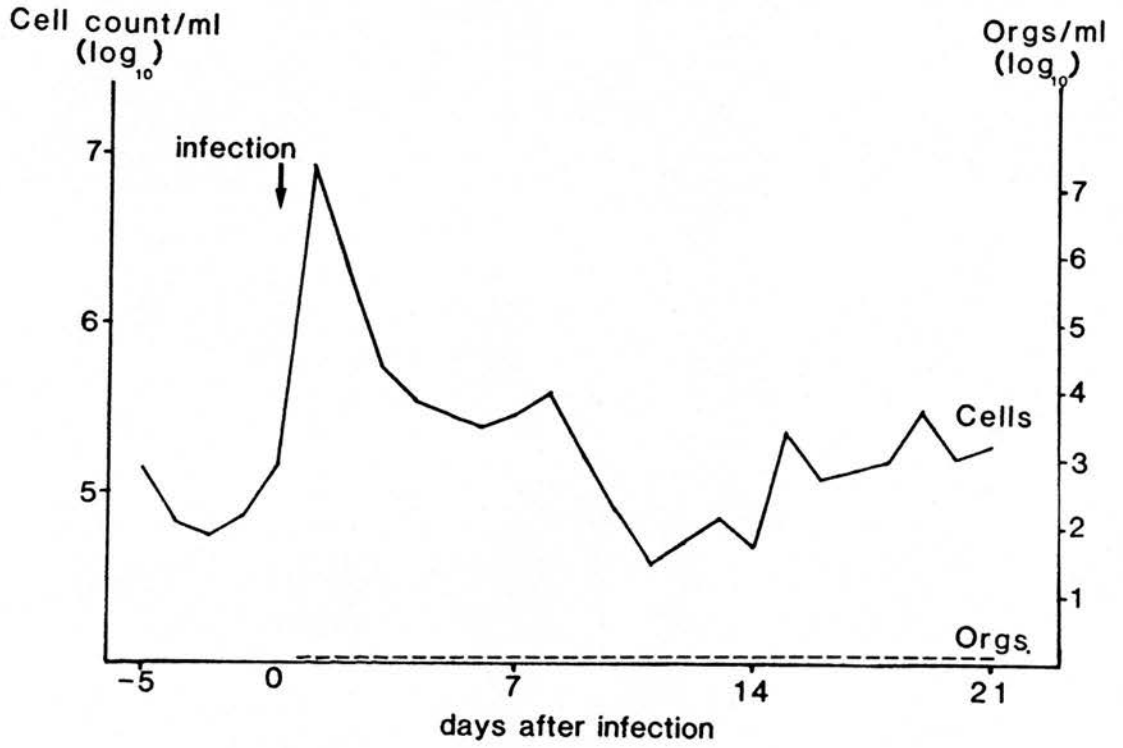


Fig. 5.16

Effect of A.laidlawii in the bovine mammary gland.



have the advantages of relatively low cost and ready availability and should be able to demonstrate any differences in the virulence of ureaplasma isolates. The isolates tested in this system consisted of five strains associated with disease and three strains from normal animals.

In the first test system used, cell culture, two criteria were used to select the cell type to be examined:

(i) Low passage cell cultures only were used to avoid any adventitious but undetected mycoplasmal contaminants, since a high percentage of established cell lines are contaminated by mycoplasmas: in contrast very few primary or secondary cell cultures are so affected.

(ii) the cell cultures used should all be derived from sheep tissues.

Four of the six cell cultures used were readily available. The other two, ovine uterus and vaginal epithelium, were produced specifically for these studies, because it was felt that, as most of the ureaplasmas examined had been isolated from the urogenital tract, these two cell cultures would be more likely to support their growth in vitro.

Two of the cell cultures, STh and FLK were found to be refractory to infection with all eight isolates tested and were excluded from the study at an early stage. Three other cell types FLT, FLS and SV supported growth of three isolates on one occasion, but two attempts to repeat this failed, in that ureaplasmas could neither be recovered from

the cells nor detected by bisbenzamide staining. It is difficult to determine why there should be this variation in infectivity, the organisms were all at the same stage of growth and at the same titre on each occasion. It was unlikely to be due to a growth inhibitor in the serum component of the medium, as the same batch of foetal bovine serum was used throughout. Thangavelu, Erno and Therkelsen (1979) reported that a least  $10^4$  or  $10^5$  mycoplasmas must be present before they can be detected in cell culture by bisbenzamide staining, which may explain why ureaplasmas were not seen. Theoretically, however, only one organism is required to be present for its detection by cultural means, so even low numbers of ureaplasmas should have been detected by culture of the cells. It has been suggested that mycoplasmas lose some of their pathogenicity or infectivity on prolonged storage or that they may become attenuated after continuous subculture in artificial media (Couch, Cate and Chanock, 1964; Smith, Chanock, Friedwald and Alford, 1967). This may explain why the three cell cultures could not be infected at the second or third attempts, as the strains had undergone two more passages since the first successful infection. However, the failure of the original stock culture to infect these cells on the third attempt failed to confirm this supposition. One other possible explanation was that the different batches of cells used varied in their sensitivity to infection with these three strains of ureaplasma. Different cell types have been shown to vary in their susceptibility to human ureaplasmas (Mazzali and Taylor-Robinson, 1971) but there appears to be



no evidence in the literature that demonstrates differences in sensitivity between different batches of the same cell type.

One cell culture type which was sensitive to all eight ureaplasma strains was the ovine uterus cells. A minimal CPE was produced by all strains, the onset and development of the CPE was similar, the only difference noted was that onset of the CPE was quicker in those cultures which received higher numbers of ureaplasmas.

A similar but more marked CPE was seen in cell cultures in which the medium was not replaced. This was presumably due to either the alkaline medium pH or the toxic metabolites produced by ureaplasmas or both. This suggests that the CPE seen in cultures in which the medium was changed regularly was caused by the same factors but was less marked because of the periodic removal of causal factors.

Observation of broth cultures have shown that if ureaplasmas are not subcultured soon after the onset of the increase in pH produced during growth, they quickly die (Ford, 1962) and this probably explains why ureaplasmas could not be recovered from cell cultures in which the medium was not changed.

The rise of pH in broth cultures of ureaplasmas is related to the degradation of urea in the medium to carbon dioxide and ammonia (Shepard, 1967). Although the culture medium alone preserved the viability of ureaplasmas for up to four days, the organisms did not reach the titres obtained when they were grown in the presence of cells. It is possible that low levels of urea were present in the

serum component of the culture medium which allowed limited multiplication of the ureaplasmas in cell-free medium. As growth of ureaplasmas was much better in the ovine uterus cell cultures, it is reasonable to assume that the cells were providing some factor(s), which was beneficial to the ureaplasmas.

Masover, Namba and Hayflick (1976), observed that the ability of human ureaplasmas to hydrolyse urea was lost or altered after they had been grown in human lung fibroblast cultures. This was confirmed by Shepard (1979), who suggested that the cells supplied factor(s) in vivo which the urea supplied in vitro.

Ureaplasmas of human origin have been shown to produce a toxin or toxic product during growth in vitro (Shepard and Masover, 1979). The exact nature of this product is still controversial, but it is possible that the ammonia produced during metabolism may be involved. Ammonia has been shown to be toxic for cells and tissues in vivo (MacLaren, 1969) and therefore it is possible that the ammonia produced by the ureaplasmas in this study was responsible for the CPE observed.

Although, infection of ovine uterus cells was successfully established, the fact that all the isolates tested produced very similar and mild cytopathic effects, and no differences were apparent in the numbers of cells infected, indicated this system was ineffective as a means of differentiating strains according to virulence. Organ cultures have been used to study both human (Taylor-Robinson and Carney, 1974) and bovine (Stalheim,

Proctor and Gallagher, 1976) ureaplasmas, and they appear to be a good model for investigations on the pathogenicity of ureaplasmas.

In the present studies only three strains were successfully investigated in Fallopian tube organ cultures. The recoveries of ureaplasmas from medium containing organ cultures were similar to those obtained from cell culture studies, i.e. ureaplasmas were recovered at higher titres and for longer periods from medium containing organ cultures than from medium alone and that ureaplasmas persisted longer in cultures in which the medium was changed daily than in cultures where the medium was not changed. Ciliary activity also ceased much more quickly in cultures where the medium was not changed, which again suggests the possible accumulation of a toxic product with a cilia-stopping effect. A similar effect was reported by Stalheim et al (1976), who found that ureaplasmas from the human genital tract and bovine genital and respiratory tracts were all capable of stopping ciliary activity in bovine uterine tube organ cultures 24 - 144 hours after inoculation. The cilia stopping effect could also be induced by adding to the organ cultures medium containing non-viable ureaplasmas, which again suggests the production of a toxin by the ureaplasmas. In this study, ciliostasis was not apparent in the infected cultures where the medium was changed regularly, suggesting that the repeated changes prevented the build up of the toxin and thereby prevented damage to the organ cultures.

From the results obtained in this work and by Stalheim et al. (1976) it is clear that the outcome of the experimental infections of organ cultures with ureaplasmas is dependent on the methods employed.

All three strains tested gave similar findings, and since it appeared that significant differences between strains were unlikely to be revealed by this system no further studies were undertaken. However, the system did demonstrate that toxic substance(s) produced by ureaplasmas during metabolism may accumulate and cause ciliostasis. If this toxin is also produced in vivo, localised cellular damage may occur, which, in the case of the urogenital tract, could cause, or predispose to, the production of vulvitis or vulvovaginitis.

Embryonated hens' eggs were frequently used in early studies on mycoplasmas and, before suitable culture media had been developed for human ureaplasmas, eggs were used for subculture of the organisms (Shepard, 1958). The first passage of ovine ureaplasmas in the yolk sac of embryonated hens' eggs suggested that the system had potential as a means of distinguishing between strains, as three of the eight inoculated strains were still recoverable ten days after inoculation. However, further passage of these strains led to a gradual decline in numbers of ureaplasmas recovered. No macroscopic lesions were seen in any of the membranes surrounding the embryo nor in the yolk sac itself, and it was felt that the three strains which were recoverable were merely persisting in the yolk sac and becoming diluted with each passage.

Other than studies involving the mouse mammary gland there are only two known reports on the use of small laboratory animals for the study of ureaplasma infections, both of these concern the human ureaplasma U.urealyticum. Friedlander and Braude (1974) injected U.urealyticum at titres of  $10^6$  to  $10^8$  ccu/ml into the bladder or kidneys of rats; bladder stones were produced in 78% of animals. Kraus, Jacobs, Chandler and Arum (1977) implanted subcutaneous tissue cavities into mice and guinea pigs in a study involving two strains of U.urealyticum. One strain persisted for more than four months in mice and for four weeks in guinea pigs. The other strain was detected only on day one in the mouse and not at all in the guinea pig. Although the work of Friedlander and Braude (1974) is interesting suggesting a possible link between ureaplasmas and the formation of bladder stones, there is no evidence of a causal relationship in man. The studies of Kraus et al. (1977) produced, as the authors themselves point out, a very artificial situation, in that organisms normally associated with mucosal surfaces were transposed to a very different environment. The effects produced at this site may be completely different from those elicited in their normal habitat.

The four animal species used in the present work were readily available and the routes of inoculation used to infect them were mainly those which would bring the ureaplasmas into contact with the anatomical sites from which they are normally isolated i.e. mucosal surfaces.

However, excluding the intramammary route in mice, none of the animal species tested became infected by any route of inoculation with any of the eight ureaplasma strains tested. The concomitant injection of hog gastric mucin into the peritoneal cavity of mice appeared to increase the invasiveness of the organisms but did not allow infection to become established. Thus ovine ureaplasmas appear to be extremely host specific.

Despite the failure to infect mice by other routes, intramammary injections, with the eight strains tested did produce colonisation and mastitis. These findings are similar to those of Howard et al. (1975), who found that all nine bovine and human strains tested produced mastitis. Two of the bovine and three of the human strains were isolated from the urogenital tract, but information on their association with disease was not given so that assessment of the mouse mastitis model as an indicator of ureaplasma pathogenicity cannot be made from this work. Another study which used the same model system did, however, detect differences in the pathogenicity of six mycoplasma species and these correlated with their pathogenicity for cattle (Anderson, Howard and Gourlay, 1976). These authors could detect no differences in the histological response of the mammary gland to the different mycoplasma species but differences were detected between strains of the same species which paralleled their pathogenicity for cattle. This model would therefore seem to have the ability to differentiate between virulent and non-virulent mycoplasmas.

Five of the ovine strains tested were associated with disease and three were isolated from normal ewes. The histological responses and the growth curves of all eight strains in the mouse mammary gland were very similar and no differences could be detected between strains in the response of the gland. The histological changes produced by the ovine ureaplasmas closely resembled those described for bovine and human ureaplasmas and bovine mycoplasmas in the same system (Howard et al., 1975 ; Anderson et al., 1976). The latter authors were unable to determine whether the mycoplasma species had multiplied in the gland or merely persisted: in the present study six strains were recovered at higher titres on day three than were inoculated, indicating that multiplication had occurred.

The mouse mammary gland thus provided an environment in which ureaplasmas could multiply and produce a histological response. However, differences between strains in this response were so slight that its value as a means of comparing strains for virulence could not be assessed. It is, of course, possible that the strains did not vary greatly in virulence.

The final model system tested was the bovine mastitis model. This system has been used previously to assess the pathogenicity of human and bovine ureaplasmas (Howard, Gourlay and Brownlie, 1973). By measuring their ability to produce mastitis in cows it was shown that ureaplasmas isolated from cattle could be separated into virulent and non-virulent strains.



This system does not meet two of the conditions proposed at the beginning of this discussion - those of low cost and ready availability. However, the other systems tested, although generally able to demonstrate the potential pathogenicity of ureaplasmas in vivo and in vitro, were unable to determine any possible difference in virulence between strains. As Howard et al. (1973) have shown that the bovine mastitis model is capable of detecting such differences, it was decided to investigate the effects of ovine ureaplasmas in this system.

Four of the seven ovine ureaplasmas tested produced mastitis, as indicated by persistent high milk cell counts. Three of these strains (A801/1, 7860/1 and 7790/2) had been isolated from cases of vulvovaginitis and the fourth strain (US1) was isolated from the semen of an infertile tup with orchitis. The other three strains which did not produce mastitis had been isolated from the vagina of apparently normal ewes.

Howard et al. (1973) found that strains of ureaplasma not of bovine origin could not cause infection in the mammary gland of cows. This led them to suggest that the strains were either avirulent or, more probably, that some host specific factors were involved which prevented such strains from causing infection in cows. However, the finding in the present work that ovine ureaplasmas were able to infect and multiply in both the bovine and murine mammary glands suggests that host specific factors, which appear to affect organisms inoculated by other routes, do not play a



significant role in the mammary gland. The fact that very low levels only of inhibitory activity were found in the whey samples examined may be another reason why the ovine ureaplasmas were able to persist in the bovine mammary gland for long periods. Gourlay et al. (1972) detected high titre of MI antibody in the whey from two quarters infected with bovine ureaplasmas and associated this with the ability of the cow to control and resolve the infection in these quarters. In the present study whey sampled from the three quarters infected with the strains which produced little (US33 and US50) or no effect (US55) did not have higher antibody levels than the whey from the quarters infected with the mastitis producing strains. Therefore it appears that it is differences in the ability of these strains to initiate infection, rather than differences in host response which determines whether or not mastitis will be produced.

The murine mammary gland appeared more susceptible than the bovine to infection with ovine ureaplasmas as all strains tested produced mastitis in mice, compared with four of seven in the bovine mammary gland. Howard et al. (1975) reported a similar situation with bovine and human ureaplasmas. One possible explanation for this difference in susceptibility is that the cows were milked twice daily, and lactation continued for long periods after inoculation with ureaplasmas, so that the udder was continually being flushed through with milk. In the mouse model, the offspring were removed one hour prior to inoculation and milk production

declined very quickly thereafter; few glands were still found to be lactating six days after infection, at which time viable ureaplasmas could no longer be recovered from the glands. If the toxic product which seems to be produced by ureaplasmas in vitro is also produced in vivo it would accumulate in the non-milked mouse mammary gland and elicit a quicker and more severe response of shorter duration than in the cow where the continued secretion of milk would cause dilution of any toxic substance produced.

It may be that some ovine ureaplasma strains, e.g. the three strains which did not cause mastitis in the bovine udder, produce lower levels of this proposed toxic product and if constant dilution of these low levels is occurring little or no response may be elicited in the mammary gland.

From the in vitro systems tested little information was obtained on possible differences in virulence between strains of ovine ureaplasmas. However, the cytopathic effect observed in the cell culture work suggested a potential pathogenicity. This pathogenicity was also demonstrated in the mouse mammary gland model, in which all strains produced mastitis. However, only the studies using the bovine udder were able to differentiate between the ureaplasma strains. The four strains which produced mastitis in this system had all been associated with disease whereas the three strains which did not derived from normal animals.

While the production of mastitis in cows demonstrates that ovine ureaplasmas may vary in virulence, the ability of

these strains to produce disease in the ovine urogenital tract must be examined.

CHAPTER SIXINVESTIGATIONS ON THE IN VIVO PATHOGENICITY  
OF OVINE UREAPLASMAS.

Ureaplasmas have been associated with infertility, abortion and endometritis in humans (Taylor-Robinson and McCormack, 1980) and with infertility and granular vulvitis in cattle (Doig et al., 1979). In the U.S.A. and Canada there have been reports of their association with infertility and granular vulvitis in sheep (Doig & Ruhnke, 1977; Livingston et al., 1978). A high percentage of normal and fertile sheep in Britain have been shown to harbour ureaplasmas in the urogenital tract (McCaughey et al., 1979; Jones and Rae, 1979). In the present study however, ureaplasmas were isolated from significantly more ewes with vulvovaginitis than from clinically normal ewes. Experimental infection of the bovine udder has demonstrated the presence of virulent and avirulent isolates of ovine ureaplasmas suggesting a potentially pathogenic role for these organisms. However, proof of pathogenicity according to Koch's postulates requires reproduction of the associated condition in the relevant host animal.

MATERIALS AND METHODS

Forty-five unmated Scottish Blackface gimmers from a single flock were used. Vaginal swabs were taken on three occasions at weekly intervals, prior to the start of the experiment, and examined for the presence of mycoplasmas and bacteria. Additional swabs were also taken at the

final pre-experimental sampling to check for the presence of chlamydiae and viruses. Three Scottish Blackface rams were tested for suitability by examination of preputial swabs and semen, collected by electroejaculation, for the presence of mycoplasma, bacteria, viruses and chlamydiae: the quality of the ejaculate was also examined. Only one ram was found to be suitable and retained for the experiment.

#### Experimental Design

The ewes were randomly divided into three groups and kept in separate fields under the same husbandry and dietary conditions. Group 1 comprised a non-infected group of 20 ewes which served as controls, Group 2 were 15 ewes which were infected with ureaplasmas per vaginam and Group 3 were 10 non-infected ewes used to determine if venereal transmission of ureaplasmas occurred. The oestrus cycle of each group of ewes was synchronised by intramuscular injections of prostaglandin (Iliren, Hoechst U.K. Ltd.) to begin at 21 day intervals. Each ewe was given 1 - 1.5 ml of prostaglandin on two occasions 10 days apart. The ram, which was fitted with a mating marker, was introduced to the synchronised ewes in each group two days after the last injection. Group 1 ewes were served first, followed 21 days later by Group 2 ewes, and after a further 21 days by Group 3 animals. Seven days before introduction of the ram the Group 2 ewes were infected with five low-passage strains of ureaplasmas per vaginam (Table 6.1). The inoculum comprised a pool in equal parts of 16 hour broth

TABLE 6.1

ORIGIN OF OVINE UREAPLASMA STRAINS USED AS INOCULUM FOR  
GROUP 2 EWES

STRAIN	SITE OF ISOLATION	CONDITION	PASSAGE LEVEL	TITRE* IN INOCULUM
US1	Semen	Infertility	6	10 <sup>5</sup>
7790/2	Vagina	Vulvovaginitis	7	10 <sup>4</sup>
7860/1	Vagina	Vulvovaginitis	6	10 <sup>4</sup>
A801/1	Vagina	Vulvovaginitis	6	10 <sup>6</sup>
6CT <sup>+</sup>	Tonsil	Normal	9	10 <sup>5</sup>

\* ccu/0.2 ml.

+ belongs to same serotype (IX) as strain 1202  
(Livingston et al., 1978)

A pool containing 2 ml of each strain was used as inoculum,  
this contained 10<sup>5</sup> ccu/0.2ml.

cultures of each strain. A cotton wool swab dipped into this pool was rubbed vigorously over the vulval and vaginal epithelium of each ewe, a separate swab being used for each animal. The ewes in Groups 1 and 3 were treated similarly with sterile culture broth.

All three groups were mated sequentially with the same ram, and the following parameters measured in each group; conception rates, gestational period, lambing weights and lambing percentages.

The ewes in Group 2 were examined daily until day seven for the development of vulvitis. Swabs were also taken daily for mycoplasmal and bacterial examination and to determine the numbers of ureaplasmas present. Thereafter, examinations and swabbings were at weekly intervals until day 37 and then at two week intervals until the experiment was terminated. Examination of control ewes was performed at weekly intervals for four weeks then at two week intervals.

Preputial swabs and semen were collected from the ram immediately before introduction to the Group 2 ewes and examined for the presence of mycoplasmas and bacteria. Preputial swabs were also collected from the ram for the first seven days after he had been introduced to Group 2.

Twenty-one days later the ram was transferred to the Group 3 ewes. Vaginal swabs were taken from these ewes as soon as the ram's marker had indicated mating had occurred, then at weekly and two week intervals as described for Group 2. Visual examination of the vulvar epithelium was also made at these intervals.

Serum samples were taken from all ewes and the ram at two week intervals and tested for ureaplasma antibody by the metabolism inhibition and indirect fluorescent antibody tests.

At parturition the number of lambs produced, their weight and sex were recorded for each group. Nasal, ocular, rectal and vaginal or preputial swabs were taken from the lambs and a vaginal swab from the ewe. Pieces of placenta, when available, were also taken. Swabbing of the lambs and the ewes continued at two week intervals until the lambs were three months of age.

The ram remained with the Group 3 ewes until midway through their gestational period, when he was slaughtered. The urogenital tract was recovered and several sites sampled for the presence of mycoplasmas and ureaplasmas. Urine collected by aspiration from the intact bladder was also sampled.

To investigate the possibility of an ascending infection in the urogenital tract, caesarian section was performed on six ewes, three from each of Groups 2 and 3. These ewes were selected on the basis of high ureaplasma numbers in the vagina at 140 days of gestation. Nasal, ocular and preputial or vaginal swabs were collected from the lambs as they were removed from the uterus, uterine fluid and pieces of placenta were also sampled.

#### RESULTS

Ureaplasmas were not isolated from any vaginal swab taken in the three weeks prior to the start of the



experiment. A.laidlawii was isolated from two ewes at the first sampling only and from another ewe at the final pre-experimental swabbing. Viruses or chlamydiae were not cultured from any ewe.

Ureaplasmas were isolated on all three samplings from preputial swabs taken from two of the three rams screened for this study. However, the semen samples of all three rams were negative for ureaplasmas. The quality of the semen produced by each ram was good, being of a creamy consistency in which the spermatozoa demonstrated good motility and numbers. Thus the third ram (X558) was selected for use.

All ewes of Group 1 were served by the ram within 13 days of his introduction. No ureaplasmas or mycoplasmas were isolated from any of these ewes during gestation, nor was there any evidence of development of vulvitis. Other than a transient swelling of the vulvar epithelium noted in most ewes the vulva appeared normal (Fig 6.1). A semen sample and preputial swab taken from the ram immediately before introduction to the Group 2 ewes did not yield any ureaplasmas, but A.laidlawii was isolated from the preputial swab.

Ureaplasmas at titres in excess of the inoculum titre were recovered from all Group 2 ewes on each of the seven days following infection. The mean ureaplasma titre achieved from swabs taken just prior to the introduction of the ram was  $10^{5.3}$  ccu/ml. On the day the ram was introduced, 11 of the 15 ewes in this group were observed to have vulvitis. The vulvitis was characterised by marked

hyperaemia of the vulvar epithelium (Fig 6.2). Swelling of the vulva was also seen in some ewes in the first seven days of infection but this effect was generally transient, persisting in only two ewes until 105 days after infection. Small white vesicles on the anterior vulvar epithelium of three ewes persisted until 17 dai in two and until parturition in the third. The vulvitis was seen in nine ewes at 63 dai and in seven ewes at 105 and 140 dai (Table 6.2).

To avoid undue stress on the ewes no further sampling or examinations were performed until immediately after parturition. Although swabs were taken at this time the effects of lambing obscured assessment of the presence of vulvitis. However, at 30 days after parturition four of the ewes still had a mild vulvitis which persisted in two until the end of the experiment, 293 days after initial infection.

Ureaplasmas were recoverable from all 15 ewes at 63 dai but from only 12 at 105 and 140 dai (Table 6.2). At parturition only nine animals were positive. The average titre of ureaplasmas recovered from positive ewes throughout gestation fell from  $10^{5.3}$  ccu/ml at seven dai to  $10^{3.7}$  ccu/ml at 140 dai. At parturition the titre increased to  $10^{5.3}$  ccu/ml. After parturition the isolation of ureaplasmas was more intermittent. Thirty days after lambing ureaplasmas were recovered from only four ewes, all of which had vulvitis; 60 days later ureaplasmas were recovered from eight animals, only two of which had a mild vulvitis. At the final swabbing 120 days after lambing

Fig 6.1

Appearance of the vulvar epithelium in non-infected Group 1 ewes.

Fig 6.2

Appearance of the vulvar epithelium in artificially infected Group 2 ewes, seven days after infection. Epithelium is markedly hyperaemic.



TABLE 6.2

## MICROBIOLOGICAL AND CLINICAL FINDINGS IN GROUP 2 EWES (N=15)

	Days							
	after infection				after parturition			
	7	63	105	140	P*	30	90	120
No. +ve for ureaplasma	15	15	12	12	9	4	8	5
No. with vulvitis	11	9	7	7	ND	4	2	2
Mean isolation titre of ureaplasma <sup>+</sup>	5.3	4.6	2.9	3.7	5.3	5.2	4.0	4.2

\* samples taken within 8 hours of parturition (P).

ND examination of vulva not possible due to effects of lambing.

+ expressed as  $\log_{10}$  ccu/ml. Positive animals only.

five ewes were still positive for ureaplasmas. Titres in excess of  $10^6$  ccu/ml were found in the two ewes which still had vulvitis at this time, compared with  $10^3$  to  $10^4$  ccu/ml in the other three ewes.

The ram mated with one ewe on the day of his introduction to Group 2. A preputial swab taken after this mating yielded ureaplasmas at a titre of  $10^4$  ccu/ml. Thereafter ureaplasmas were consistently isolated from the prepuce of the ram.

The ram mated with all Group 3 ewes within 13 days and vaginal swabs were taken from each ewe as they were seen to have been marked by the ram. On day 13 ureaplasmas were recovered from all but one ewe (463) at titres in excess of  $10^6$  ccu/ml. The initial isolation titre of ureaplasmas from ewe 463 was  $10^4$  ccu/ml, this increased to  $10^7$  ccu/ml at 42 days but this animal was negative at 56 days and remained so throughout gestation. Ewe 460 which had an initial titre of  $10^7$  ccu/ml yielded declining titres throughout gestation reaching  $10^3$  ccu/ml at 69 days after mating and was negative at 83 days and remained so. The other eight ewes all yielded ureaplasmas up to the 140th day of gestation, but two of these were negative at parturition. However, when samples were collected 15 days after parturition only four ewes were found to be positive. These yielded ureaplasmas until 30 days after parturition but only two were positive at the last sampling at 240 days after mating (Table 6.3).

Vulvitis similar to that seen in the Group 2 ewes was observed in four of the Group 3 ewes 13 days after mating.

TABLE 6.3

## MICROBIOLOGICAL AND CLINICAL FINDINGS IN GROUP 3 EWES (N=10)

	Days							
	after infection				after parturition			
	13	63	105	140	P <sup>*</sup>	15	30	90
No. +ve for ureaplasma	10	10	8	8	6	4	4	2
No. with vulvitis	4	4	6	6	ND	4	4	2
Mean isolation titre <sup>+</sup> of ureaplasma	5.8	4.9	4.1	4.0	4.3	4.0	3.7	4.5

Symbols as for Table 6.2



Two more developed the condition 105 days after mating, and all six still had vulvitis and yielded ureaplasmas at 140 days gestation. Four continued to exhibit a mild vulvitis at 15 and 30 days after parturition. At the final sampling only two ewes still had vulvitis; ureaplasmas were recovered from both (Table 6.3).

Of the six ewes selected to investigate the possibility of an ascending ureaplasma infection four had vulvitis (Table 6.4). The ewes were operated on when the gestational age of the foetus was calculated to be 143 days. Vaginal swabs taken at this time yielded ureaplasmas at titres ranging from  $10^4$  to  $10^7$  ccu/ml. Ten lambs, five male and five female were produced. Ureaplasmas were not isolated from any sample taken from the lambs nor from the placentae, uterine fluids or tissues.

Sera collected from the three groups of ewes and the ram throughout gestation and from the ewes and lambs after parturition did not demonstrate any antibodies to ureaplasmas by the metabolism inhibition and indirect fluorescent antibody tests.

Of the 20 ewes in Group 1, 16 produced viable lambs. The gestational period of these ewes ranged from 139 - 152 days. Two ewes (365C and 526C) aborted in the last four weeks of pregnancy. Placental smears made from ewe 365C demonstrated the presence of a chlamydial infection. Ureaplasmas were not isolated from this ewe nor from the aborted foetus or placenta. A vaginal swab taken from ewe 526C shortly after she aborted yielded ureaplasmas at a titre of  $10^4$  ccu/ml. The placenta, nasal and vaginal swabs,



TABLE 6.4

UREAPLASMA NUMBERS IN THE VAGINA OF EWES WHICH UNDERWENT  
CAESARIAN SECTION.

ewe no.	UREAPLASMA TITRE*	PRESENCE OF VULVITIS
Gp. 2		
556C	$10^5$	-
949C	$10^7$	+
960C	$10^4$	+
Gp. 3		
324C	$10^4$	-
341C	$10^5$	+
475C	$10^4$	+

\* ccu/0.2 ml

lung and stomach contents of the aborted lamb were all negative for ureaplasmas. When sampled two weeks later ureaplasmas were not recovered from the vagina of the ewe. The other two ewes were subsequently shown to be barren.

The lambing data for these ewes are given in Table 6.5. Twenty-one lambs, comprising five sets of twins and 11 single lambs were born from these 16 ewes, giving a lambing percentage of 130. Thirteen female and eight male lambs were born in the group. The average weight of all the lambs in this group was 3.49 kg (Table 6.6), the average weight of twin lambs being 2.88 kg and of single lambs 4.05 kg.

Placentae obtained from 11 of these ewes and swabs taken from the lambs within eight hours of birth and again at one, two, and three months of age were all negative for mycoplasmas and ureaplasmas.

In Group 2, 14 of the 15 ewes produced viable lambs, (Table 6.5), the other ewe was barren. These ewes lambed down over a 13 day period and produced 20 lambs to give a lambing percentage of 143. Six sets of twins and eight single lambs were produced and comprised 12 female and eight male lambs. The average weight of all the lambs in this group was 4.14 kg the average weight of the twins was 3.62 kg and of the single lambs 5.04 kg. (Table 6.6)

Ureaplasmas were not isolated from any lamb, the experiment being terminated when the lambs were weaned. Two lambs in this group died within a week of birth from the incorrect placement of rubber rings used for

TABLE 6.5

LAMBING PERFORMANCE OF EWES IN GROUPS 1, 2 and 3.

	NO. OF EWES TO TERM	NO. OF LAMBS BORN	NO. OF LAMBS SURVIVING	LAMBING %AGE
Group 1	16	21	20	130
Group 2	14	20	18	143
Group 3	10	12	10	120

TABLE 6.6

BIRTHWEIGHTS OF LAMBS BORN TO EWES IN GROUPS 1, 2 AND 3.

	NO. OF TWIN LAMBS	NO. OF SINGLE LAMBS	MEAN WEIGHT* OF:		
			ALL LAMBS	TWINS	SINGLES
Group 1	5	11	3.49	2.88	4.05
Group 2	6	8	4.14	3.62	5.04
Group 3	3	6	4.45	4.17	4.73

\* Kilograms

castration, with consequent urethral obstruction and rupture of the bladder. Ureaplasmas were not isolated from any of the tissues sampled from these two lambs at necropsy.

All ten ewes in Group 3 went to term and produced 12 lambs (lambing percentage 120) within a period of 13 days (Table 6.5). Three sets of twins and six single lambs, comprising seven female and five male lambs were produced. The average weight of all the lambs in this group was 4.45 kg the average weight of twins was 4.17 kg and of single lambs 4.73 kg (Table 6.6).

Ureaplasmas were not isolated from eight placentae examined shortly after parturition nor from any of the swabs taken from the lambs at various times up to three months of age. However, M.conjunctivae was isolated from nasal or ocular swabs from nine lambs, M.ovipneumoniae from the nasal swab from one lamb and A.laidlawii from four preputial swabs taken from Group 2 and 3 lambs when they were four weeks old. At eight weeks of age M.conjunctivae was isolated from 13 lambs, M.ovipneumoniae from three lambs and A.laidlawii from the prepuce of three and vagina of four lambs.

#### DISCUSSION

The association of ureaplasmas with ovine granular vulvitis was first reported by Doig and Ruhnke (1977). They reproduced the disease in two lambs and one ewe by inoculation into the vulva of an isolate obtained from the affected flock, and suggested that the vulvitis may have

been responsible for the poor conception rate noted in the flock. In this study, the inoculation of five ureaplasma isolates obtained from the ovine urogenital tract (Chapter Four) into the vulva of 15 virgin ewes produced vulvitis in 11. Venereal transmission of the organism to a further ten ewes was demonstrated and resulted in the development of vulvitis in six animals.

The ewes in all three experimental groups conceived on their first oestrus cycle within a period of 13 days. Two ewes in the control group and one from each of the two infected groups were subsequently shown to be barren, an acceptable proportion for the age and breed of sheep used. The fact that the majority of these sheep mated and conceived whilst they had vulvitis does not support the suggestion of Doig and Ruhnke (1977) that a ureaplasma-associated vulvitis can affect conception rates. However, the disease these workers described was a 'granular vulvitis', whereas no granularity of the vulvar epithelium was evident in the vulvitis reproduced in this study.

Ball and McCaughey (1982) described a vulvitis, similar to the condition reproduced in this study, in ewes experimentally inoculated with a ureaplasma isolate obtained from an outbreak of vulvitis. However, the vulvitis produced was transient and the vulvar epithelium had returned to normal by seven days after inoculation. In contrast, the vulvitis reproduced in the present study persisted in 13 animals, in both infected groups, until 140 dai and was still present, in milder form, in eight ewes 30

days after lambing and in four at 90 days after lambing (243 dai). The ewes in this study were virgin gimmers and had been shown to be negative for ureaplasmas and mycoplasmas on three separate occasions prior to the beginning of the experiment. Ball and McCaughey (1982) used multiparous, non-pregnant ewes for their study and it is possible, as they suggest, that immunity resulting from a previous ureaplasma infection may have been responsible for the transient nature of the vulvitis seen in their experimental studies. The long-term persistence of the vulvitis and the failure to detect any systemic antibodies in any of the ewes at any time throughout their gestational or post-parturient period suggests that the animals used in the present study had never previously experienced infection with ureaplasmas.

An association between vaginal colonisation with U.urealyticum and low birth weights in infants has been noted by Braun et al. (1971) in a prospective study involving 484 infants. They noted that the average birth weight of babies born to women from whom U.urealyticum had been isolated was 202 grams less than that of babies born to mothers who were culturally negative for U.urealyticum. However, the authors suggest that factors other than simple colonisation, such as invasion by the ureaplasmas, may have more effect on determining the birth weight.

Livingston and Gauer (1982) examined the effect of ureaplasma infection in ewes on the outcome of pregnancy. Ewes free from vaginal colonisation with ureaplasmas were allocated to two groups of ten animals. One group was

mated with a ram known to be free from ureaplasma infection, the other was mated with a ram which had been infected intraurethrally into the bladder with ureaplasmas. Each ewe of the second group became infected following mating. The average birthweight of the lambs from the control group was 0.64 kg greater than that from the infected group, and the average weights of twin lambs and single lambs in the control group were higher than those in the ureaplasma infected group.

No such association between vaginal ureaplasma infection and low birth weight was found in this study. In fact the reverse situation was true, the average weights of the lambs born to the experimentally infected and venereally infected groups being respectively 0.65 kg and 0.96 kg heavier than the average weight of lambs born to the control group. The same ram was used for all three groups, so the data obtained from the control group serve as a base-line for the other two groups and indicate that a vaginal ureaplasma infection has no effect on the birth weight of lambs. The results obtained by Livingston and Gauer (1982) may have been due to the use of two different rams.

From the survey detailed earlier (Chapter Four) it was clear that ureaplasmas are rarely isolated from the upper genital tract of sheep. In this study the opportunity to obtain material from the upper genital tract of ewes known to be colonised with large numbers of ureaplasmas in the lower genital tract supported the survey findings. Ureaplasmas were not recovered from the upper



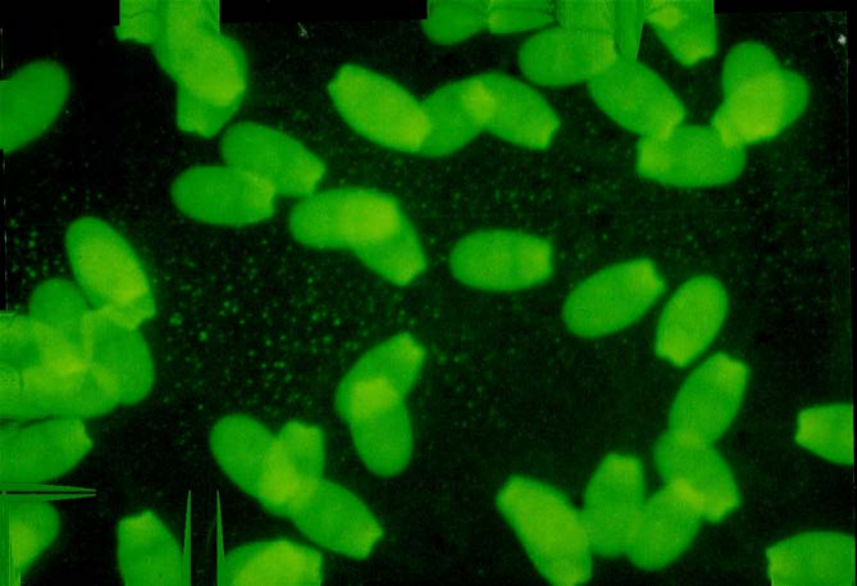
genital tract of any of the six ewes examined. It is reasonable to assume that if ureaplasmas do affect birthweight, as suggested by Braun et al. (1971) and Livingston and Gauer (1982), then their effects will be elicited in the uterus as the foetus is growing. If the ureaplasmas gain entrance to the uterus then it must presumably occur at a time when the cervix is open i.e. at mating. All ewes in Group 2 had high titres of ureaplasma in the vulva at the time of mating (mean  $10^{5.8}$  ccu/ml) when access to the uterus was available. At the time the ram was introduced to the Group 3 ewes ureaplasmas in excess of  $10^4$  ccu/ml were present in the semen and so would be introduced into the cervix at mating. Livingston and Gauer (1982) also suggested that the presence of ureaplasmas may cause reproductive losses at conception or during the early stages of pregnancy. There was however, no evidence from the present study to suggest that ureaplasmas affect the outcome of pregnancy; of the 25 ewes which had vaginal infections with ureaplasmas only one was subsequently shown to be barren, the other 24 producing 42 lambs. This compares favourably with the control group in which two ewes were found to be barren in the absence of ureaplasma infection.

Semen samples collected from the ram after he had mated with the Group 2 ewes were stained using a DNA specific stain, bisbenzimidazole (Hoechst 33258) (Chen, 1977). This demonstrated the presence of ureaplasmas in close proximity to the spermatozoa (Fig 6.3). Gnarpe and Friberg (1973) demonstrated the attachment of U.urealyticum

Fig 6.3

Semen, collected by electroejaculation, from ram X558 after mating with the ureaplasma infected Group 2 ewes. Large numbers of ureaplasmas are present.

bisbenzamide stain                      x300



to sperm obtained from men with reproductive failure, indicating that the organism may enter the uterus with the sperm and interfere with conception. In the study by Livingston and Gauer (1982) two of the ewes which mated with the ram infected with ureaplasmas failed to conceive on their first oestrus cycle: the authors suggested that ureaplasma introduced into the uterus by the semen may have killed the fertilised ovum before implantation causing the ewes to recycle. The same ram was mated with these two ewes again: one conceived on the next oestrus cycle, and the other on the following (third) cycle. Ureaplasmas were still present in the ram's semen at these times and were therefore just as likely to have interfered with conception on these occasions as they were suggested to do on the first cycle. As separate rams were used for both groups it is possible that the vigour or the quality of the rams' spermatozoa may have had more bearing on the conception rate than the presence or absence of ureaplasma. In the present studies the use of the same ram for all three groups avoided this criticism.

The ram, like the ewes examined, showed no evidence of ascending infection of the urogenital tract with ureaplasmas. Ureaplasmas were recovered only from the terminal part of the urethra, from a preputial swab and from semen collected immediately before the ram was slaughtered. The samples taken from other sites were negative. These results would indicate that the semen only becomes infected with ureaplasmas as it passes through the urethra during ejaculation. The prepuce appears to be the

main site of colonisation and it is probable that the distal part of the urethra becomes infected from this site. In their study of men Gnarpe and Friberg (1973) were unable to determine where or when in the urogenital tract the spermatozoa they studied became infected. Taylor-Robinson, Csonka and Prentice (1977) inoculated two male volunteers intraurethrally with two strains of U.urealyticum and both developed urethritis. Fractionation of semen from one subject demonstrated that the highest concentrations of ureaplasma were found in the prostatic portion of the ejaculated semen and this led to the suggestion that the ureaplasmas had infected the prostate. However, the possibility still exists that the ureaplasmas were present only in the urethra and the semen became infected as the ejaculate travelled along the urethra. The differences in numbers of ureaplasmas found between the initial, prostatic portion, and latter portions of the ejaculate may be explained by a dilution effect as different secretions became incorporated in the semen.

Taylor-Robinson, Thomas and Dawson (1969) isolated ureaplasmas from preputial washings and seminal fluids of eight bulls. The urogenital tract of two of these bulls was sampled soon after slaughter; ureaplasmas were not isolated from the testicles, vas deferens or the mucosal lining of the urethra.

These two reports and the failure to isolate ureaplasmas from the upper urogenital tract of the ram in this study would suggest that ureaplasmas colonise only the distal part of the urethra or the preputial cavity and

that semen only becomes infected during ejaculation.

It is apparent from this study that venereal transmission is the most likely route by which ureaplasmas are transmitted. All the ewes in Group 3 became infected with ureaplasmas after mating. In Northern Ireland, Ball, McCaughey and Irwin (1984) studied the importance of natural mating on the transmission of ureaplasmas in a flock of sheep. Four of the ewes and one of the rams used in their study were infected with ureaplasma before the ram was introduced to the flock. By the time the ram was removed seven weeks later a further 21 ewes had become infected, confirming the importance of venereal transmission. Genital disease was not seen in any of the ewes throughout the period of the study.

Although venereal transmission is probably the most likely route by which ureaplasmas are spread, the isolation of ureaplasmas from unmated animals (Taylor-Robinson et al., 1969; Jones and Rae, 1979) suggests that contact with infected animals may be another means of transmission.

The lambs produced by the two infected groups were sampled until they were three months of age. Ureaplasmas were not isolated from any lamb, throughout this period. These findings are in agreement with those of Livingston and Gauer (1982) who failed to isolate ureaplasmas from lambs born to ewes with a vaginal ureaplasma infection. The lambs in that study were sampled for only three weeks after birth. In contrast, Ball et al. (1984) isolated ureaplasmas from the urogenital tract of six lambs, one to two weeks after birth. The ewes which produced these lambs

were all infected with ureaplasmas.

The discrepancy between the results of this study and that of Ball et al. (1984) may be explained perhaps by differences in the husbandry of the ewes. In the present study the ewes were kept on grass and were not housed during their pregnancy except for 24 or 48 hours as they lambed, when they were kept in a wooden stall partitioned by straw bales. The only other time they were together was for swabbing and examination. The report of Ball et al. (1984), although not absolutely clear, suggested that the ewes were housed during the winter, the period when the ewes would be lambing. The lambs would therefore be in closer contact for longer periods with ureaplasma infected ewes and therefore more likely to become infected themselves. No information on housing is given in the report of Livingston and Gauer (1982).

The results of the study conducted by Jones and Rae (1979) also indicates that close contact may be involved in the spread of ureaplasmas. One of the flocks in their report contained 39 Finnish-Landrace ewes, 23 of which yielded ureaplasmas from vaginal swabs taken two months after birth; 29 of 31 ewe lambs born to these ewes were positive for ureaplasmas at three months of age. These ewes and lambs were housed throughout their weaning period (unpublished observation) and it is possible that the close contact the lambs had with the infected ewes increased the probability of the lambs becoming infected and positive for ureaplasmas at three months of age.

The increasing isolation of M.ovipneumoniae and



M.conjunctivae from the nasal and ocular swabs from the lambs agrees with the findings of Jones, Buxton and Harker (1979) who demonstrated that the isolation of these two species increased with the age of the lamb. However, the isolation rates of M.conjunctivae in that study peaked at 25 to 35 days whereas in the present study recovery rates at 56 days were 83% higher than those at 28 days.

M.ovipneumoniae and M.conjunctivae are commonly associated with the respiratory tract of sheep and the airborne route is probably the most likely means by which lambs acquired infection.

The production of vulvitis in 11 of 15 ewes infected with a mixture of five ureaplasma isolates indicates that pathogenic strains of ovine ureaplasma exist in Britain. Venereal transmission of infection was demonstrated, with subsequent development of vulvitis in some ewes. Ureaplasmas were isolated from all ewes with vulvitis but also from many in which vulvitis did not develop. The vulvitis had no effect on conception rates, or on birthweight of the lambs. Although other mycoplasma species were recovered from the lambs, ureaplasmas were not recovered during the three months after birth. Six ewes and the ram with which they mated failed to yield ureaplasmas from the upper genital tract despite the presence of large numbers of organisms in the lower genital tract of these animals.



CHAPTER SEVENSEROLOGICAL STUDIES WITH OVINE UREAPLASMAS

Ureaplasmas of human origin have been shown to be serologically heterogeneous, and a number of distinct serotypes have been found to exist (Black, 1970, 1973; Lin, Kendrick and Kass, 1972). These serotypes are now included in a single species, Ureaplasma urealyticum (Shepard, et al., 1974). Recent studies using polyacrylamide gel and DNA homology techniques have shown these serotypes to comprise of two clusters of similar but not identical strains (Howard, Pocock and Gourlay, 1981; Christiansen, Black and Freundt, 1981).

Bovine ureaplasmas have also been shown to be serologically heterogeneous (Taylor-Robinson et al., 1969; Howard and Gourlay, 1972). Serological studies on bovine ureaplasmas, using antisera prepared in rabbits, demonstrated the presence of three clusters of similar strains; eight strains were proposed as representatives of the serological diversity of the bovine ureaplasmas (Howard, Gourlay and Collins, 1975). In a later report Howard and Gourlay (1981) demonstrated that antisera, prepared in gnotobiotic calves against a representative strain from each of the three clusters recognised in the previous study, were capable of identifying a further 110 bovine isolates.

The serology of ovine ureaplasmas has been less extensively studied. Livingston et al. (1978), in the U.S.A., used a complement-dependent mycoplasmacidal test

with rabbit antisera to examine 19 ureaplasma isolates; they identified nine serological groups. Kotani, Nagatomo and Ogata (1980) in Japan used growth inhibition and metabolism inhibition tests to study 23 caprine and 21 ovine isolates and found these could be divided into two serological groups unrelated to the species of origin.

The studies described in this Chapter were undertaken to resolve, if possible, the discrepancies in findings between American and Japanese workers and to clarify the serology of ovine ureaplasmas.

#### MATERIALS AND METHODS

The growth inhibition (GI), indirect immunofluorescence antibody (IFA) and agar gel double diffusion (AGDD) tests have been described previously (Chapter Two).

The metabolism-inhibition (MI) test of Purcell, Taylor-Robinson, Wong and Chanock (1966) was used but with several modifications. The test was carried out in microtitre plates (Flow Laboratories) using UB as diluent. Sera were diluted 1 in 5 in PBS and inactivated at 56°C for 30 min. before testing. Antigens were overnight broth cultures diluted to contain  $10^2$  and  $10^3$  ccu/0.2 ml. Antigen titrations were included in each test and results were recorded when the dilution used in the test caused a pH change in the growth medium. Initially, varying concentrations of guinea pig serum between 2 and 10% were included in the growth medium, but as the concentration appeared to have little effect on the result, a level of 2%

was finally adopted. Antibody titre was taken to be the reciprocal of the highest dilution of serum which prevented a colour change in the growth medium.

The mycoplasmacidal (MC) test employed was that described by Lin et al. (1972). To 25 $\mu$ l of serial doubling dilutions of antisera in microtitre plates was added 25 $\mu$ l of an overnight broth culture of the ureaplasmas under test diluted to contain  $10^2$  and  $10^3$  ccu/0.2ml. To this was added 50 $\mu$ l of a 1 in 20 dilution of fresh guinea pig complement in PBS. The plates were sealed and incubated for one hour at 37°C. Wells containing ureaplasmas only and ureaplasmas plus complement were included in each test as controls.

After incubation 100 $\mu$ l of growth medium (UB) was added to all wells, the plates were resealed and incubated at 37°C until a colour change was observed in the antigen plus complement control wells. Antibody titre was expressed as the reciprocal of the highest antiserum dilution that prevented a colour change in the growth medium.

The polyacrylamide gel electrophoresis (PAGE) procedure used was a modification of the technique described by Laemmli (1970). Polyacrylamide slab gels, 0.75 mm thick and containing 11% acrylamide were prepared and polymerised by the addition of 0.025% N,N,N,N-tetramethylethylenediamine (TEMED, BDH Pharmaceuticals) and 0.05% ammonium persulphate (BDH Pharmaceuticals). Gels were prepared between glass slides to a length of 12.5 cms.

Antigen preparations were adjusted to contain 40 µg of protein. Ethanolic precipitation of the proteins was carried out by the addition of four volumes of ethanol to one of antigen and leaving the suspension at 4°C overnight. The solutions were then centrifuged at 15,000g for 10 minutes, the ethanol was decanted and the pellets dried under vacuum. Laemmli buffer containing sodium dodecyl sulphate (SDS) and 2 mercaptoethanol was added in 40 µl volumes to the pellets which were then heated in a water bath at 100°C for 90 seconds. Each preparation was loaded onto the gel and electrophoresis was performed at room temperature for 3-4 hours at 13 milliamps and 80 volts. Gels were stained by:(i) - soaking in Coomassie Brilliant Blue (R) in trichloroacetic acid:acetic acid:methanol (0.4:1:7.4:50) for 2 hours at room temperature with continuous gentle agitation and then treating them with a solution containing 7% acetic acid and 23% ethanol to remove background staining or (ii) silver nitrate using the method described by Morrissey (1981).

The preparation of ureaplasma antigens and the production of the hyperimmune sera used in these tests has been described previously (Chapter Two).

Antisera to 10 ureaplasma isolates obtained during the survey (Chapter Four) and to strain 1202, which was kindly supplied by Dr. C.W. Livingston (U.S.A.) were produced in rabbits. The sources of these strains, which were selected on a geographical basis, are given in Table 7.1.

TABLE 7.1

ORIGIN OF OVINE UREAPLASMA STRAINS USED TO PREPARE  
ANTISERUM IN RABBITS.

STRAIN	SITE OF ISOLATION	CONDITION	COUNTRY OF ORIGIN
US1	Semen	Infertility	Scotland
US31	Vagina	Normal	"
US50	Vagina	Vulvovaginitis	"
US55	Vagina	Vulvovaginitis	"
7790/2	Vagina	Vulvovaginitis	England
7860/1	Vagina	Vulvovaginitis	"
A801/6	Vagina	Vulvovaginitis	"
B111	Semen	Normal	Scotland
B165/1	Vagina	Vulvovaginitis	"
B174/2	Vagina	Vulvovaginitis	"
1202	Uterus	Infertility	U.S.A.*

\* kindly supplied by Dr. C.W. Livingston, Texas, U.S.A.

Four strains 7860/1, A801/1, US1 and 6CT were used to prepare antisera in specific pathogen-free lambs. The first three were isolated from the ovine urogenital tract in the course of this study (Chapter Four); the fourth, 6CT, was isolated by Dr. G.E. Jones at the Moredun Research Institute, from tonsillar tissue of a lamb. Each isolate was cloned at least three times before antiserum production. Prior to use in the IFA test each serum was absorbed with 10 mg per ml of lyophilised UB to reduce the effects of non-specific fluorescence caused by antibodies against growth medium components.

## RESULTS

### (i) GI test:

Forty isolates were tested against the 11 antisera prepared in rabbits. Zones of inhibition were small, being only 1-1.5 mm in width against homologous antigens. Zones of this size made satisfactory interpretation of the results difficult. A modification of the GI test, in which wells were cut into the agar and filled with antiserum, failed to increase the zones of growth inhibition. The poor zones of inhibition were presumed to be due to lack of potency of the antisera.

Attempts to improve potency by extending the vaccination schedule to include a further four intravenous inoculations at weekly intervals failed to provide any increase in the size of the zones obtained.

(ii) Mycoplasmacidal test:

Although antibody titres were detectable by the MC test, there were inconsistencies between tests. The test depends on changes of pH in the growth medium as indicated by phenol red. Changes in pH (colour) within plates were frequently sporadic, however, making interpretation of results difficult. It has been suggested that the ammonia produced from urea by ureaplasmas can interfere with the complement fixation pathway (Lin et al., 1972) and this may explain the inconsistent results obtained. Reducing the concentration or entirely omitting urea resulted in very poor ureaplasma growth: this test was therefore discarded in favour of the MI test.

(iii) Metabolism-inhibition test:

The results obtained with 40 isolates and 11 rabbit antisera in the MI test are given in Table 7.2.

The strains formed three groups. The largest (Group A), comprised 33 strains amongst which there were many cross-reactions. Two other groups, B and C, containing five and two strains respectively, were both homogeneous and each was distinct from the other two groups. Homologous titres ranged from 1 in 640 to 1 in 5120. All strains in Group A cross-reacted with at least one other strain within the group but there was an indication of a polarisation of the strains in this group into two sets of similar but not identical strains.

(iv) Indirect immunofluorescence test:

The same 40 isolates were tested against the 11 rabbit antisera, reciprocal antibody titres are given in

TABLE 7.2

TITRES OBTAINED BY METABOLISM-INHIBITION OF ANTISERA, PREPARED IN RABBITS,  
TO 11 OVINE UREAPLASMA STRAINS TESTED AGAINST 40 OVINE ISOLATES.

STRAIN	ANTISERUM AGAINST										
	B111	US1	US31	US50	US55	7790/2	7860/1	B165/1	B174/2	A801/6	1202
B111	<u>5120</u>	5120	1280	320	320	40		80	20		
B41/1	2560	2560	640	320	40	160					
B62/2	2560	2560	160	1280	160	160			80		
B62/3	2560	1280	40	80	40						
US1	5120	<u>5120</u>	320	1280	80	320	20	40	20		
US33	640	1280	320	640		160					
US60	640	1280	80	320	20						
US31	2560	640	<u>2560</u>	80	40	160	80	320	160		
US33	640	2560	320	160	320	320					
US41	2560	320	640	80	320	160		320			
US10	2560	1280	640		320	160		160	160		
US65	2560	2560	320	1280	320	160					
B193	2560	2560	320	1280	320	160			80		
US50	2560	640	320	<u>2560</u>	80	640	40				
US39	1280	320	320	1280	80						
US11	2560	320	640	1280	40	160		80			
D223	640	640	320		80	640					
US55	2560	640	320	1280	<u>640</u>	80		20	20		
D3184	640	2560	1280	1280	80	160					
7790/2		640	320	320	320	<u>1280</u>	320				
7790/1	640	640	160	320	20	640					
B248/1	640	640	160		160	80	1280	2560			
B248/2		640	1280				1280	2560	320		
D1973				320			320	2560	640		
B248/4				640			320	320	320		
7860/1		40	320	640		160	<u>2560</u>	1280	160		
D1998				160	40		1280		160		
D2562				320			1280	1280			
D3092							1280		160		
B165/1		80	20	80			80	<u>5120</u>			
B236/1							320	640	640		
B236/2							160	640	40		
B174/2							320	40	<u>1280</u>		
A801/1										1280	
A801/3										640	
A801/4										640	
A801/5										640	
A801/6										<u>1280</u>	
6CT											5120
1202											<u>5120</u>

Homologous titres are underlined.



Table 7.3. With the exception of antiserum to US55 homologous titres ranged from 1 in 40 to 1 in 160. The strains fell within the same three groups identified by the MI test. Group A comprised 33 strains which demonstrated varying cross-reactivity between themselves: antibody titres to heterologous strains ranged from 1 in 5 to 1 in 160. Two of these strains (US1 and B111) appeared to be identical. Group B contained five very similar strains which exhibited cross-reaction titres of 1 in 80 to 1 in 160 within the group but no cross-reactions with strains from the other two groups. Group C comprised the two strains 6CT and 1202, which appeared identical and distinct from the other two groups.

(v) Agar-gel double diffusion:

Twenty-four strains were tested against the 11 rabbit antisera. These strains were selected from the three groups demonstrated by the MI and IFA test, 20 strains derived from Group A, three from Group B and one from Group C.

Unabsorbed antisera produced many precipitin lines against medium components. Treating the antisera with lyophilised culture medium eliminated these lines, although several treatments were required for some antisera.

All 11 antisera gave similar precipitin lines against the 24 isolates tested and differed only in the intensity of the lines produced. The reactions obtained between antiserum to US1 (Group A) and antigens from two strains of Group A, and one each from Groups B and C are shown in Fig.7.1. Although these strains were derived from

TABLE 7.3

TITRES OBTAINED BY IMMUNOFLUORESCENCE OF ANTISERA, PREPARED IN RABBITS, TO 11 OVINE UREAPLASMA STRAINS TESTED AGAINST 40 OVINE ISOLATES.

STRAIN	ANTISERUM AGAINST										
	B111	US1	US31	US50	US55	7790/2	7860/1	B165/1	B174/2	A801/6	1202
B111	<u>160</u>	160	40	10	10			5			
B41/1	80	80	20	10		20					
B62/2	80	80	5	40	5	20					
B62/3	80	40									
US1	160	<u>160</u>	10	40		40		5			
US33	20	40	10	20	10	20					
US60	20	40		10	5	5					
US31	80	20	<u>80</u>		10	20	20	10	5		
US33	20	80	10	5	10	40					
US41	80	10	20		10	40		5			
US10	80	40	20		5	20		5	10		
US65	80	80	10	40	5	20					
B193	80	80	10	40	10	20					
US50	80	20	10	<u>80</u>	20	80					
US39	40	10	10	40							
US11	80	10	20	40		20		5			
D223	20	20	10			80					
US55	80	10	10	40	<u>20</u>	5			5		
D3184	20	80	40	40		10					
7790/2		20	10	10	10	<u>160</u>	20				
7790/1	20	80	5			<u>80</u>					
B248/1	80	20	10	10	5	10	80				
B248/2		20	40				80	40	10		
D1973				10			20	20	20		
B248/4				20			20	40	10		
7860/1			10	20		20	<u>160</u>	40	5		
D1998				5			80	40	10		
D2562				10			80	5			
D3092							80	40	5		
B165/1							5	<u>80</u>			
B236/1							20	10	20		
B236/2							20	20	5		
B174/2							20		<u>40</u>		
A801/1										160	
A801/3										80	
A801/4										80	
A801/5										80	
A801/6										<u>160</u>	
6CT											160
1202											<u>160</u>

Homologous titres are underlined.

Fig 7.1

Result of agar gel double diffusion test.

US1 antiserum in centre well

- 1 = Swine serum (used in culture medium)
- 2 = Ureaplasma broth
- 3 = US1 )
- 4 = 7860/1 )
- 5 = A801/1 ) antigen
- 6 = 6CT )



different groups according to the MI and IFA tests a specific precipitin line was common to all strains.

The MI and IFA tests, which used rabbit antisera clearly identified three serological groups within the 40 strains of ovine ureaplasmas tested. However, variable intragroup cross-reactions were demonstrated by the members of Group A; confident classification of an isolate as a member of this group would thus require the use of antisera against several strains of the group. A similar situation has been described for bovine ureaplasmas by Howard and Gourlay (1981). They overcame this problem by preparing antisera against three representative strains in gnotobiotic calves; with these antisera they were able to classify all 110 bovine ureaplasma isolates tested.

To determine if a similar situation operated with ovine ureaplasmas antisera to four representative strains were produced in SPF lambs. Two strains from Group A (US1 and 7860/1) were chosen because of their apparent dissimilarity by MI and IFA results: the representative strain for Group B was A801/6, and for Group C, 6CT was selected. Import regulations prohibited the inoculation of the pathogenic American isolate, strain 1202, into any animal other than rabbits. Thus 6CT, the only other member of Group C, was used, although this strain was unrepresentative as it had been isolated from the ovine respiratory tract. The preparation of antisera in lambs has been described in Chapter Two.

The antisera produced in lambs yielded very low titres (< 1 in 20) against homologous antigens in the MI test. However, all antisera gave titres of 1 in 160 to 1 in 320 to their homologous antigens in the IFA test, and further typing of isolates was therefore performed only with this test. In addition to the 40 isolates tested previously a further 58 isolates\* from the United Kingdom and eight from the U.S.A. were tested by the IFA test. A 1 in 40 dilution of each ovine antiserum, was absorbed with growth medium as before, and used in the tests at that dilution.

All 106 isolates reacted with at least one of the four antisera (Table 7.4). Eighty-nine isolates reacted with both US1 and 7860/1 antisera, 12 reacted with A801/6 antiserum and five with 6CT antiserum. Some Group A strains fluoresced slightly with antiserum to A801/6 but when retested with a 1 in 80 dilution of the antiserum this weak reaction was invariably eliminated. In contrast, US1 and 7860/1 antisera showed no decrease in the intensity of the fluorescence with these Group A strains at this higher dilution (Fig. 7.1). No differences between the groups were apparent in the derivation of strains, either according to anatomical site or with any associated disease.

#### Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed on 22 isolates selected on the basis of findings in the MI and IFA tests. Difficulties were again encountered in the preparation of adequate quantities of antigen protein and with several strains

\* see Appendix B for details of ureaplasma isolates.

TABLE 7.4

## IDENTIFICATION OF OVINE UREAPLASMA ISOLATES

STATUS OF ANIMAL	NO. OF STRAINS FLUORESCING WITH ANTISERA TO:		
	Gp A (US1:7860/1)	Gp B (A801/6)	Gp C (6CT)
Diseased *	32	7	1
Normal	57	5	4
TOTAL	89	12	5

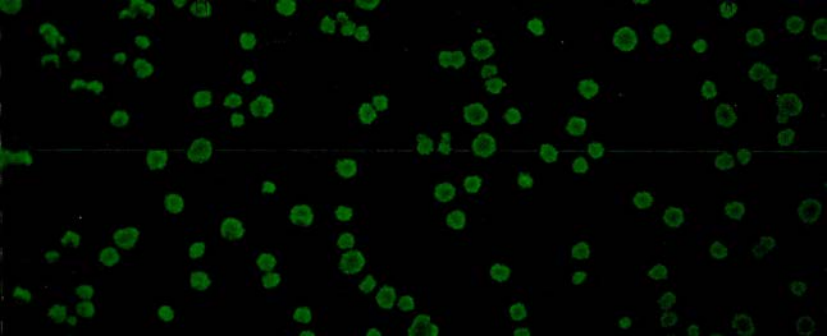
\* Isolates obtained from cases of: vulvovaginitis, orchitis, balanoposthitis, granular posthitis or from the semen of rams associated with infertility.

Fig 7.2

Indirect immunofluorescent staining of strain 7860/1 using lamb antisera prepared against strain US1.

Colonies had been grown at 37°C for two days prior to testing.





there was insufficient protein to allow confident interpretation of the results. The results are shown in Figs 7.3a and b.

Two major protein bands of approximately 78,000 and 55,000 MW were present in most tracks. These corresponded with similar bands produced by uninoculated growth medium and are therefore presumed to be medium contaminants associated with the ureaplasmas cells. Two groupings could be distinguished. The first of these, all Group A strains, comprised, US1 and 7860/1 which possessed bands b, c and d, B1206/3 shared bands b and c with these strains, US31, US50 and US55 shared band b and C with the previous strains and in addition had three bands f, g and h. None of the Group B strains resolved. The Group C strains 6CT, 1202, G700/1 and G700/2 all possessed a polypeptide of approximately 93,000 MW, band a, which did not appear in any of the other strains.

Strain B1206/2 and B1206/6 were the only strains in which a protein yield of more than 1 mg/ml was attained (actual content was 3.6 mg/ml and 5.4 mg/ml). Although no other organisms could be isolated from this sample, the number and intensity of the bands produced suggested the presence of a contaminating organism.

#### DISCUSSION

The two serological tests, the MI and IFA, which were successfully employed in the present study gave very similar results in that each test identified three groups within the 40 isolates examined with rabbit antiserum. The

Fig 7.3a

Polyacrylamide gel electrophoresis of ovine ureaplasmas.  
Gel stained with silver nitrate.

<u>Group A</u>	<u>Track</u>	<u>Peptide bands present</u>
7860/1	2	b, c & d
B62/2	5	-
B111	6	d
B1206/1	8	a'
B1206/2	9	contaminating organism present
B1206/3	10	b & c
B1206/6	11	contaminating organism present
US60	12	-

<u>Group B</u>		
A801/2	7	-

<u>Group C</u>		
6CT	3	a & a'
1202	4	a & a'

Track 13 contains uninoculated ureaplasma broth.

Fig 7.3b

<u>Group A</u>	<u>Track</u>	<u>Peptide bands present</u>
US31	2	b, c, f, g & h
US50	3	b, c, f, g & h
B174/2	4	-
B165/1	8	-
US55	9	b, c, f, g & h
US60	10	-
US1	11	b, c, & d

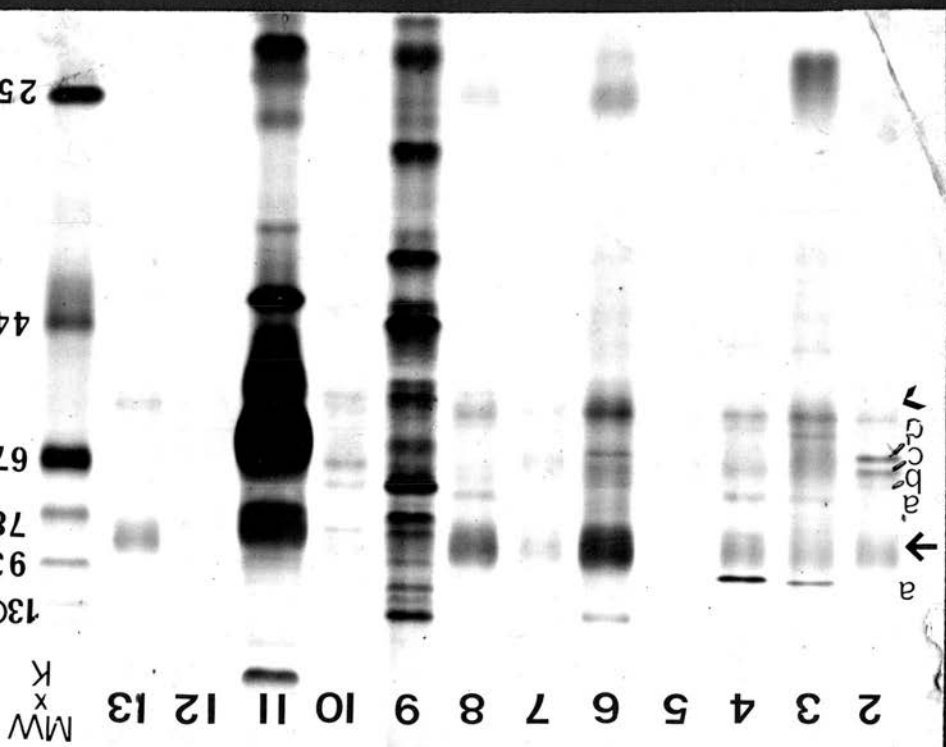
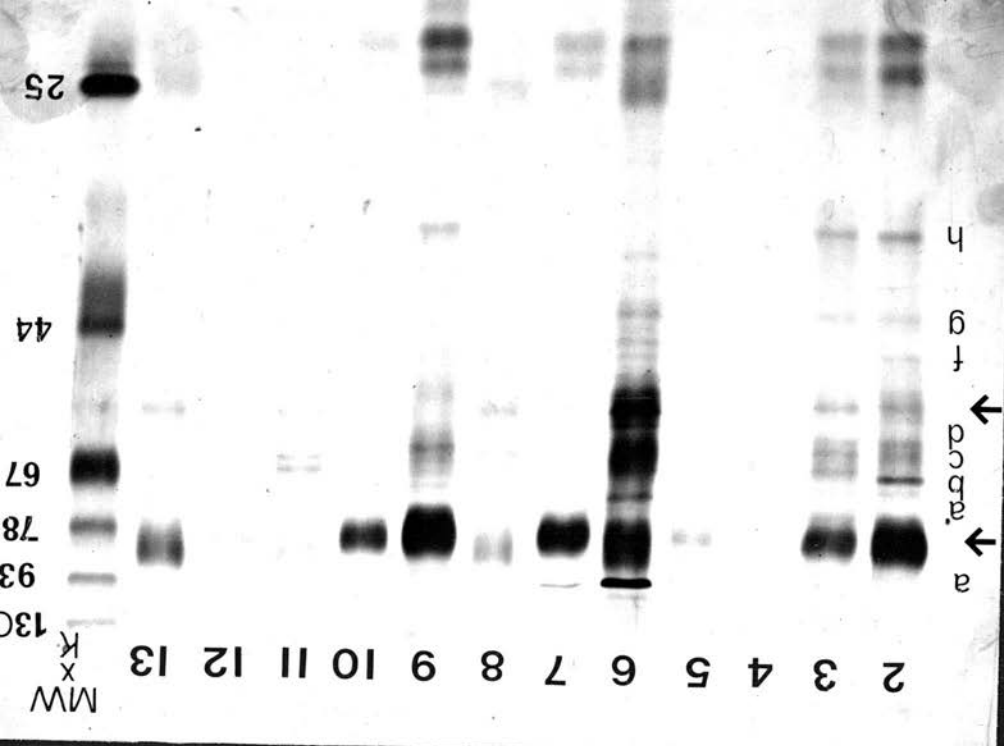
<u>Group B</u>		
A801/6	5	-
A801/3	12	-

<u>Group C</u>		
G700/1	6	a & a'
9700/2	7	a

Track 13 contains uninoculated ureaplasma broth.

Arrows indicate probable medium contaminants.



MI test detected a greater degree of cross-reaction amongst the strains of Group A, which contained 33 strains, than the IFA test did. This may have been due to differences in the sensitivities of the tests or it may reflect differences in the type of antibody detected by each test. The MI test measures inhibition of ureaplasma growth indirectly via the inhibition of metabolic activity. The specific antibodies responsible for inhibition of metabolism appear to be complement dependent, although the role of complement in the MI test remains controversial (Kenny, 1979). The antibodies involved in the IFA test are those directed against surface components only.

The heterogeneity exhibited by the strains of ovine ureaplasma tested, especially those in Group A, would necessitate the use of several antisera to ensure confident serotyping of all isolates. Howard and Gourlay (1981) suggested that rabbit antisera normally used for serotyping bovine ureaplasmas by the IFA test, recognised different epitopes on the ureaplasma cell compared with those recognised by antisera prepared in the natural host.

The results of this study suggest a similar phenomenon with ovine ureaplasmas. Group A was the most heterogeneous of the three groups by both MI and IFA tests, and was therefore represented in further studies by two strains (US1 and 7860/1) which were chosen for their dissimilarity. However, antisera prepared in lambs against these two strains failed to distinguish them and nearly identical results were obtained with both with all other Group A strains. Strain US1 therefore was finally selected

as the representative strain of Group A. The use of lamb antisera, against the three representative strains US1, A801/6 and 6CT, in the IFA test confirmed the existence of three groups within the 106 ureaplasma isolates examined. Eight of the nine serotypes proposed by Livingston et al. (1978) could be placed in Group A.

Kotani et al. (1980) examined 21 ovine strains, including eight of Livingston's serotypes, by the MI and GI tests using rabbit antisera, and distinguished only two groups, one of which contained 20 isolates and the other the single isolate, strain 1202. The proportions of this division are similar to Groups A and C of the present study. Absence of a third group (Group B of this study) could be explained by the relatively low prevalence of Group B strains and the small number of strains examined by the Japanese workers.

The assignment of eight of the serotypes proposed by Livingston et al. (1978) into a single group by MI and IFA tests in this study and by the MI and GI tests in the Japanese study suggests that the MC test used by Livingston et al. (1978) is more sensitive than these other tests and reveals minor antigenic differences which may be misleading in the determination of serological relationships.

It was unfortunate that the attempts to use the MC test in this study failed, due to its poor reproducibility as the test would have allowed direct comparison with the results obtained by Livingston et al. (1978)

The GI test has been successfully used by Howard, Gourlay and Collins (1978) and Ogata, Kotani, and Yamamoto

(1979) in the study of bovine ureaplasmas and results were obtained comparable with those from the MI test. The GI test is very specific, simple to perform and interpret but it is also very insensitive and requires the use of potent antiserum (Clyde 1964). The main problem encountered throughout this serological study was the production of sufficient ureaplasma antigen. The ureaplasma protein harvested from five or ten litres of culture was generally less than one mg per ml. This limiting factor was probably largely responsible for the low potency of the antisera produced and the failure to obtain results with some strains in the PAGE studies.

The AGDD test does not appear to have been applied previously to ovine ureaplasmas. The tests performed in this study demonstrated similarities rather than differences between strains. One major precipitin line was shared by all antigen suspensions and little difference was noted in the number of other lines produced. Nagatomo, Kotani, Ogata and Shimizu (1980) found representative strains of the three bovine serogroups to share a single common antigen by the AGDD test. This common antigen was also shared with human, feline, caprine, canine and fowl ureaplasmas and indicates that ureaplasmas have a common antigenic component at the generic level. Although ovine ureaplasmas have not been compared directly with those of other species by AGDD the finding of Kotani *et al.* (1980) that ovine and caprine ureaplasmas appear to possess a common antigen, as detected by the MI and GI, tests would suggest that ovine ureaplasmas also possess the antigen common to the ureaplasmas of other species.

Both AGDD and GI tests are relatively insensitive. The positive results obtained with the gel test contrasted with the uninterpretable results of the GI test and can perhaps be explained by the fact that both membrane and cytoplasmic antigens would operate in the AGDD test since the antigen suspensions were disrupted by ultrasonication. In the GI test only membrane antigens are available for interaction with the antiserum.

The PAGE studies showed the Group C strains to possess a common major polypeptide of approximately 93,000 MW which was absent in the Group A strains tested. Unfortunately, electrophoretic patterns were not obtained from Group B strains, due to the paucity of antigenic material harvested.

The polyseptides of US1 and 7860/1, which resolved, were identical, supporting the similarity between these strains which had been demonstrated previously by the IFA test using ovine antisera. Other Group A strains had both shared and unshared polypeptides, an observation that supports the heterogeneity demonstrated in this group by serological methods.

Thus PAGE, like AGDD, was more useful in demonstrating similarities than differences within Group A, although it did reveal differences between Groups A and C.

Sayed and Kenny (1980) examined a representative strain from each of the eight proposed serotypes of U. urealyticum and found that although the polypeptide patterns were very similar, each serotype possessed a



unique polypeptide which was present in a major amount. However, as only one strain from each serotype was examined they were unable to determine whether this polypeptide corresponded with the antigenic component responsible for differences in serotype.

More recently, Howard Pocock and Gourlay (1981) examined the polypeptides of human and bovine ureaplasmas. The human strains showed similarities with each other, as did the bovine strains, but the two groups could be distinguished from one another. They also noted that the polypeptide patterns of the bovine strains could, to some extent, be related to serogroup.

The demonstration in the present study of a major polypeptide shared by four of the five Group C strains, but seen in no other strain examined, suggests that this polypeptide may be related to serogrouping according to the MI and IFA tests.

No association between ureaplasma serotype and disease has been shown to exist for U.urealyticum. Although experimental inoculation of serotype five strains resulted in the development of urethritis in two volunteers (Taylor-Robinson et al., 1977) epidemiological surveys have failed to demonstrate the predominance of any one serotype in disease, (Lin et al., 1972; Black, 1974; Lin, Radnay, Kendrick, Rasner and Kass, 1978)

Howard and Gourlay (1981) found no association between any of the three serogroups of bovine ureaplasmas defined by calf antisera and specific disease conditions but suggested that the capacity of rabbit antisera to

recognise many more serotypes might enable identification of any association between disease and serotype. In these present studies the examination of 40 ovine strains using rabbit antisera in the MI test revealed no association between disease and serotype.

At the inception of this work the only ovine ureaplasma to have been ascribed a pathogenic role was strain 1202, which belonged to serotype IX of the groups described by Livingston et al. (1978). In the early stages of this study strain 1202 was clearly distinct, serologically from all other ovine urogenital ureaplasmas examined, but as the work progressed four other strains were found, using lamb antisera, to cross-react with strain 1202. These were accordingly assigned to its serogroup; none of them was associated with disease, one being isolated from the tonsillar tissue of a lamb and the other three from the semen of healthy and fertile rams. It would appear therefore that there is no association between ovine ureaplasma serotype and pathogenicity.

CHAPTER EIGHTGENERAL DISCUSSION

At the start of this study evidence was accumulating that mycoplasmas, especially ureaplasmas, may be involved in pathological conditions of the human and bovine urogenital tracts. At that time little information was available on the mycoplasmas associated with the ovine urogenital tract and this study was initiated to determine the incidence, distribution and potential pathogenicity of such mycoplasmas.

Any investigation involving the isolation and cultivation of mycoplasmas is very dependent on the quality of the growth media utilised. The media used for the isolation of glycolytic and arginine-hydrolysing mycoplasma (OB and AB) were previously described by Jones et al (1976) and had been used successfully for several years for the routine isolation of ovine and bovine mycoplasmas. The media in use for the isolation of ureaplasmas, TB2 and TA2 (Jones, 1978), were capable of supporting the growth of ureaplasmas for a few passages but were not suitable for continued cultivation of the organisms. Similar problems have been encountered with human ureaplasmas and Furness (1973) suggested that newly isolated ureaplasma cells contain a pool of essential metabolites which support normal growth of the organism in artificial media until they become depleted. Thereafter, this pool is either replenished too slowly for growth or another less efficient metabolic pathway becomes active.

The modifications to TB2 and TA2 described in Chapter

Three provided media which were satisfactory for the isolation and cultivation of ovine ureaplasmas. However, the problem of devising a medium for the production of bulk antigens for use in serological studies was not resolved. Very low antigen yields were obtained from five or ten litre volumes of culture. This problem also occurs in the preparation of antigens from human ureaplasma cultures (Masover, Sawyer and Hayflick, 1976). The reason for the poor yields of ureaplasma from fluid media is unknown, but it is well-recognised that once the titre of ureaplasma reaches  $10^6 - 10^8$  ccu/ml growth of the organism ceases (Shepard and Masover, 1979). The possibility that the high pH produced by the ammonia released during hydrolysis of urea was responsible for the cessation of growth was ruled out by the findings of Razin, Masover and Hayflick (1977) who demonstrated that keeping the pH low by incubating the cultures in  $CO_2$  did not result in increased titres. Similarly, in the present work although buffering the media with MES and HEPES maintained a lower pH it failed to result in an increased yield of ureaplasmas.

Furness (1973) suggested that the cessation of growth was due to a toxic factor which accumulated in the media. The identity of the toxic factor was not known but it was shown to be thermostable and slowly dialyzable and produced by all the human ureaplasma strains tested. This factor was capable of preventing the growth not only of the strain producing it but also other strains, and Furness (1973) suggested that the production of a toxic factor was a characteristic of all ureaplasmas.

If a similar toxic factor is produced by ovine ureaplasmas, as seems likely, attempts to remove it by dialysis failed to increase the yield of ureaplasmas (Chapter Three). The dialysate was not toxic to ovine ureaplasmas at 24 or 48 hours incubation. That the toxic substance did not permeate through the dialysis tubing, or did so only in very small amounts, suggests that the substance has a high molecular weight and is not simply  $H^+$  or  $NH_4^+$  ions as has been suggested (Ford and McDonald, 1967; Shepard and Lunceford, 1967).

Recently, U.urealyticum has been reported to produce extracellular proteases which are active against human IgA1 (Robertson, Stemler and Stemke, 1984). A similar but more active enzyme has also been shown to be produced by the type strain of U.diversum (Kilian and Freundt, 1984). Although no proteases have as yet been identified from ovine ureaplasmas it is possible that they may be present within the cell, and as the pool of metabolites within the cell are depleted these proteases are released into the culture media during late log or early stationary phase of growth as cell death occurs. As these enzymes accumulate they may have a lytic effect on the protein components of the ureaplasma cell membrane, leading to death of the cell. The susceptibility of mycoplasma membrane proteins to proteolytic enzymes has been noted and utilised in biochemical studies. Kahane and Marchesi (1973) used pronase to isolate membrane proteins from M.pneumoniae and found that even at low concentrations the enzyme was capable of causing lysis of some cells.

Proteases other than that specific for IgA1 have been shown to exist in ureaplasmas. Watanabe, Mishima and Horikawa (1973) demonstrated that three strains of U.urealyticum were capable of digesting horse serum proteins, and all eight serotype strains of U.urealyticum were found to possess aminopeptidase activity: serotypes VII and VIII were also capable of releasing amino acids from peptides (Vinther and Black, 1974).

Differences have been demonstrated in the activity against IgA of the proteases produced by U.diversum (Kilian and Freundt, 1984), with one strain producing an enzyme capable of causing almost complete degradation of both human and bovine IgA. If other proteases are released by ureaplasmas, and these vary in activity as has been shown for the U.diversum IgA protease, then this could explain both the early cessation of growth and the poor yields of antigenic material in bulk cultures, and also the poor results obtained with some of the ovine strains in the PAGE studies. Amar, Rottem, and Razin (1974), cited by Razin (1979), found that prolonged treatment of mycoplasma membranes with pronase resulted in the production of peptides which were not resolved by polyacrylamide gels and concluded that the enzyme produced peptides which were too small to be retained in the gel. Although proteolytic enzymes have not yet been shown to exist in ovine ureaplasmas, their occurrence in ureaplasmas of other hosts suggests that they do.

In the present PAGE studies none of the Group B strains demonstrated polypeptide bands on gels. If these

strains from Group B produce proteases which are more proteolytic than those from Groups A and C, it may be that their proteins had already been degraded to peptides and were therefore not detectable in the gel.

#### Incidence of Mycoplasmas in the Ovine Urogenital Tract

The results of the survey (Chapter Four) demonstrated that several species of mycoplasma may occur in the ovine urogenital tract. However, with the exception of M.capricolum, M.ovipneumoniae and Ureaplasma spp. there is little evidence to suggest that the other species isolated namely M.arginini, A.axanthum, A.laidlawii and A.oculi are significant pathogens.

##### (i) Mycoplasmas

Although M.ovipneumoniae has been shown to be pathogenic in the ovine respiratory tract (Sullivan, St.George and Horsfall, 1973; Foggie, Jones and Buxton, 1976; Jones, 1978) the infrequency of its isolation from the ovine urogenital tract suggests it is unlikely to have a significant pathogenic role at this site.

The association of M.capricolum with several ovine disease conditions and the experimental reproduction of some of these has been discussed previously (Chapter Four), these reports indicate that M.capricolum can be a particularly virulent mycoplasma. However, M.capricolum has also recently been isolated from the tonsillar tissue and the middle and external ears of clinically normal goats and from the mites found in these ear canals (Cottew and



Yeats, 1981, 1982 ). This raises the possibility that some animals may be carriers of the organism for long periods and that their association with disease may only become apparent following a period of stress, such as pregnancy or parturition.

The effect of stress on the activation of a latent mycoplasma infection has been suggested as one means by which M.mycooides subsp. mycooides (M.mycooides) the causative agent of contagious bovine pleuropneumonia (CBPP) is transmitted. It is known that cattle recovered from CBPP may become carriers by forming sequestra in their lungs which contain viable M.mycooides organisms (Windsor and Masiga, 1977). Studies performed by these authors failed to verify that the stresses they investigated could lead to the breakdown of these sequestra with liberation of the M.mycooides. However, they concluded that other stresses or several stresses acting together, could nonetheless cause reactivation of the infection and play an occasional role in the transmission of CBPP. A similar situation may have existed in the flock from which M.capricolum was isolated with an inapparent infection being activated by the stresses of pregnancy.

The infrequency with which the above mycoplasma species were isolated from the urogenital tract in this study would indicate that this site is not their preferred habitat and none of them appeared to be the causative agent of the disease with which they were encountered.



(ii) Ureaplasmas

The association of ureaplasmas with vulvovaginitis noted in this study was worthy of further examination, not only because of the earlier findings of Doig and Ruhnke (1977) on the association of ureaplasmas with granular vulvitis in sheep but also because of the information which had accumulated during the period of this study on the association of U.diversum with various reproductive disorders in cattle.

Initial reports associating ureaplasmas with diseases of the bovine urogenital tract were made by Anderson (1974) and Ruhnke et al. (1978). Subsequently, the organism was isolated from 100% of acute cases of bovine granular vulvitis (BGV) (Doig et al., 1979). First service conception rates of cows with this form of disease were less than 30%. The disease was reproduced in virgin heifers by infecting the vulvar epithelium with ureaplasmas isolated from natural outbreaks (Doig, Ruhnke and Palmer, 1980a). The organisms could be recovered from the vulva of experimentally-infected animals for up to seven months, but there was no evidence of an ascending infection of the urogenital tract. Inoculation of the organism directly into the uterus resulted in 14 of 16 heifers developing granular vulvitis three to four days later; endometritis developed in six and salpingitis in eight. Ureaplasmas were recoverable from the uterus up to day seven following inoculation but not thereafter (Doig et al., 1980b). Similarly, Ball et al. (1981) in Northern Ireland inoculated a strain of ureaplasma obtained from the bladder

and urethra of a cow into the uterus of five cows; however, apart from the development of an exudate of polymorphonuclear neutrophils within the uterus of one cow, no effects were noted.

The above studies demonstrated that ureaplasmas can cause BGV, and if they gain entry to the uterus they are capable of producing disease. However, the studies of Langford (1975) and Ball and McCaughey (1978) indicated that the uterus in cows is rarely infected with ureaplasmas and, with the exception of the few reported isolations of ureaplasmas from aborted foetuses, there is little evidence that ureaplasmas can naturally gain access to the upper urogenital tract. The findings of Doig et al. (1980b) that ureaplasmas could only be recovered from the uterus up to seven days after infection indicates that the organisms are quickly cleared from this site, which may explain the relative infrequency with which the organism is isolated from the upper urogenital tract.

U.urealyticum is frequently found in the vagina of women and although they are occasionally associated with endometritis, salpingitis and infertility (Cassell and Cole, 1981) and with low birthweight infants (Braun et al. 1971), there are relatively few reports of its isolation from the upper urogenital tract. This infrequent isolation rate is in contrast to the numerous reports of other mycoplasma species in the upper tract (see e.g. review by Taylor-Robinson and McCormack, 1979). This difference in isolation rate between the lower and upper urogenital tract of humans and cattle also appears to be true for sheep.

Ureaplasmas were not isolated from any of the 235 upper urogenital samples examined but 43 of 174 vaginal swabs yielded ureaplasmas, 38 of which were from cases of vulvovaginitis.

The proposal of Doig and Ruhnke (1977) that the ureaplasma associated granular vulvitis seen in their study may have been the cause of the low conception rate observed in the flock was further investigated by Livingston and Gauer (1982). Their findings led them to suggest that a urogenital ureaplasma infection could affect conception rates, although the presence of vulvitis in their ewes was not reported. This contrasts with the results from the in vivo pathogenicity experiment discussed in Chapter Six, where vulvitis was produced following both vulvar inoculation and venereal transmission of ovine ureaplasmas but no effect was noted on the fertility of the ewes.

Vulvitis in sheep has been reproduced by vulvar inoculation of ureaplasmas (Doig and Ruhnke, 1977; Ball and McCaughey, 1982) but has not previously been reported following venereal transmission of the organism, although venereal transmission has been suggested as the most likely route by which the organism is spread (Ball et al., 1984). The effect of a naturally acquired ureaplasma infection on the fertility of ewes in Northern Ireland was studied by McCaughey, Ball and Irwin (1981) and Ball et al. (1984) and they also concluded that the presence of ureaplasmas in the vulva had no effect on the fertility of the ewe.

Differences in lambing percentages or lamb birthweights were not found in the pathogenicity experiment

described in Chapter Six. Livingston and Gauer (1982) noted that the birthweights of lambs born to ewes with a vaginal ureaplasma infection were significantly lower than those of lambs produced by ewes free of infection and attributed this difference to the presence of the ureaplasmas. Simple vaginal colonisation by ureaplasmas is unlikely to have any effect on birthweight. In order to produce such a difference the ureaplasmas must have entered the uterus and elicited an effect on the developing conceptus. As the foetus is ultimately dependent on a properly functioning placenta for growth any malfunction or decreased placental activity would adversely affect the growth of the foetus.

Miller, Ruhnke, Doig, Poitras and Palmer (1983) demonstrated that inoculation of U.diversum into the amniotic cavity of cows, between 4.5 and 8.5 months gestation, resulted in transfer of the organism to the chorioallantois with the development of placentitis. Of four cows inoculated, two subsequently aborted, the other two produced live but weak calves, one of which died within one hour of birth. Ureaplasmas were isolated from the lungs of three of the calves and from all four placentae. The isolation of U.urealyticum from a case of placentitis which resulted in abortion has also been reported by Kundsinn, Driscoll and Ming (1967). The mechanism of abortion in these two reports was not determined but the impairment of placental function as a result of placentitis may have resulted in abortion or the birth of weak calves. The presence of an in utero ureaplasma infection being

responsible for the production of weakly calves is another possible explanation, as the organisms were isolated from the lungs of three of the four calves produced in the study by Miller et al. (1983)

The possibility that a ureaplasma-associated placentitis may have been responsible for the lower birthweight lambs produced by infected ewes in the study by Livingston and Gauer (1982) was not examined.

In the present study, ureaplasmas were isolated from only one of 17 placentae, sampled at parturition or following caesarean section, despite the presence of ureaplasmas in the vulvovaginal canal (Chapter Six). This finding, together with the failure to isolate ureaplasmas from the upper urogenital tract in the survey (Chapter Four) and the very infrequent reports of the isolation of ureaplasmas from this site (Livingston and Gauer, 1978; McCaughey and Ball, 1981) suggests that ureaplasmas either do not gain entry to the ovine uterus or, if they do, they are quickly cleared before uterine infection can be initiated.

Therefore, although the experimental inoculation of ureaplasmas into the bovine and ovine uterus can affect reproductive efficiency (Miller et al., 1983; Livingston and Gauer, 1978) no evidence was obtained during this study to suggest that ureaplasmas naturally gain entrance to the uterus and affect reproduction.

Ball and McCaughey (1984) investigated various methods of eliminating a genital ureaplasma infection in sheep and found that a single intramuscular inoculation of

long-acting tetracycline eliminated infection in 14 out of 19 ewes and eight out of eight rams; a second inoculation eliminated infection from a further three ewes. Therefore, even if ureaplasmas are able to enter the uterus and initiate an infection resulting in infertility, low birthweight or abortion, the infrequency with which they are isolated from the uterus together with the ease with which they can be eliminated from the ovine urogenital tract (Ball and McCaughey, 1984) suggests that they are unlikely to be an important cause of economic loss to the sheep industry.

#### Pathogenicity Factors of Ureaplasmas

The in vitro studies described in Chapter Five demonstrated that all ovine ureaplasma strains tested could attach to and produce a similar CPE in ovine uterus cell cultures. How strong the attachment between the ureaplasmas and cells was could not be determined using the techniques employed. Attachment to host cells is of primary importance in the pathogenicity of many mycoplasma species e.g. M.pneumoniae (Lipman and Clyde, 1969; Lipman, Clyde and Denny, 1969; Collier and Clyde, 1971) and possibly M.gallisepticum (Banai, Kahane, Feldner and Razin, 1981; Kahane, Granek and Reisch-Saada, 1984). In addition many mycoplasma species have been shown to produce toxic products such as hydrogen peroxide (Whittlestone, 1972) and ammonia (Rodwell and Mitchell, 1979) and it may be that production of such a substance is important in pathogenicity especially if there is an intimate



association between the mycoplasma and host cells.

All ureaplasmas produce ammonia in vitro (Shepard and Masover, 1979) and the production of a haemolysin by human, bovine, simian and canine strains of ureaplasmas has also been demonstrated and although this haemolysin is thought to be a peroxide this has not been conclusively proven (Manchee and Taylor-Robinson, 1970; Black, 1973). Although little information is available on the attachment of animal ureaplasmas to cells, limited studies have been carried out with U.urealyticum. Shepard (1957) described the close association of U.urealyticum with urethral epithelial cells taken from cases of non-gonococcal urethritis. Subsequently, agar colonies of six strains of U.urealyticum were shown to adsorb Hela cell suspensions, some more vigorously than others; attachment of the cells to the colonies was tenacious and resisted vigorous washing (Manchee and Taylor-Robinson, 1969). Differences have also been observed in the ability of U.urealyticum to adsorb to red blood cells; Black (1974) tested eight serotype strains with erythrocytes from a variety of species and found only serotype III to be capable of haemadsorption. The mechanism(s) by which ureaplasmas attach to host cells is not known but pretreatment of U.urealyticum cells with trypsin allowed a persistent infection of W1-38 human embryonic lung fibroblasts to be established (Masover, Palant, Zerrudo and Hayflick, 1977). Previous attempts to infect these cells had met with limited success as the infections were only of short duration. The trypsin treatment allowed a more

intimate association between the membranes of the ureaplasmas and cells, suggesting that close membrane-to-membrane contact is required before persistent infection occurs. If the toxic product produced by ureaplasmas in vitro is also produced in vivo, the close association ureaplasmas appear to have with their host cells will lead to a local accumulation of this product which may damage the host cells thus predisposing them to disease. The production of proteases by human and bovine ureaplasmas, recently demonstrated by Kilian and Freundt (1984) may also have a role to play in the pathogenicity of these organisms. All eighteen strains of U.urealyticum examined produced this enzyme, active against human IgA1, whereas none of three strains of U.diversum had this ability. However, the type strain of U.diversum (A417) produced a much more active enzyme which was capable of extensively degrading both human and bovine IgA.

IgA is the predominant immunoglobulin in colostrum and other external secretions of man and other species (Schultze and Heremans, 1966; Tomasi and Bienenstock, 1968). Of the two subclasses of IgA (IgA1 and IgA2), produced by humans, IgA1 comprises as much as 40% of the total IgA in mucous secretions (Weir, 1977). Several bacterial pathogens that attack the human mucous membranes, e.g. Neisseria gonorrhoeae, Haemophilus influenzae and Streptococcus pneumoniae have been shown to produce IgA1 protease, and the available evidence suggests that these enzymes are important in determining virulence of these organisms (Plaut, 1983; Kilian, Thomson, Petersen and



Bleeg, 1983). Watson and Lascelles (1971) demonstrated the presence of IgA in vaginal washings of sheep and cattle, bovine milk, and in oviductal and ovarian follicular fluids and seminal plasma of sheep. These observations with other Ureaplasma spp., namely production of IgA protease(s) and interstrain variation in this capacity, provide a possible explanation for the variations of virulence shown by ovine ureaplasma strains in the bovine mammary gland (Chapter Five).

Livingston and Gauer (1982) stated that one of their ureaplasma isolates, strain 1202, which was associated with reduced conception rates and low birthweights, was "the most invasive, and therefore the most likely ovine ureaplasma isolate to have pathogenic properties". If protease production is a feature of ureaplasmas of all animal hosts, but varies in degree between strains, then the particular "invasiveness" of strain 1202 might be related to this property. Other strains which may produce less active protease would presumably be inhibited by the IgA present in the ovine urogenital tract. Extending this hypothesis the finding in the present work that strains belonging to the same serogroup as 1202 can be isolated from healthy animals would suggest that proteolytic activity is a function of the individual strains within a serogroup rather than of the serogroup. The occurrence of this activity within ovine and bovine ureaplasmas should be further studied to determine the possible role these proteases may have in the pathogenesis of diseases attributed to these organisms.

From the survey (Chapter Four) it was evident that ureaplasmas were associated with vulvovaginitis, but under experimental conditions only a mild vulvitis was reproduced. Although no one species of bacteria was consistently isolated from these natural cases of vulvovaginitis, bacterial involvement in the field disease was a possibility. It seems more likely therefore, that ureaplasmas are only capable of causing mild disease, and that this can predispose to a more severe condition involving other opportunistic bacteria.

The potentiating effect of other mycoplasma species for subsequent bacterial infection has been described. The inoculation of M.pulmonis and Pasteurella pneumotropica into mice showed that the organisms had an additive effect, and it appears that both are involved in pneumonia in mice (Brennan, Fritz and Flynn, 1969). M.gallisepticum (Fabricant, 1969) and M.synoviae (Springer, Luskus and Pourciau, 1974) have been shown to predispose chickens to infection with Escherichia coli and infectious bronchitis virus. Studies on atypical pneumonia in sheep (Jones, Gilmour and Rae, 1978; Gilmour, Jones and Rae, 1979) indicated that M.ovipneumoniae and P.haemolytica are both involved in the disease. The endobronchial inoculation of M.ovipneumoniae alone reproduced pneumonia but the extent of the condition was increased when both organisms were inoculated in combination. Similarly, Gourlay, Howard, Thomas and Stott (1976) produced an exudative bronchopneumonia in calves following endobronchial inoculation of lung homogenates obtained from the pneumonic

lungs of calves: M.dispar and U.diversum were two of the organisms isolated from this homogenate. Inoculation of cloned cultures of these organisms into gnotobiotic calves, however, produced only a subclinical pneumonia involving 2 to 10% of the lung (Howard, Gourlay Thomas and Stott, 1976). This led to the suggestion that the low grade subclinical pneumonia produced by the mycoplasmas predisposes to a more severe pneumonia in which other organisms are involved. It is possible therefore that ureaplasmas have a similar role in the ovine urogenital tract to that suggested for them in the bovine respiratory tract.

The difference in isolation rate of ureaplasmas from normal sheep in this study compared with that from intensively stocked flocks (Jones and Rae, 1979) was perhaps due to differences in the husbandry of the animals, and may be due to transmission of the ureaplasmas by direct contact, which in intensively stocked flocks, is greatly increased, or by bringing animals into more frequent contact with ureaplasma infected urine which is probably the most likely source of infection (Livingston and Gauer, 1975).

#### Serology of Ureaplasmas

The results reported in Chapter Seven confirm the heterogeneity of ovine ureaplasmas demonstrated by earlier studies (Livingston et al., 1978); Kotani et al., 1980). However, the existence of a third, previously unreported serological group was demonstrated. The advantages of

using a combination of serological methods has been shown since the MI and IFA tests gave similar results whereas the GI and MC tests did not provide any information to clarify the serology of ovine ureaplasmas. Use in the IFA test of antisera prepared in SPF lambs against representative strains from each of the three recognised groups enabled all 106 isolates examined to be assigned to one of the three groups, a situation analogous to that described for bovine ureaplasmas by Howard and Gourlay (1981).

The SPF lambs produced only low levels of metabolism-inhibiting antibody against ovine ureaplasmas compared with those produced by rabbits. A similar situation was described by Cole and Ward (1973) who reported that antisera against M.arthritis prepared in mice and rats contained low levels of metabolism-inhibiting and growth-inhibiting antibodies, compared with antisera produced against non-murine mycoplasma species or with antisera against M.arthritis prepared in other animal species. Howard and Pocock (1983) reported that U.diversum elicited a poor metabolism-inhibiting antibody response in calves compared with that generated by U.urealyticum and ovine and caprine ureaplasmas in the same host, and suggested that these low titres may be related to ureaplasma pathogenicity and host specificity. The metabolism-inhibiting antibody titres to ovine ureaplasmas obtained in their study (1 in 2560 - 1 in 5120) compared with the low titres obtained from lambs in this study (1 in 20 or less) supports the suggestion that host specificity is certainly involved.

The PAGE studies did not demonstrate major differences between strains from the three serological groupings, identified by MI and IFA tests, but they did show the presence of a polypeptide which was present only in the Group C strains.

Using antisera prepared in calves to human, bovine, ovine and caprine ureaplasmas Howard and Pocock (1983) demonstrated that the 12 ovine and 2 caprine strains studied constituted two groups that were not related to the animal species from which they had been isolated. These 14 strains could not be classified absolutely with either the human or bovine strains but they appeared to have closer affinities to the bovine strains. In the same report polypeptides from the various ureaplasma strains were compared by growing the organisms in the presence of [<sup>35</sup>S] methionine to allow good resolution of the polypeptides in polyacrylamide gels. The results indicated that most ovine ureaplasma strains possessed similar polypeptides but the polypeptide pattern of two strains, one of which was 1202, were distinct from the others. The polypeptide patterns of all the ovine strains were distinct from the two U.urealyticum and three U.diversum strains also examined.

Earlier studies conducted by Howard, Pocock and Gourlay (1978) had shown that the G + C content of the DNA of two strains of ovine ureaplasmas (30.6 & 31.4 moles%) were similar to that of U.diversum (mean 29.6 moles%) but higher than that of a human ureaplasma strain (27.6 moles%). These results, and the ones reported above

suggested that the ovine strains should not be classified with U.urealyticum.

Although no relationship, either serologically or by polypeptide analysis, was evident between bovine ureaplasmas and the larger group of ovine strains, the two ovine strains in Howard and Pocock's (1983) study, which constituted a distinct group, appeared to possess some polypeptides similar in size to those seen in U.diversum. These similarities, together with the earlier observation that the G + C content of the DNA from ovine strains was similar to that of U.diversum strains, suggested to these authors that the ovine strains could not be regarded as a separate species and that they might represent additional subgroup(s) of U.diversum.

In conclusion, it is apparent that several species of mycoplasma can be isolated from the ovine urogenital tract but with the exception of Ureaplasma sp. none of them are common inhabitants of the tract and likely to be pathogenic at this site. The pathogenic effects of Ureaplasma sp. in the urogenital tract of the ewe appear to be mild and do not affect the reproductive efficiency of the animal. The possibility that the mild vulvitis produced may predispose to a more severe condition involving other micro-organisms should not be excluded. Three groups of ovine ureaplasmas have been demonstrated by serological methods but no association between serotype and disease has been shown to exist. The taxonomic position of ovine ureaplasmas in relation to those of other animal species has not been

fully elucidated and further studies involving immunological and biochemical techniques should be undertaken to resolve this situation.



## REFERENCES

- Aktan, M., Güley, M. and Doguer, M. (1955) [Abortion in sheep following immunization with contaminated sheep pox vaccine.] *Türk Veteriner Hekimleri Derneği Dergisi* 25, 2463-2477, *Veterinary Bulletin* (1956) 26, 258.
- Al-Aubaidi, J.M., Dardiri, A.H. and Fabricant, J. (1972) Biochemical characterisation and antigenic relationship of Mycoplasma mycoides subsp. mycoides Freundt and Mycoplasma mycoides subsp. capri (Edward) Freundt. *International Journal of Systematic Bacteriology* 22, 155-164.
- Al-Aubaidi, J.M., Dardiri, A.H., Muscoplatt, C.C. and McCauley, E.H. (1973) Identification and characterisation of Acholeplasma oculosi spec. nov. from the eyes of goats with keratoconjunctivitis. *Cornell Veterinarian* 63, 117-129.
- Al-Aubaidi, J.M., Ernø, H. and Al-Shammary, J. (1978) Recovery and identification of Acholeplasma oculi from camels. *Zentralblatt für Bakteriologie und Hygiene, I. Abt. Orig. A.* 241, 260-261.
- Alley, M.R., Quinlan, J.R. and Clarke, J.K. (1975) The prevalence of Mycoplasma ovipneumoniae and Mycoplasma arginini in the respiratory tract in sheep. *New Zealand Veterinary Journal* 23, 137-141.
- Amar, A., Rottem, S. and Razin, S. (1974) Characterization of the mycoplasma membrane proteins: IV. Disposition of proteins in the membrane. *Biochimica et Biophysica Acta* 352, 228-244.
- Anderson, N.G. (1974) Mycoplasma genital tract infections in Cattle. *Canadian Veterinary Journal* 15, 95.
- Anderson, J.C., Howard, C.J. and Gourlay, R.N. (1976) Experimental mycoplasma mastitis in mice. *Infection and Immunity* 13, 1205-1208.
- Arbuckle, J.B.R. and Bonson, M.D. (1980) The isolation of Acholeplasma oculi from an outbreak of ovine keratoconjunctivitis. *Veterinary Record* 106, 15.
- Ball, H.J., Neill, S.D., Ellis, W.A., O'Brien, J.J. and Ferguson, H.W. (1978) The isolation of mycoplasma from bovine fetuses and their dams. *British Veterinary Journal* 134, 584-589.
- Ball, H.J. and McCaughey, W.J. (1979) Distribution of mycoplasmas within the urogenital tract of the cow. *Veterinary Record* 104, 482-483.



- Ball, H.J., McCaughey, W.J., Mackie, D.P. and Pearson, G.R. (1981) Experimental genital infection of heifers with ureaplasmas. *Research in Veterinary Science* 30, 312-317.
- Ball, H.J. and McCaughey, W.J. (1982) Experimental production of vulvitis in ewes with a ureaplasma isolate. *Veterinary Record* 110, 581.
- Ball, H.J. and McCaughey, W.J. (1984) Investigations into the elimination of ureaplasmas from the uro-genital tract of ewes. *British Veterinary Journal* 140, 292-299.
- Ball, H.J., McCaughey, W.J. and Irwin, D. (1984) Persistence of ureaplasma genital infection in naturally-infected ewes. *British Veterinary Journal* 140, 347-353.
- Banai, M., Kahane, I., Feldner, J. and Razin, S. (1981) Attachment of killed Mycoplasma gallisepticum cells and membranes to erythrocytes. *Infection and Immunity* 34, 422-427.
- Barile, M.F., Del Giudice, R.A., Carski, T.R., Gibbs, C.J. and Morris, J.A. (1968) Isolation and characterisation of Mycoplasma arginini: spec. nov. Proceedings of the Society for Experimental Biology and Medicine 129, 489-494.
- Barile, M.F., Del Giudice, R.A. and Tully, J.G. (1972) Isolation and characterisation of M. conjuncivae sp.n. from sheep and goats with keratoconjunctivitis. *Infection and Immunity* 5, 70-76.
- Bar-Moshe, B., Goldberg-Weiss, S., Steiff, R. and Israeli, D. (1974) The incidence of mycoplasma in cattle, sheep and goats in Israel. *Refuah Veterinaria* 31, 1-12.
- Black, F.T. (1970) Serological methods for classification of human T-mycoplasmas. Proceedings of the Vth International Congress of Infectious Diseases, Vienna. 407-411.
- Black, F.T. (1973) Modifications of the growth inhibition test and its application to human T-mycoplasmas. *International Journal of Systematic Bacteriology* 23, 65-66.
- Black, F.T. (1973) Biological and physical properties of human T-mycoplasmas. *Annals of the New York Academy of Sciences* 225, 131-143.
- Black, F.T. (1974) PhD Thesis, University of Aarhus, Denmark.

- Boughton, E. and Jones, J.M.W. (1983) Infection of sheep in Great Britain with Mycoplasma capricolum. Veterinary Record 113, 603.
- Braun, P., Lee, Y.H., Klein, J.O., Marcy, S.M., Klein, T.A., Charles, D., Levy, P. and Kass, E.H. (1971) Birth weight and genital mycoplasmas in pregnancy. New England Journal of Medicine 284, 167-171.
- Brennan, P.C., Fritz, T.E. and Flynn, R.J. (1969) Role of Pasteurella pneumotropica and Mycoplasma pulmonis in murine pneumonia. Journal of Bacteriology 97, 337-349.
- Bridré, J. and Donatien, A. (1923) Le microbe de l'agalaxie contagieuse et sa culture in vitro. Compte Rendu des Seances. Academie des Sciences, Paris 177, 841-843.
- Brotherston, J.G. (1968) Report of the Animal Diseases Research Association, Edinburgh. 1967-68, p. 26.
- Buxton, A. and Fraser, G. (1977) Animal Microbiology. Vol. 1. Blackwell Scientific Publications, Oxford.
- Carmichael, L.E., St. George, T.D., Sullivan, N.D. and Horsfall, N. (1972) Isolation, propagation and characterization studies of an ovine mycoplasma responsible for proliferative interstitial pneumonia. Cornell Veterinarian 62, 654-679.
- Cassell, G.H. and Cole, B.C. (1981) Mycoplasmas as agents of human disease. New England Journal of Medicine 304, 80-89.
- Chandler, R.L. (1970) Experimental bacterial mastitis in the mouse. Journal of Medical Microbiology 3, 273-282.
- Chen, T.R. (1977) In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 3358 stain. Experimental Cell Research 104, 255-262.
- Christiansen, C., Black, F.T. and Freundt, E.A. (1981) Hybridization experiments with deoxyribonucleic acid from Ureaplasma urealyticum servovars 1 to Vlll. International Journal of Systematic Bacteriology 31, 259-262.
- Clyde, W.A. (1964) Mycoplasma species identification based upon growth inhibition by specific antisera. Journal of Immunology 92, 958-965.
- Cole, B.C. and Ward, J.R. (1973) Detection and characterisation of defective mycoplasmacidal antibody produced by rodents against Mycoplasma arthritidis. Infection and Immunity 8, 199-207.

- Collier, A.M. and Clyde, W.A. (1971) Relationships between M.pneumoniae and human respiratory epithelium. Infection and Immunity 3, 694-701.
- 
- Cordy, D., Adler, H.E. and Yamamoto, R. (1955) A pathogenic pleuropneumonia-like organism from goats Cornell Veterinarian 45, 50-68.
- Cottew, G.S. (1970) In: The Role of Mycoplasmas and L-Forms of Bacteria in Disease. J.T. Sharp, ed. Thomas, Springfield, Illinois, p. 198-211.
- Cottew, G.S. (1974) The mycoplasmas of sheep and goats. INSERM, 33, 357-362.
- Cottew, G.S. (1979) Caprine-ovine mycoplasmas. In: The Mycoplasmas. Vol II. J.G. Tully and R.F. Whitcomb, eds. Academic Press, New York: p. 103-132.
- Cottew, G.S., Lloyd, I.C., Parsonson, I.M. and Hore, D.E. (1974) Isolation of a mycoplasma from vulvovaginitis in sheep. Australian Veterinary Journal 50, 576-577.
- Cottew, G.S. and Yeats, F.R. (1981) Occurrence of mycoplasmas in clinically normal goats. Australian Veterinary Journal 57, 52-53.
- Cottew, G.S. and Yeats, F.R. (1982) Mycoplasmas and mites in the ears of clinically normal goats. Australian Veterinary Journal 59, 77-81.
- Couch, R.B., Cate, T.R. and Chanock, R.M. (1964) Infection with artificially propagated Eaton agent (Mycoplasma pneumoniae). Implications for development of attenuated vaccine for cold-agglutinin positive pneumonia. Journal of the American Medical Association 187, 442-447.
- Da Massa, A.J., Brooks, D.L., Adler, H.E. and Watt (1983) Caprine mycoplasmosis: acute pulmonary disease in newborn kids given Mycoplasma capricolum orally. Australian Veterinary Journal 60, 125-126.
- Dighero, M.W., Bradstreet, C.M.P. and Andrews, B.E. (1970) Dried paper discs for serological identification of human mycoplasmas. Journal of Applied Bacteriology 33, 750-757.
- Doig, P.A. and Ruhnke, H.L. (1977) Isolation of ureaplasma from sheep with granular vulvitis. Veterinary Record 100, 179-180.
- Doig, P.A. and Ruhnke, H.L. (1978) Infertility in dairy cows - new agent incriminated. Highlights of Ontario Agricultural Research 2, 10-13

- Doig, P.A., Ruhnke, H.L., MacKay, A.L. and Palmer, N.C. (1979). Bovine granular vulvitis associated with ureaplasma infection. Canadian Veterinary Journal 20, 89-94.
- Doig, P.A., Ruhnke, H.L. and Palmer, N.C. (1980a) Experimental bovine genital ureaplasmosis: 1. Granular vulvitis following vulvar inoculation, Canadian Journal of Comparative Medicine 44, 252-258.
- Doig, P.A., Ruhnke, H.L. and Palmer, N.C. (1980b) Experimental bovine genital ureaplasmosis: 2. Granular vulvitis, endometritis and salpingitis following uterine inoculation. Canadian Journal of Comparative Medicine 44, 259-266.
- Edward, D.G. ff. and Freundt, E.A. (1967) Proposal for Mollicutes as name of the class established for the order Mycoplasmatales. International Journal of Systematic Bacteriology 17, 267-268.
- Ernø, H., Freundt, E.A., Krogsgaard-Jensen, A. and Rosendal, S. (1972) The identification of an organism isolated from maned sheep as Mycoplasma Mycoides subsp. mycoides. Acta Veterinaria Scandinavica 13, 263-265.
- Evans, H.B. and Wells, P.W. (1979) A mouse model of Pasteurella haemolytica infection and its use in assessment of the efficacy of P. haemolytica vaccines. Research in Veterinary Science. 27, 213-217.
- Fabricant, J. (1969) Avian mycoplasmas. In the Mycoplasmatales and L-phase of Bacteria. L. Hayflick ed. Appleton-Century-Crofts, New York, 621-641.
- Foggie, A. and Angus K.W. (1972) Observation on the distribution of Mycoplasma arginini as a respiratory tract infection in sheep and its pathogenicity for specific pathogen free lambs. Veterinary Record 90, 312-313.
- Foggie, A., Jones, G.E. and Buxton, D. (1976) The experimental infection of specific pathogen free lambs with Mycoplasma ovipneumoniae. Research in Veterinary Science 21, 28-35.
- Ford, D.K. (1962) Culture of human genital "T" strain pleuropneumonia-like organisms. Journal of Bacteriology 84, 1028-1034.
- Ford, D.K. and MacDonald, J. (1967) Influence of urea on the growth of T-strain mycoplasmas. Journal of Bacteriology 93, 1509-1512.

- Ford, D.K., McCandlish, K.L. and Gronlund, A.F. (1970) Metabolism of  $^{14}\text{C}$ -urea by T-strain mycoplasma. *Journal of Bacteriology* 102, 605-606.
- Freundt, E.A. (1983) In: *Methods in Mycoplasmaology*. Vol 1. S. Razin and J.G. Tully, eds. Academic Press, New York: 9-14.
- Freundt, E.A., Andrews, B.E., Ernø, H., Kunze, M. and Black, F.T. (1973) The sensitivity of Mycoplasmatales to sodium-polyanethol-sulfonate and digitonin. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene: I Abt. Orig. A* 225, 104-112.
- Freundt, E.A. and Edward, D.G. ff (1979) Classification and Taxonomy. In: *The Mycoplasmas*. Vol I. M.F. Barile and S. Razin, eds. Academic Press, New York: 1-42.
- Friedlander, A.M. and Braude, A. (1974) Production of bladder stones by human T-mycoplasmas. *Nature* 247, 67-69.
- Furness, G. (1973) T-mycoplasmas. Factors effecting their growth, colonial morphology and assay on agar. *Journal of Infectious Diseases* 128, 703-709.
- Gilmour, J.S., Jones, G.E. and Rae, A.G. (1979) Experimental studies of chronic pneumonia of sheep. *Comparative Immunology, Microbiology and Infectious Disease* 1, 285-293.
- Gnarpe, H. and Friberg, J. (1973) T-mycoplasmas on spermatozoa and infertility. *Nature (London)* 245, 97-98.
- Gourlay, R.N., Howard, C.J. and Brownlie, J. (1972) The production of mastitis in cows by the intramammary inoculation of T-mycoplasmas. *Journal of Hygiene, Cambridge* 70, 511-521.
- Gourlay, R.N., Brownlie, J. and Howard, C.J. (1973) Isolation of T-mycoplasmas from goats, and the production of subclinical mastitis in goats by the intramammary inoculation of human T-mycoplasmas. *Journal of General Microbiology* 76, 251-254.
- Gourlay, R.N., Howard, C.J., Thomas, L.H. and Stott, E.J. (1976) Experimentally produced calf pneumonia. *Research in Veterinary Science* 20, 167-173.
- Gourlay, R.N., Howard, C.J. and Brownlie, J. (1979) Inoculation of *Acholeplasma laidlawii* strains and *Acholeplasma axanthum* into the bovine mammary gland. *Veterinary Record* 105, 100-102.



- Hart, R., MacKay, J.M.K., McVittie, C.R. and Mellor, D.J. (1971) A technique for the derivation of lambs by hysterectomy. *British Veterinary Journal* 127, 419-424.
- Hendley, J.O. and Alfred, E.N. (1972) Prolonged stationary phase growth of T-strain mycoplasmas in broth culture. *Infection and Immunity* 5, 164-168.
- Hayflick, L. (1965) Tissue cultures and mycoplasmas. *Texas Reproductive Biology and Medicine* 23, 285-303.
- Hoare, M. (1969) A survey of the incidence of mycoplasma infection in the oviducts of dairy cows. *Veterinary Record* 85, 351-355.
- Howard, C.J. and Gourlay, R.N. (1972) Serology of bovine T-mycoplasmas. *British Veterinary Journal* 128, xxvii-xi.
- Howard, C.J., Gourlay, R.N. and Brownlie, J. (1973) The virulence of T-mycoplasmas isolated from various animal species, assayed by intramammary inoculation in cattle. *Journal of Hygiene* 71, 163-170.
- Howard, C.J., Anderson, J.C., Gourlay, R.N. and Taylor-Robinson, D. (1975) Production of mastitis in mice with human and bovine ureaplasmas (T-mycoplasmas). *Journal of Medical Microbiology* 8, 523-529.
- Howard, C.J., Gourlay, R.N. and Collins, J. (1975) Serological comparison between twenty-five bovine ureaplasma (T-mycoplasma) strains by immunofluorescence. *International Journal of Systematic Bacteriology* 25, 155-159.
- Howard, C.J., Gourlay, R.N., Thomas, L.H. and Stott, E.J. (1976) Induction of pneumonia in gnotobiotic calves following inoculation of *Mycoplasma dispar* and ureaplasmas (T-mycoplasmas) *Research in Veterinary Science* 21, 227-231.
- Howard, C.J., Gourlay, R.N. and Collins, J. (1978) Serological studies with bovine ureaplasmas (T-mycoplasmas). *International Journal of Systematic Bacteriology* 28, 473-477.
- Howard, C.J., Pocock, D.H. and Gourlay, R.N. (1978) Base composition of deoxyribonucleic acid from ureaplasmas isolated from various animal species. *International Journal of Systematic Bacteriology* 28, 599-601.
- Howard, C.J. and Gourlay, R.N. (1981) Identification of ureaplasmas from cattle using antisera prepared in gnotobiotic calves. *Journal of General Microbiology* 126, 365-370.

- Howard, C.J., Pocock, D.H. and Gourlay, R.N. (1981) Polyacrylamide gel electrophoretic comparison of the polypeptides from Ureaplasma spp. isolated from cattle and humans. International Journal of Systematic Bacteriology 32, 128-130.
- Howard, C.J. and Pocock, D.H. (1983) Comparison of ureaplasmas from sheep and goats with Ureaplasma diversum and U.urealyticum. Journal of General Microbiology 129, 3197-3202.
- Jain, N.C., Jasper, D.E. and Dellinger, J.D. (1969) Experimental bovine mastitis due to mycoplasma. Cornell Veterinarian 59, 10-28.
- Jones, G.E. (1978) PhD Thesis, University of Edinburgh.
- Jones, G.E., Foggie, A., Mould, D.L. and Livitt, S. (1976) The comparison and characterization of glycolytic mycoplasmas isolated from the respiratory tract of sheep. Journal of Medical Microbiology 9, 39-52.
- Jones, G.E., Gilmour, J.S. and Rae, A.G. (1978) Endobronchial inoculation of sheep with pneumonic lung tissue suspensions and with the bacteria and mycoplasmas isolated from them. Journal of Comparative Pathology 88, 85-96.
- Jones, G.E., Buxton, D. and Harker, D.B. (1979) Respiratory infections in housed sheep with particular reference to mycoplasmas. Veterinary Microbiology 4, 47-60.
- Jones, G.E. and Rae, A.G. (1979) Ureaplasmas in sheep. Veterinary Record 104, 266.
- Jones, G.E., Rae, A.G., Holmes, R.G., Lister, S.A., Jones, J.M.W., Grater, G.S. and Richards, N. (1983) Isolation of exotic mycoplasmas from sheep in England. Veterinary Record 113, 540.
- Jurmanova, K. and Krejci, J. (1971) The isolation of M.arginini from calves with pneumonia in Czechoslovakia. Veterinary Record 89, 585-586.
- Jurmanova, K., Cerna, J., Sisak, F. and Hajkova, M. (1975) Experimental mycoplasma infection of the respiratory tract of calves. Proceeding of the 3rd Conference on Taxonomy and Physiology of Animal Mycoplasmas, Brno. In Vitro V CSSR. 4, 167-173.
- Jurmanova, K. and Sterbova, J. (1977) Correlation between impaired spermatozoan motility and mycoplasma findings in bull semen. Veterinary Record 100, 157-158.

- Kahane, I. and Marchesi, V.T. (1973) Studies in the orientation of proteins in mycoplasma and erythrocyte membranes. *Annals of the New York Academy of Sciences* 225, 38-45.
- Kahane, I., Granek, J. and Reisch-Saada, A. (1984) The adhesins of Mycoplasma gallisepticum and M.pneumoniae. *Annales de Microbiologie (Institute Pasteur)* 135A, 25-32.
- Kaklamanis, E., Thomas, L., Stavropoulos, K., Borman, I. and Boshwitz, C. (1969) Mycoplasmacidal action of normal tissue extracts. *Nature (London)* 221, 860-862.
- Kelly, J.I., Jones, G.E. and Hunter, A.G. (1983) Isolation of Mycoplasma bovoculi and Acholeplasma oculi from outbreaks of keratoconjunctivitis. *Veterinary Record* 112, 482.
- Kenny, G.E. (1979) Antigenic determinants. In: *The Mycoplasmas*. Vol. 1. M.F. Barile and S. Razin, eds. Academic Press, New York: 351-384.
- Kenny, G.E. and Cartwright, F.D. (1977) Effect of urea concentration on growth of Ureaplasma urealyticum (T-strain mycoplasma). *Journal of Bacteriology* 132, 144-150.
- Kilain, M., Thomsen, B., Petersen, T.E. and Bleeg, H.S. (1983) Occurrence and nature of bacterial IgA proteases. *Annals of the New York Academy of Sciences* 409, 612-624.
- Kilain, M. and Freundt, E.A. (1984) Exclusive occurrence of an extracellular protease capable of cleaving the hinge region of human immunoglobulin A1 in strains of Ureaplasma urealyticum. *Israeli Journal of Medical Sciences* 20, 938-941.
- Kotani, H., Nagatomo, H. and Ogata, M. (1980). Isolation and serological comparison of ureaplasmas from goats and sheep. *Japanese Journal of Veterinary Science* 42, 31-40.
- Krauss, H. and Wandera, J.G. (1970) Isolation and properties of mycoplasma from the respiratory tract of sheep with jaagsiekte in Kenya. *Journal of Comparative Pathology* 80, 389-397.
- Krauss, S.J., Jacobs, N.F., Chandler, F.W. and Arum, E.S. (1977) Experimental animal infections with Mycoplasma hominis and Ureaplasma urealyticum. *Infection and Immunity* 16, 302-309.
- Kuksa, F. and Gois, M. (1975) Diagnosis and differentiation of porcine mycoplasmas by the growth-inhibition test. *Zentralblatt für Veterinary Medizin B* 22, 850-855.



- Kumar, A. and Pathak, R.C. (1979) Isolation and characterization of some members of mycoplasma and acholeplasma from the uterus of sheep and goats. *Indian Journal of Experimental Biology* 16, 1011-1013.
- Kundsinn, R.B., Driscoll, S.G. and Ming, P.L. (1967) T-strain of mycoplasma associated with human reproductive failure. *Science* 157, 1573-1574.
- Kundsinn, R.B. and Driscoll, S.G. (1970) Mycoplasmas and human reproductive failure. *Surgery, Gynecology and Obstetrics* 131, 89-92.
- Kundsinn, R.B., Parreno, A. and Poulin, S. (1978) Significance of appropriate techniques and media for isolation and identification of Ureaplasma urealyticum from clinical specimens. *Journal of Clinical Microbiology* 8, 445-453.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680-685.
- Langford, E.V. (1974) Mycoplasma infections in the bovine genital tract. *Canadian Veterinary Journal* 15, 300-301.
- Langford, E.V. (1975) Mycoplasma species recovered from the reproductive tracts of Western Canadian cows. *Canadian Journal of Comparative Medicine* 39, 133-138.
- Langford, E.V., Jurmanova, K. and Stalheim, O.H.V. (1978) Mycoplasma infection of the bovine urogenital system. *Zentralblatt für Bakteriologie und Hygiene, 1. Abt. Orig. A.* 241, 264.
- Leach, R.H. (1970) The occurrence of Mycoplasma arginini in several animal hosts. *Veterinary Record* 87, 319-320.
- Leach, R.H., Cottew, G.S., Andrews, B.E. and Powell, D.G. (1976) Atypical mycoplasmas from sheep in Great Britain and Australia identified as Mycoplasma ovipneumoniae. *Veterinary Record* 98, 377-379.
- Lin, J.S., Kendrick, M.I., and Kass, E.H. (1972) Serological typing of human genital T-mycoplasmas by a complement-dependent mycoplasmacidal test. *Journal of Infectious diseases* 126, 658-663.
- Lin, J.S., Radnay, K., Kendrick, M.I., Rosner, B. and Kass, E.H. (1978) Serological studies of human genital mycoplasmas: Distribution of titres of mycoplasmacidal antibody to Ureaplasma urealyticum and Mycoplasma hominis in pregnant women. *Journal of Infectious Diseases* 137, 266-273.

- Lipman, R.P. and Clyde, W.A. (1969) The interrelationship of virulence, cytoadsorption and peroxide formation in Mycoplasma pneumoniae. Proceedings of the Society of Experimental Biology and Medicine. 131, 1163-1167.
- Lipman, R.P., Clyde, W.A. and Denny, F.W. (1969) Characteristics of virulent, attenuated and avirulent Mycoplasma pneumoniae strains. Journal of Bacteriology 100, 1037-1043.
- Livingston, C.W. (1972) Isolation of T-strain of mycoplasma from Texas feedlot cattle. American Journal of Veterinary Research 33, 1925-1929.
- Livingston, C.W. and Gauer, B.B. (1975) Isolation of T-strain mycoplasma from sheep and goats in Texas. American Journal of Veterinary Research, 36, 313-314.
- Livingston, C.W. and Gauer, B.B. (1978) Isolation of mycoplasma and ureaplasma from sheep and goats in West Texas (1970-1978). Proceedings of the Annual Meeting of the United States Animal Health Association 82, 389-398.
- Livingston, C.W., Gauer, B.B. and Shelton, M. (1978) A specific ureaplasma serotype associated with ovine uterine infection. American Journal of Veterinary Research 39, 1699-1701.
- Livingston, C.W. and Gauer, B.B. (1982) Effect of venereal transmission on the reproductive efficiency of ewes. American Journal of Veterinary Research 43, 1190-1193.
- Manchee, R. and Taylor-Robinson, D. (1969) Enhanced growth of T-strain mycoplasmas with N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid buffer. Journal of Bacteriology 100, 78-85.
- Manchee, R. and Taylor-Robinson, D. (1970) Lysis and protection of erythrocytes by T-mycoplasmas. Journal of Medical Microbiology 3, 539-546.
- Mårdh, P.A. and Weström, L. (1970) T-mycoplasmas in the genito-urinary tract of the female. Acta Pathologica et Microbiologica Scandinavica; Sect. B. 78, 376-374.
- Marmion, B.P. (1967) The mycoplasmas: new information on their properties and their pathogenicity for man. In Recent Advances in Medical Microbiology. 170-253. Ed. A.P. Waterson, Churchill, London.
- Masover, G.K. and Hayflick, L. (1973) The growth of T-strain mycoplasmas in media without added urea. Annals of the New York Academy of Sciences 225, 118-130.

- Masover, G.K., Benson, J.R. and Hayflick, L. (1974) Growth of T-strain mycoplasmas in medium without added urea. Effect of trace amounts of urea and of a urease inhibitor. *Journal of Bacteriology* 117, 765-774.
- Masover, G.K., Namba, M. and Hayflick, L. (1976) Cytotoxic effect of a T-strain mycoplasma (*Ureaplasma urealyticum*) on cultured normal human cells (WI-38). *Experimental Cell Research* 99, 363-374.
- Masover, G.K., Sawyer, E.J. and Hayflick, L. (1976) Urea-hydrolyzing activity of a T-strain mycoplasma: *Ureaplasma urealyticum*. *Journal of Bacteriology* 152, 581-587.
- Masover, G.K., Palant, M., Zerrudo, Z. and Hayflick, L. (1977) Interaction of *Ureaplasma urealyticum* with eucaryotic cells in vitro. In: *Nongonococcal Urethritis and Related Infections*. D. Hobson and K. Holmes, eds. American Society for Microbiology. Washington: 364-369.
- Masover, G.K., Razin, S. and Hayflick, L. (1977) Effects of carbon dioxide, urea, and ammonia on growth of *Ureaplasma urealyticum* (T-strain mycoplasma). *Journal of Bacteriology* 130, 292-296.
- Mazzali, R. and Taylor-Robinson, D. (1971) The behaviour of T-mycoplasmas in tissue culture. *Journal of Medical Microbiology* 4, 125-138.
- McCaughey, W.J., Ball, H.J. and Irwin D. (1979) *Ureaplasma spp.* isolated from ewes in Northern Ireland. *Veterinary Record* 104, 397-398.
- McCaughey, W.J. and Ball, H.J. (1981) Distribution of ureaplasmas in the urogenital tract of ewes. *Veterinary Record* 109, 472.
- McCaughey, W.J., Ball, H.J. and Irwin, D. (1981) Isolation of ureaplasma from ewes following synchronised mating. *Irish Veterinary Journal* 35, 210-213.
- McIlwain, P.K. and Bolin, F.M. (1967) A mycoplasma associated with ovine orchitis. *American Journal of Veterinary Research* 28, 885-887.
- MacLaren, D.M. (1969) The significance of urease in proteus pyelonephritis: A histological and biochemical study. *Journal of Pathology* 97, 43-49.
- Miller, R.B., Ruhnke, H.L., Doig, R.A., Poitras, B.J. and Palmer, N.C. (1983). The effects of *Ureaplasma diversum* inoculated into the amniotic cavity in cows. *Theriogenology* 20, 367-374.

- Moorthy, A.R.S., Kirchoff, H., Heitmann, J., Plumer, G.V. and Spradbrow, P.B. (1977) Isolation of Acholeplasma oculi from diseased horses in Australia. Veterinary Microbiology 2, 253-256.
- Morgan, J.F., Morton, H.J. and Parker, R.C. (1950) Nutrition of animal cells in tissue culture: initial studies on synthetic medium. Proceedings of the Society for Experimental Biology and Medicine 73, 1-8.
- Morrisey, J.H. (1981) Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. Analytical Biochemistry 117, 307-310.
- Morton, H.E. and Roberts, R.J. (1967) Production of anti-mycoplasma (PPLO) antibodies in rabbits. Proceedings of the Society for Experimental Biology and Medicine 125, 538-543.
- Nagatomo, H., Kotani, H., Ogata, M. and Shimizu, T. (1980) Serological studies of bovine ureaplasmas by agar-gel precipitation test. Japanese Journal of Veterinary Science 42, 9-17.
- Nocard, E., and Roux, E.R., (1898) Le Microbe de la péripneumoniae. Annales de Institute Pasteur 12, 240-262.
- Ogata, M., Kotani, H. and Yamamoto, K. (1979) Serological comparison of bovine ureaplasmas. Japanese Journal of Veterinary Science 41, 629-637.
- Olson, N.O., Seymour, W.R., Boothe, A.D. and Dozza, L. (1960) Characteristics of pleuropneumonia-like organism isolated from the genital and respiratory tracts of cattle. Annals of the New York Academy of Sciences 79, 677-685.
- Onoviran, O., Truscott, R.B., Fish, N.A., Barker, A.V. and Ruhnke, H.L. (1975) The recovery of mycoplasmas from the genital tracts of bulls in artificial breeding units in Ontario. Canadian Journal of Comparative Medicine 39, 474-475.
- Ouchterlony, O. (1948) In vitro method for testing the toxic producing capacity of diphtheria bacteria. Acta Pathologica et Microbiologica, Scandinavica 25, 186-191.
- Panangala, V.S., Fish, N.A., and Barnum, D.A. (1978) Microflora of the cervico-vaginal mucus of repeat breeder cows. Canadian Veterinary Journal 19, 83-89.
- Plaut, A.G. (1983) The IgA1 protease of pathogenic bacteria. Annual Review of Microbiology 37, 603-622.

- Perreau, P. and Breard, A. (1979) Caprine mycoplasmosis due to Mycoplasma capricolum. Comparative Immunology, Microbiology and Infectious Diseases 2, 87-97.
- Purcell, R.H., Taylor-Robinson, D., Wong, D. and Chanock, R.M. (1966) Colour test for the measurement of antibody to T-strain mycoplasmas. Journal of Bacteriology 92, 1-12.
- Razin, S. (1979) Membrane Proteins. In: The Mycoplasmas. Vol. I. M.F. Barile and S. Razin, eds. Academic Press, New York: 289-322.
- Razin, S., Masover, G.K. and Hayflick, L. (1977) Physiology of ureaplasmas. In: Nongonococcal Urethritis and Related Infections. D. Hobson and K.K. Homes, eds. American Society for Microbiology, Washington, D.C. 358-363.
- Razin, S., Masover, G.K., Palant, M. and Hayflick, L. (1977) Morphology of Ureaplasma urealyticum (T-mycoplasma) organisms and colonies. Journal of Bacteriology 130, 464-471.
- Robertson, J.A. (1978) Bromothymol blue broth: Improved medium for detection of Ureaplasma urealyticum (T-strain mycoplasma). Journal of Clinical Microbiology 7, 127-132.
- Robertson, J.A., Stemler, M.E. and Stemke, G.W. (1984) Immunoglobulin A protease activity of Ureaplasma urealyticum. Journal of Clinical Microbiology 19, 225-258.
- Rodwell, A.W. and Mitchell, A. (1979) Nutrition, growth and reproduction. In: The Mycoplasmas. Vol. I. M.F. Barile and S. Razin, eds. Academic Press, New York: 103-140.
- Romano, N. and Romano, F. (1968) [Finding and significance of mycoplasmas in inflammatory diseases of the vaginal tract.] Giornale di Malattie infettive C.parassitarie 20, 585-591.
- Romano, N., Ajello, F., Massenti, M.F. and Scarlata, G. (1975) Effect of L-histidine and various buffers on colony size and morphology of a T-strain of mycoplasma. Bollettin Institute Sieroter, Milan 54, 292-295.
- Rosendal, S. and Black, F.T. (1972) Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta Pathologica et Microbiologica Scandinavica 80B, 615-622.



- Ruhnke, H.L., Doig, P.A., MacKay, A.L., Gagnon, A. and Kierstead, M. (1978) Isolation of ureaplasma from bovine granular vulvitis. *Canadian Journal of Comparative Medicine* 42, 151-155.
- Sayed, I.A. and Kenny, G.E. (1980) Comparison of the proteins and polypeptides of the eight serotypes of *Ureaplasma urealyticum* by isoelectric focusing and sodium-dodecyl sulphate polyacrylamide gel electrophoresis. *International Journal of Systematic Bacteriology* 30, 33-41.
- Schultze, H.E. and Heremans, J.F. (1966) *Molecular Biology of Human Proteins with Special Reference to Plasma Proteins*, Vol. I. Elsevier, Amsterdam, p. 838.
- Senterfit, L.B. (1983) Serological identification of mycoplasmas. In: *Methods in Mycoplasmaology Vol. I*. S. Razin and J.G. Tully, eds. Academic Press, New York, 401-404.
- Shepard, M.C. (1954) The recovery of pleuropneumonia-like organisms from negro men with and without nongonococcal urethritis. *American Journal of Syphilis* 38, 113-124.
- Shepard, M.C. (1957) Visualisation and morphology of pleuropneumonia-like organisms in clinical material. *Journal of Bacteriology* 73, 162-171.
- Shepard, M.C. (1958) Growth and development of T-strain pleuropneumonia-like organisms in human epidermoid carcinoma cells (Hela). *Journal of Bacteriology* 75, 351-355.
- Shepard, M.C. (1967) Cultivation and properties of T-strains of mycoplasma associated with nongonococcal urethritis. *Annals of the New York Academy of Sciences* 143, 505-514.
- Shepard, M.C. (1979) Special features of ureaplasmas. In: *The Mycoplasmas*. Vol. I. M.F. Barile and S. Razin, eds. Academic Press, New York: 451-494.
- Shepard, M.C. and Lunceford, C.D. (1967) Occurrence of urease in T-strains of mycoplasma. *Journal of Bacteriology* 93, 1513-1520.
- Shepard, M.C. and Howard, D.R. (1970) Identification of "T" mycoplasmas in primary agar cultures by means of a direct test for urease. *Annals of the New York Academy of Sciences* 174, 809-819.
- Shepard, M.C. and Lunceford, C.D. (1970) Urease color test medium U-9 for the detection and identification of "T" mycoplasmas in clinical material. *Applied Microbiology* 20, 539-543.

- Shepard, M.C. and Lunceford, C.D. (1975) Unusual colonies of Ureaplasma urealyticum (T-mycoplasmas) in primary agar cultures of certain urine specimens. Journal of Clinical Microbiology 2, 456-458.
- Shepard, M.C., Lunceford, C.D., Ford, D.K., Purcell, R.H., Taylor-Robinson, D., Razin, S. and Black, F.T. (1974) Ureaplasma urealyticum gen. nov., sp. nov: Proposed nomenclature for the human T (T-strain) mycoplasma. International Journal of Systematic Bacteriology 24, 160-171.
- Shepard, M.C. and Lunceford, C.D. (1976) Differential agar medium (A7) for identification of Ureaplasma urealyticum (human T-mycoplasmas) in primary cultures of clinical material. Journal of Clinical Microbiology 3, 613-625.
- Shepard, M.C. and Masover, G.K. (1979) In: The Mycoplasmas. Vol I. M.F. Barile and S. Razin eds. Academic Press, New York: 451-494.
- Shurin, P.A., Alpert, S., Rosner, D., Driscoll, S.G., Lee, Y.H., McCormack, W.M., Santamarina, B.A.G. and Kass, E.H. (1975). Chorioamnionitis of the new-born infant with genital mycoplasmas. New England Journal of Medicine 293, 5-8.
- Smith, G.R. (1958) Experimental infections of Pasteurella haemolytica in mice and their use in demonstrating passive immunity. Journal of Comparative Pathology 68, 455-468.
- Smith, C.B., Chanock, R.M., Friedwald, W.T. and Alford, R.H. (1967) Mycoplasma pneumoniae infection in volunteers. Annals of the New York Academy of Sciences 143, 471-483.
- Springer, W.T., Luskus, C. and Pourciau, S.S. (1974) Infectious bronchitis and mixed infections of Mycoplasma synoviae and Escherichia coli in gnotobiotic chickens. I. Synergistic role in the air-sacculitis syndrome. Infection and Immunity 10, 578-579.
- Stalheim, O.H.V., Proctor, S.J. and Gallagher, J.E. (1976). Growth and effects of ureaplasmas (T-mycoplasmas) in bovine oviductal organ cultures. Infection and Immunity 13, 915-925.
- Stemke, G.W., Stemler, M.E. and Robertson, J.A. (1984) Growth characteristics of ureaplasmas from animal and human sources. Israel Journal of Medical Sciences 20, 935-937.
- Stipkovits, L., Romvary, J., Nagy, Z., Boden, L. and Varga, L. (1974). Studies on the pathogenicity of Acholeplasma axanthum in swine. Journal of Hygiene 72, 289-296.

- Subcommittee on the Taxonomy of Mycoplasmas. (1979) International Journal of Systematic Bacteriology 29, 172-180.
- Sullivan, N.D., St. George, T.D. and Horsfall, N. (1973) A proliferative interstitial pneumonia of sheep associated with mycoplasma infection: 2. The experimental exposure of young lambs to infection. Australian Veterinary Journal 49, 63-68.
- Swanepoel, R., Efstratiou, S. and Blackburn, K. (1977) Mycoplasma capricolum associated with arthritis in sheep. Veterinary Record 101, 446-447.
- Talavera Boto, J. (1980) Contagious agalactia of sheep and goats in Spain. Identification of three species of mycoplasma associated with clinical cases of contagious agalactia. Comunicaciones INIS serie: Higiene y Sanidad animal No.4.
- Taylor-Robinson, D. (1979) Pathogenicity of ureaplasmas for animals and man. Zentralblatt für Bakteriologie und Hygiene, 1. Abt. Orig.A.245, 150-163.
- Taylor-Robinson, D. (1983) Serological identification of mycoplasmas. In: Methods in Mycoplasmaology Vol. V. S. Razin and J.G. Tully, eds. Academic Press, New York, 411-417.
- Taylor-Robinson, D., Purcell, R.H., Wong, D.C. and Chanock, R.M. (1966) A color test for the measurement of antibody to certain Mycoplasma species based upon the inhibition of acid production. Journal of Hygiene 64, 91-104.
- Taylor-Robinson, D. and Manchee, R.J. (1967) Spermadsorption and spermagglutination by mycoplasmas. Nature 215, 484-487.
- Taylor-Robinson, D., Haig, D.A. and Williams, M.H. (1967) Bovine T-strain mycoplasmas. Annals of the New York Academy of Sciences 143, 517-518.
- Taylor-Robinson, D., Thomas, M. and Dawson, P.L. (1969) The isolation of T-mycoplasmas from the urogenital tract of bulls. Journal of Medical Microbiology 2, 527-533.
- Taylor-Robinson, D. and Carney, F.E. (1974) Growth and effects of mycoplasmas in Fallopian tube organ cultures. British Journal of Venereal Diseases 50, 212-216.
- Taylor-Robinson, D., Csonka, G.W. and Prentice, M.J. (1977) Human intraurethral inoculation of ureaplasmas. Quarterly Journal of Medicine 46, 309-326.



- Taylor-Robinson, D., Purcell, R.H., London, W.T., and Sly, D.L. (1978) Urethral infection of chimpanzees by Ureaplasma urealyticum. Journal of Medical Microbiology 11, 197-202.
- Taylor-Robinson, D. and McCormack, W.M. (1979) Mycoplasmas in human genitourinary infections. In: The Mycoplasmas. Vol II. J.G. Tully and R.F. Whitcomb, eds. Academic Press, New York: 307-366.
- Taylor-Robinson, D. and McCormack, W.M. (1980). The genital mycoplasmas. New England Journal of Medicine 302, 1063-1067.
- Thangavelu, M., Erno, H. and Therkelsen, A.J. (1979) Detection of mycoplasma contamination in tissue cultures by fluorescence microscopy. Human Genetics 47, 199-202.
- Tiwana, J.S. and Singh, N. (1982) Isolation of Acholeplasma oculi from genital lesions in sheep. Veterinary Record 111, 417.
- Tomasi, T.B. and Bienenstack, J. (1968) Secretory Immunoglobulins. In: Advances in Immunology. Vol. 9. F.J. Dixon and H.G. Kunkel, eds. Academic Press, New York, p. 1-96.
- Tully, J.G. (1979) Special features of the acholeplasmas. In: The Mycoplasmas, Vol. I. M.F. Barile and S. Razin, eds. Academic Press, New York: 431-446.
- Tully, J.G. and Rose, D.L. (1983) Sterility and quality control of mycoplasma culture media. In: Methods in Mycoplasmaology Vol. I. S. Razin and J.G. Tully, eds. Academic Press, New York, 121-125.
- Vinther, O. and Black, F.T. (1974) Aminopeptidase activity of Ureaplasma urealyticum. Acta Pathologica et Microbiologica Scandinavica 82B, 917-918.
- Washburn, L.R. and Somerson, N.L. (1979) Lipoproteins as substitutes for serum in mycoplasma culture medium. Journal of Clinical Microbiology 10, 586-589.
- Watanabe, T., Mishima, K. and Horikawa, T. (1973) Proteolytic activities of human mycoplasmas. Japanese Journal of Microbiology 17, 151-153.
- Watson, D.L. and Lascelles, A.K. (1971) IgA in the body fluids of sheep and cattle. Research in Veterinary Science 12, 503-507.
- Watson, W.A., Cottew, G.S., Erdag, O. and Arisoy F. (1968) The pathogenicity of mycoplasma organisms isolated from sheep and goats in Turkey. Journal of Comparative Pathology 78, 283-291.

- Weir, D.M. (1977) Immunology. An Outline for Students of Medicine and Biology. Churchill Livingstone, Edinburgh, 30-87.
- Whittlestone, P. (1972) Pathogenicity of mycoplasmas in animals. In: Microbial Pathogenicity in Man and Animals. Cambridge University Press, 217-250.
- Windsor, D.G., Edward, D.G. ff. and Trigwell, J.A. (1975) A solid medium for culture and indentification of human T-mycoplasmas. Journal of Medical Microbiology 8, 183-188.
- Windsor, G.D. and Trigwell, J.A. (1976) Prolonged survival of Ureaplasma urealyticum in liquid culture. Journal of Medical Microbiology 9, 101-103.
- Windsor, R.S. and Masiga, W.N. (1977) Investigations into the role of carrier animals in the spread of contagious bovine pleuropneumonia. Research in Veterinary Science 23, 224-229.

APPENDIX A

Source and designation of mycoplasma strains used as antigens for the production of hyperimmune sera in rabbits.

SPECIES	TYPE STRAIN	SOURCE
<u>M.arginini</u>	G230	Brain of scrapie infected mouse.
<u>M.capricolum</u>	California kid	Joint of a goat with arthritis.
<u>M.conjunctivae</u>	HRC581	Conjunctival scrapings from a sheep with keratoconjunctivitis.
<u>M.ovipneumniae</u>	Y98	Lung of a sheep with pneumonia.
<u>A.axanthum</u>	S743	Murine leukaemia (Friend) tissue culture line.
<u>A.laidlawii</u>	PG8	Sewage.
<u>A.oculi</u>	19L	Eye of goat with keratoconjunctivitis.

Strains were kindly supplied by Dr.H.Erno, FAO/WHO Collaborating Centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Denmark.

## APPENDIX B

Source and serological grouping, by immunofluorescence, of 58 ureaplasma strains isolated in the United Kingdom.

DESIGNATION	SOURCE	SEROLOGICAL GROUP		
		A	B	C
4924/1	normal vagina	+		
/2	vulvovaginitis	+		
4777/2	normal vagina	+		
5003/3	" "	+		
/6	" "	+		
5135/2	vulvovaginitis	+		
5156/3	normal vagina	+		
/4	" "	+		
/8	" "	+		
/11	" "	+		
/12	" "	+		
/13	" "	+		
/14	" "	+		
5229/1	vulvovaginitis	+		
/3	" "	+		
5302	" "	+		
5327/1	normal vagina	+		
/2	" "	+		
5735/1	vulvovaginitis	+		
/2	" "	+		
5955/1	" "	+		
/3	" "	+		
/4	" "	+		
/5	" "	+		
5963/2	normal vagina	+		
/3	vulvovaginitis	+		
/5	" "	+		
E2768	normal semen	+		
E2831	" "	+		
E2974	" "	+		
E3321	vulvovaginitis	+		
E3967	semen of infertile tup	+		
E4068	vulvovaginitis	+		
E4069/1	" "	+		
/2	" "	+		
/3	" "	+		
E4097	semen of infertile tup	+		
E4133/1	normal vagina	+		
/2	" "	+		
/3	vulvovaginitis	+		
/4	" "	+		
E4151/1	" "	+		
/2	" "	+		
E4423/3	normal semen	+		
/6	" "	+		
/7	" "	+		
E4514	" "	+		
E4068	vulvovaginitis		+	
E4097/1	normal vagina		+	
/3	" "		+	
E4133/1	vulvovaginitis		+	
/2	" "		+	
/3	normal vagina		+	
/4	" "		+	
G700/1	normal semen			+
/3	" "			+
/4	" "			+
/6	" "			+

# Short Communications

## Isolation of exotic mycoplasmas from sheep in England

G. E. JONES, A. G. RAE, *Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH*, R. G. HOLMES, S. A. LISTER, J. M. W. JONES, *Winchester Veterinary Investigation Centre, Ichen Abbas, Winchester, Hampshire*, G. S. GRATER, N. RICHARDS, *49 Endless Street, Salisbury, Wiltshire*

*Veterinary Record* (1983) **113**, 540

TWO mycoplasmas not hitherto recorded in Britain have been isolated from sheep in the south of England. The source of the isolates was a group of about 120 housed ewes which were part of a flock of 2250 mainly greyface ewes.

At tupping in September 1982, several animals in this group were seen to have blood on their hindquarters, which further investigation revealed to have originated from one particular Suffolk ram. This animal was found to have a grossly swollen penis, was removed, treated with antibiotic and made an uneventful recovery.

No further abnormalities were noted in the ewes until February 1983, some two to four weeks before lambing, when an outbreak of vulvovaginitis occurred. Lesions started just below the tail as blisters or abscesses and spread ventrally, becoming more haemorrhagic and causing the vulvar lips to swell. Bleeding occurred when the shallow scabs were removed.

The anterior vagina showed marked hyperaemia and mild inflammation, but detailed vaginoscopy was not performed because of the proximity of lambing. The ventral tail surface also became affected in some cases, apparently from contact with vulvar lesions.

The lesions persisted for 10 to 14 days; treatment with penicillin and streptomycin over three days appeared to reduce the severity but not the duration of the disease. No systemic effects were noted. By early March nearly half the group had been affected.

The subsequent lambing percentage in the affected ewes was normal at about 170 per cent and no disease was observed in the lambs born to these animals.

Vulvar scabs from three ewes were examined for viruses by electron microscopy, with negative results. Bacteriological culture of these samples yielded *Staphylococcus epidermidis* and *Streptococcus faecalis*; two also contained low numbers of *Staph aureus*. Examination for mycoplasmas, performed initially on six samples of vulvar scabs placed in Eaton's broth, was according to the methodology of Jones and others (1978). Four of the samples yielded a vigorously growing glycolytic mycoplasma at titres of  $10^4$  to more than  $10^6$  colour changing units per 0.2 ml.

Clones from the four positive samples have since been identified by the growth inhibition (Clyde 1964) and indirect epi-immunofluorescent antibody (Rosendal and Black 1972) tests as *Mycoplasma capricolum*. Furthermore, these clones have been shown to be capable of metabolising both glucose and arginine, a feature of only five known mycoplasma species including *M capricolum*.

Further clones from three of the four positive samples have been identified by the growth inhibition and immunofluorescent antibody tests to be *Acholeplasma axanthum*, although antiserum to *A modicum* produced some fluorescence with the clones in the immunofluorescent antibody test. The resistance of these clones to 1.5 per cent digitonin and their positive reactions in the arbutin and aesculin tests support the

serological results (Tully 1979). The identities of these isolates and clones have been confirmed at the Central Veterinary Laboratory, Weybridge. Further cloning and examination of the samples is continuing.

Three further samples including a preputial swab from the affected ram were taken at a later date, but gross bacterial contamination thwarted efforts at mycoplasma isolation.

*M capricolum* has been isolated only from sheep and goats. It has been recorded in Australia, France, India, Spain, Sweden, USA and Zimbabwe, and reports indicate that it is a particularly virulent mycoplasma which may cause polyarthritides (Swanepoel and others 1977), a contagious agalactia-like syndrome (Perreau and Breard 1979, Talavera Boto 1980) and pneumonia and death in kids (Da Massa and others 1983).

*A axanthum* has been isolated in a number of countries including East Germany, Hungary, Japan and the USA, and from several hosts, principally cattle but also pigs, horses and birds (Tully 1979). *A axanthum* has not, as far as is known, been isolated before from sheep. Experimental studies in young pigs suggested the organism to be mildly pathogenic (Stipkovits and others 1974).

Neither *M capricolum* nor *A axanthum* has previously been associated with diseases of the urogenital tract, and they may not have been responsible for the cases of balanoposthitis and vulvovaginitis seen by the authors. However, if the disease in the ewes originated from the tup, its long incubation period of approximately four months is consistent with a mycoplasmal aetiology.

Although the mycoplasmas were isolated from indigenous sheep in this flock, these may not have been the original source of infection. No exotic breeds were present on the farm at the time of sampling, but 18 rams of exotic breeds or crosses of such breeds had been used there between 1979 and 1981. If the mycoplasma isolates originated from these animals, then infection by them remained inapparent for at least one year.

There was no particular evidence of polyarthritides, mastitis or pneumonia in the flock during this time, which suggests that the strain(s) of *M capricolum* involved was limited to the urogenital tract as predilection site and that it was not markedly infectious, or that infection with it did not necessarily result in overt disease.

Cottew and Yeats (1982) have recently found in Australia that healthy goats may harbour several pathogenic species of mycoplasma, including *M capricolum*, in the external ear canal.

Further work is required to establish the pathogenicity of *M capricolum* (and perhaps *A axanthum*) in sheep, to determine which and what proportion of animals exposed to infection may become carriers, and to develop improved means of identifying such animals.

## References

- CLYDE, W. A., Jr (1964) *Journal of Immunology* **92**, 958  
 COTTEW, G. S. & YEATS, F. R. (1982) *Australian Veterinary Journal* **59**, 77  
 DA MASSA, A. J., BROOKS, D. L., ADLER, H. E. & WATT, D. E. (1983) *Australian Veterinary Journal* **60**, 125  
 JONES, G. E., GILMOUR, J. S. & RAE, A. (1978) *Journal of Comparative Pathology* **88**, 85  
 PERREAU, P. & BREARD, A. (1979) *Comparative Immunology, Microbiology and Infectious Diseases* **2**, 87  
 ROSENDAL, S. & BLACK, F. T. (1972) *Acta Pathologica et Microbiologica Scandinavica, Section B* **80**, 615  
 STIPKOVITS, L., ROMVARY, J., NAGY, Z., BODON, L. & VARGA, L. (1974) *Journal of Hygiene* **72**, 289  
 SWANEPOEL, R., EFSTRATIOU, S. & BLACKBURN, N. K. (1977) *Veterinary Record* **101**, 446  
 TALAVERA BOTO, J. (1980) Comunicaciones INIA Serie: Higiene y sanidad animal No 4  
 TULLY, J. G. (1979) *The Mycoplasmas I: Cell Biology*. Eds M. F. Barile and S. Razin. New York, Academic Press, p431

# Letters

## Ovine ureaplasmas and bovine mastitis

From Mr A. G. Rae

SIR, — In a recent report by D. P. Mackie and H. J. Ball (*VR*, March 17, p271) two ovine ureaplasmas were shown experimentally to produce mastitis in the bovine mammary gland, thus demonstrating the potential of this model as an indicator of the virulence of mycoplasmas. This letter summarises results obtained in this institute using the same model system.

Seven strains of ovine ureaplasma were tested: these were derived from vaginal swabs taken from ewes with vulvovaginitis (A801/1, 7790/2 and 7860/1), an aborted ewe (US33), a clinically normal ewe (US50), a barren ewe (US55) and from semen collected from an infertile tup with orchitis (US1).

Also included were a bovine ureaplasma strain (A417C) known to induce mastitis (Gourlay and others 1972) and *Acholeplasma laidlawii* which has been shown to be non-pathogenic in this model (Jain and others 1969).

Two ovine strains, the bovine strain A417C and *A. laidlawii* were each inoculated into separate udder quarters of two cows; the remaining three ovine strains and the A417C strain were inoculated into separate quarters of a third cow.

The cows, which were in early lactation, were milked twice daily; samples from the first milking were used to determine numbers of cells (measured electronically) and of ureaplasmas and acholeplasmas (Jones 1978) until 21 days after inoculation.

All ureaplasmas became established and multiplied in the udder and all except one (US55) caused milk cell counts to rise to greater than  $10^6$  cells/ml.

Four strains (A801/1, 7790/2, 7860/1 and US1) persisted in the milk at titres of  $10^4$  to  $10^6$  colour changing units/ml throughout the period of observation, and cell counts in the quarters infected with these strains also remained high (the mean for all four strains over 21 days was  $10^{6.4}$  cells/ml).

Cell counts with strain US1 were greater than  $10^7$  cells/ml from nine to 15 days after inoculation and this rise was accompanied by the appearance of clots in the milk at days 12 and 13. Two strains (US33 and US50) could be recovered from milk only until day 11; cell counts in the quarters inoculated with them fell to pre-inoculation levels by days 12 to 14.

Bovine strain A417C also caused persistently raised cell counts and was reisolated throughout the 21 days.

There was no response to the *A. laidlawii* infection other than a transient increase in cell counts on the two days following inoculation, and the organism was not reisolated at any time. No other mycoplasmas or bacteria were isolated from the milk before or during the experiment.

All four strains which produced mastitis (A801/1, 7790/2, 7860/1 and US1) were isolated from sheep with vulvovaginitis or orchitis. The three strains which produced little (US33 and US50) or no (US55) effect in the bovine udder were isolated from the

apparently normal vaginas of a ewe which had aborted, a clinically normal ewe and a barren ewe respectively.

These findings support those of Mackie and Ball and indicate that the bovine udder model is capable of differentiating between ureaplasmas associated with disease and others presumably less pathogenic. However, more strains should be tested to confirm these findings.

A. G. RAE  
 Moredun Research Institute  
 408 Gilmerton Road  
 Edinburgh

### References

- GOURLAY, R. N., HOWARD, C. J. & BROWNLIE, J. (1972) *Journal of Hygiene, Cambridge* **70**, 511  
 JAIN, N. C., JASPER, D. E. & DELLINGER, J. D. (1969) *Cornell Veterinarian* **59**, 10  
 JONES, G. E. (1978) PhD thesis, University of Edinburgh