CULTURAL, BIOCHEMICAL AND ANTIGENIC CHARACTERISTICS OF CAPRINE MYCOPLASMA ISOLATED IN KENYA

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A thesis submitted in part fulfilment for the Degree of Master of Science in the University of Nairobi.

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January, 1983.

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my wife Kobilo, children Chepkangor, Kangogo, Kibet, Jerotich and Jelagat.

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ACKNOWLEDGEMENTS

I am grateful to my Supervisor Dr. P.G. Waiyaki for his constant advice, constructive criticism and encouragement throughout the carrying out of the experimental part of this study and writing of the thesis. His patience in receiving and correcting the several drafts of the manuscript is very much appreciated.

I am also grateful to the following persons who made a contribution to the success of this project. Professor E.A. Freundt, The Director FAO/WHO Collaborating Centre for Animal Mycoplasma, Aarhus Denmark for kindly granting his Institute's facilities to be used in carrying out all the experiments; Dr. Henning Ernø also of the same Institute for his advise on practical aspects of the Mycoplasma work carried out and for stimulating discussion on characterization of Mycoplasma.

I am also indebted to Dr. Mohammed Salih who helped out with some of the tests, especially the growth inhibition and growth precipitation test. I will not also forget all the technical staff of the Collaborating Centre for their expert assistance and Dr. S. Waghela, my colleague who aroused my interest in Mycoplasma; all the technical staff of the Mycoplasma Section, Veterinary Research Laboratory, Kabete and particularly Mr. Ndirangu Ndugire who sorted out all the strains.

My thanks also go to Dr. S. Chema, The Deputy Director in the Ministry of Livestock Development in Charge of Research for his encouragement and interest in helping me to finish this work.

This project was financially supported from funds provided by the German Foundation for International Development (Fieldafing) administered through the German Agricultural Team in Kenya.

Last but not least I want to thank Dr. Risto Heinonen my counterpart and co-ordinator of the Contagious Caprine Pleuropneumonia Project (CCPP), for his constant assistance throughout the carrying out of this project.

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SUMMARY

Contagious caprine pleuropneumonia (CCPP) is an acute disease of goats characterized by acute fibrinous pneumonia and pleuritis. Three Mycoplasma species namely, Mycoplasma mycoides subsp. mycoides (Y-goat), M. mycoides subsp. capri and Mycoplasma species strain F38 are the main aetiological agents.

In Kenya Mycoplasma mycoides subsp. mycoides (Y-goat) and Mycoplasma strain F38 have been clearly documented to cause CCPP. In an investigation to establish whether mycoplasma other than the two mentioned above cause CCPP in Kenya, 34 strains were isolated from CCPP cases and characterized culturally, biochemically and serologically. By the growth inhibition and indirect-epi-immunofluorescent tests, 17 of the strains were conclusively identified as Mycoplasma species strain F38, eight as Mycoplasma arginini, five as Mycoplasma ovipneumoniae and four as Mycoplasma mycoides subsp. mycoides (Y-goat).

All the F38 strains metabolized glucose aerobically and anaerobically with acid production. Stronger reactions were however, observed in cultures incubated anaerobically. Mannose and galactose were readily metabolized with acid production by all the strains except four which poorly metabolized

galactose and two which never metabolized mannose at all. Xylose was readily metabolized by 13 strains while cellobiose and sorbitol were either poorly metabolized or not at all by most of the strains. Two strains strongly metabolized these two sugars.

Of the four media tested for their ability to support growth of the F38 group, two media yielded the best growth. These were medium B (Modified Hayflick) and Newings tryptose medium. Visible colonies appeared after an average of three days following inoculation of media. The colonial morphology had the characteristic fried egg appearance of mycoplasma. Medium B without yeast extract and SP4 medium poorly supported the growth of the F38 group. Growth on SP4 medium was slow and it normally took seven days of incubation before the colonies became visible on plates. In this medium the colonies lacked the typical fried egg appearance characteristic of mycoplasma.

Hyperimmune sera produced in rabbits immunized with membranes from Mycoplasma species strain F38 were compared with sera produced by immunization with whole cells. The growth inhibition and the indirect-epi-immunofluorescent tests showed that the membrane antigen produced antisera were of lower quality than that produced with whole organism.

1. INTRODUCTION

Mycoplasma cause two most economically important diseases of goats. These are contagious caprine pleuropneumonia (CCPP) and contagious agalactia. Of the two diseases, CCPP is the most widespread and causes heavy mortality of goats. It is responsible for 80 per cent of deaths every year in countries where the disease occurs (Cottew, 1979).

Contagious caprine pleuropneumonia is an acute disease of goats characterized by acute bronchopneumonia and exudative pleuritis. Under natural conditions CCPP affects goats of all ages and sex. However, the disease has been reproduced experimentally in sheep (Rosendal, 1980). In Kenya CCPP was first reported in 1915 (Anon, 1915). At that time not much was known about its impact on goat production in Kenya. Mettam (1929) concluded after detailed studies on CCPP that it was the most prevalent and economically important disease of goats in East Africa.

The successful cultivation of Mycoplasma mycoides subsp. capri from clinical CCPP cases revealed that mycoplasma were the cause of CCPP as opposed to the earlier belief that the causative agent was a virus (Mettam,1929). Since that time, M. mycoides subsp. capri has been accepted as the cause of classical CCPP. However, other species of Mycoplasma have been isolated and proven to also cause CCPP (Ojo, 1973, 1976; MacOwan and Minette,1976).

The other species known to cause this disease are M. mycoides subsp. mycoides (Y-goat) and Mycoplasma species strain F38. In Kenya Mycoplasma mycoides subsp. mycoides and Mycoplasma species strain F38 cause CCPP. The F38 group however, has been shown to cause 80 per cent of the outbreaks studied so far. A third species, that is, Mycoplasma ovipneumoniae has also been suspected to cause CCPP in this country (Masiga and Rurangirwa, 1979).

To this author's knowledge, Mycoplasma mycoides subsp. capri is not included in the list of mycoplasma identified from CCPP cases in Kenya. Since it is a well-known pathogen of goats, a need arose to investigate and establish whether it is present in CCPP cases. In addition, it was necessary to establish the prevalence of the already identified species and other species not heretofore isolated. The above objective was achieved by the characterization of 34 Mycoplasma strains recently isolated from acute CCPP cases.

The biochemical activities of the F38 group on sugars such as cellobiose, galactose, mannose, sorbitol and xylose have not heretofore been clearly documented. Seventeen recently isolated strains and the original F38 strain were therefore, inoculated into the above mentioned sugars and incubated aerobically. In addition to this, the aerobic and anaerobic catabolism of glucose and the anaerobic reduction of tetrazolium were also investigated.

From our experience at the Veterinary Research Laboratory at Kabete, the F38 group are fastidious and slow growing when cultivated in conventional mycoplasma media. A need therefore arose to investigate a number of media not commonly used in our Laboratory for their ability to support the rapid growth of this group. Three basic mycoplasma media were investigated namely, Medium B with and without yeast extract (Ernø and Stipkovits, 1973), Newings tryptose (Muguga) and SP4 medium (Tully, Rose, Whitcomb and Wenzel, 1979). It was deemed necessary to carry out this particular aspect of the investigation since the results obtained would hopefully aid in rapid identification of the fastidious Mycoplasma species and also allow for the production of large volumes of culture required in vaccine production.

Hyperimmune serum for the identification of Mycoplasma is produced by immunization of rabbits with whole cell organisms (Ernø, Jurmanova and Leach, 1973). To this author's knowledge, experiments designed to test the quality of antisera prepared in rabbits by immunization with the F38 strain membrane antigens have not been reported. It was therefore deemed necessary to compare that type of antisera with one produced by immunization with a whole cell antigen. This objective was achieved by testing the antisera using the growth inhibition and indirect epi-immuno-fluorescent tests.

2. REVIEW OF THE LITERATURE

1. CULTURAL AND GROWTH CHARACTERISTICS.

Mycoplasma are the smallest free-living organisms that can grow in cell-free media (Razin and Knight, 1960). Unlike other bacteria, they do not possess a cell wall but are surrounded by a triple-layered bilipid membrane. They are gram negative and pleomorphic. Examination of smears stained with giemsa or those prepared for dark field microscopy show mycoplasma to be round, ovoid and ring shaped in old cultures. In young logarithmic phase cultures, some are short while others are long and branched filaments.

Growth of mycoplasma occurs along the longitudinal axis of the organism. This type of growth results in the elongation of the cell and gives rise to the formation of filaments. The other type of growth is that in which the organism increases in size and volume. Multiplication of mycoplasma is by binary transverse fission, budding and multiple release. The multiple release phenomenon occurs usually at the end of the filamentous stage when the chainlike structures that form break into independent round cells.

Some mycoplasma, for example, Mycoplasma gallisepticum, M. pneumoniae and M. pulmonis are motile. Motile species possess a club-shaped structure at one end. It is believed to aid in their motility.

When mycoplasma grow in semisolid media they produce colonies that are small (less than 1.0 mm in diameter), hemispherical, slightly raised or flattened. The central part of the colony is usually raised. This type of colonial morphology is referred to as fried-egg. Some colonies, for example, those of M. mycoides subsp. capri however, attain a diameter of 2.0 mm. The central papillae is the part of the colony where the organisms have grown into the semisolid agar while the peripheral zone is the part of the colony where the organisms have spread on the agar. Although most mycoplasma grow and show a fried-egg colonial morphology, there are some which do not. For example, Mycoplasma pneumoniae, M. ovipneumoniae, M. dispar and M. pulmonis produce smooth round colonies. In liquid media mycoplasma produce light turbidity and sometimes a tree-like vortex is observed when the medium is disturbed.

2. MEDIA AND GROWTH REQUIREMENTS.

Mycoplasma have poor catabolic and biosynthetic activities (Razin and Knight, 1960). For this reason, media intended for cultivation of these organisms are complex, exacting and well balanced. Media for cultivation of Mycoplasma are made up of five main components:-

1. A basal component consisting of infusion broth prepared from beef and liver or heart and brain

2. A protein-lipid component which acts as a source of cholesterol. Cholesterol is required by

pathogenic mycoplasma in order for them to grow in artificial media. Animal serum particularly horse serum can be used to satisfy this requirement. Goat, pig and bovine sera are also used.

3. A carbohydrate component of which glucose is the main sugar used.

4. A nucleic acid precursor component. This is supplied by deoxyribonucleic acid (DNA) added to most mycoplasma media formulations.

5. A vitamin component. This part of the medium is supplied by yeast extract.

Mycoplasma have been subdivided into two genera on the basis of cholesterol requirement for growth. In the first genus, called Mycoplasma are organisms which require incorporation of cholesterol into media so as to grow and in the other genus called Acholeplasma are organisms which do not require cholesterol for growth. Although cholesterol is essential for the growth of pathogenic Mycoplasma, bovine serum albumin fraction V is used as substitute (Washburn, John and Somerson, 1978). Another substitute for cholesterol is lipoprotein. Some investigators claim that it supports better growth of Mycoplasma than unfractionated serum (Washburn.and Somerson, 1979). Fatty acids play an important role in membrane synthesis in addition to being a source of oxidizable energy substrate. Some fatty acids and

especially oleic acid have lytic effects on mycoplasma. This effect is however neutralized by cholesterol.

Glucose is incorporated into both liquid and solid media. Some of the media which have glucose in them are Newings tryptone, Viande Foie goat medium (MacOwan, 1976) and Medium N (Ernø and Stipkovits, 1973). However, glucose is not always incorporated into all mycoplasma media, for example, Medium B (Ernø and Stipkovits, 1973). From our experience at the Veterinary Research Laboratory at Kabete, incorporation of glucose into media to be used for cultivating fastidious mycoplasma and slow growing field strains of the F38 group leads to luxurious growth. Incorporation of glucose into mycoplasma media however, leads to rapid decrease in the pH especially when they are inoculated with glucose fermenting species. This results in the death of mycoplasma (Davies, Stone and Read, 1969 ; Gourlay and Macleod, 1966). The effect is normally counteracted by use of buffers in the medium.

The nucleic acid precursor requirement for mycoplasma varies. Some require purine, pyrimidine and a ribose base, while others require uracil and thymidine. Bovine and caprine species require an exogenous source of DNA in order to grow in solid agar media. (Hayflick, 1969). Edward and Fitzgerald (1952) observed that though nucleic acid precursors were an essential ingredient of mycoplasma media, high concentrations were inhibitory for their growth. For this reason, it

is mandatory that care be taken to ensure that optimum concentrations are added. In practice a concentration of 0.2% (w/v) DNA is used.

Although the amino acid and vitamin requirements for mycoplasma are not well defined, they are nevertheless added to mycoplasma media. Edward (1955) showed that thiamine, calcium pantothenate, folic acid, biotin and choline are essential for the growth of human strains of mycoplasma. Bovine strains required riboflavin, thiamine and nicotinamide while caprine strains required biotin and pantothenate in addition to those required by the bovine strains.

Since the first successful cultivation of M. mycoides subsp. mycoides in artificial medium, several types of media have been formulated (Hayflick, 1969). The goat mycoplasma agent was claimed to have been grown in ox serum agar by Beaton (1931). Longley (1940) used Viando Foe medium and goat serum medium but did not succeed in growing the caprine pneumonia agent. Shirlaw (1949) found that Martin Bennet's medium supported the growth of M. mycoides subsp. capri. Longley (1951) achieved good growth of a caprine mycoplasma while using plain goat and sheep serum. The organism grew well in liquid and solid (inspissated) serum. On the other hand Abdulla and Lindley (1967) had limited success in growing caprine Mycoplasma in a medium composed of 10% yeast extract, liver extract

and DNA. The difficulties experienced by the above quoted workers may have arisen due to the presence in the media of high concentrations of DNA which is known to be inhibitory (Hayflick, 1969).

Edward (1951) used an ox-infusion agar medium to which peptone, 10% yeast extract 10% and 20% horse serum were added to investigate the cultural and biochemical characteristics of animal and sewage strains of mycoplasma. He found that the medium supported satisfactory growth of all the strains he studied. In the same study, different variations of the same medium were used. Best growth occurred in the semisolid medium. Tully, Rose, Whitcomb and Wenzel (1979) made similar observations when they used semisolid SP4 medium. This medium was used to isolate M. pneumoniae from sputa specimens of human patients suspected to have atypical pneumonia . In their study, more isolations were made with the semisolid than with either solid or liquid media.

Motty-Sabry and Atefa (1975) working in Egypt carried out an extensive study on five different media. Their objective was to identify a medium which would yield the highest number of isolations of mycoplasma from the genital tract of farm animals. From their study, they concluded that no one medium was satisfactory for the growth of all mycoplasma. However, they found that media to which

DNA was incorporated gave higher chances of isolating mycoplasma from cattle and buffaloes.

Other types of media have been used to grow mycoplasma. Ali (1977) investigated a pleuropneumonia-like medium (PPLO, Difco) to which 20% horse serum, 10% yeast extract and inhibitors against bacteria other than mycoplasma were added. In his study he also included medium FF74 and Medium B. Several species were grown in the media and he found that FF74 gave the best growth of M. ovipneumoniae. However, when candle jars were used, medium B proved better than either FF74 or the PPLO medium. Abdulla and Lindley (1967) working in the Sudan used three media to grow caprine mycoplasma. These media were Near East Animal Health Institute (NEAHI) with and without goat serum and pure goat serum. They found that goat serum alone supported better growth of the caprine mycoplasma than either NEAHI alone or NEAHI to which goat serum was incorporated. This finding led to the hypothesis that goat serum should be added to media intended for the isolation of caprine mycoplasma. This hypothesis was supported by the findings of MacOwan (1976b) who used Viande Foie Medium to which 50% goat serum was incorporated and successfully isolated a "new" Mycoplasma strain F38.

Razin and Cosenza and Tourtellote (1966) used two media namely, tryptose broth and modified Edward Medium and found that addition of oleic acid to the tryptose broth

induced the formation of long filaments in A. laidlawii. Their work indicated that some fatty acids are necessary for the elaboration of filamentous growth in mycoplasma.

Ernø and Stipkovits (1973) investigated three mycoplasma media namely, medium B (modified Hayflick), G.S and FF74. Medium B was found to support the growth of most of the bovine mycoplasma investigated except M. dispar and M. hyopneumoniae. M. dispar grew best in G.S medium and M. hyopneumoniae grew best in FF74. From their study, they recommended that media of simple composition be used to cultivate mycoplasma. They particularly recommended the use of medium B which they found supported the growth of other mycoplasma of human and animal origins in addition to the bovine mycoplasma they studied.

3. BIOCHEMICAL CHARACTERISTICS

Klieneberger (1936) initiated work on the biochemical characterization of Mycoplasma. In her study, Mycoplasma pulmonis was found to ferment glucose, maltose, fructose, mannose, dextrin, glycogen and salicin. Edward (1951) also investigated the biochemical characteristics of animal and sewage strains. An A. laidlawii (sewage A and B) strain fermented with acid production, the same sugars as those fermented by M. pulmonis. In addition to this, it also fermented starch. Two bovine strains (B15 and S) gave the same reactions as those given by the A. laidlawii

sewage strain. However, they differed from it in that they did not ferment fructose. Edward (1953) also investigated two caprine strains namely, the Chu and Beveridge strain and a Nigerian strain. Both strains fermented Laevulose in addition to those sugars fermented by A. laidlawii. On the other hand they did not ferment dulcitol, lactose, mannitol, galactose saccharose and salicin. Other parameters investigated included haemolysis of horse blood and liquefaction of coagulated serum. The strains digested serum and produced alpha haemolysis in horse blood. Solana and Rivera (1967) observed that the caprine strains they studied in Mexico had similar biochemical characteristics as those of the Chu and Beveridge strain. This led to the suggestion that the Mexican strains belonged to the same species as the Chu and Beveridge strain. However, the Mexican strains were identified as M. mycoides subsp. mycoides and the Chu and Beveridge strain as M. mycoides subsp. capri. El-Nasri (1967) working in the Sudan, also observed that the caprine mycoplasma he studied fermented the same sugars as those fermented by M. mycoides subsp. capri (Edward, 1953). The strains Edward (1953) and El-Nasri (1967) investigated belong to the same species except that they differ serologically in some respects (Ernø, Al-Aubaidi, Ojo, Minga and Sikdar, 1978).

Hudson, Cottew and Adler (1967) observed that M. mycoides subsp. capri produced acid in media to which galactose and sucrose were incorporated. Their work confirmed the findings of Edward (1951). Cottew, Watson, Erdag and Arisoy (1969) working in Turkey compared eight caprine mycoplasma strains including the M. mycoides subsp. capri (PG 3). Four strains gave the same biochemical reactions. They fermented glucose, produced beta haemolysis in horse and sheep blood and liquefied serum. The remaining four strains gave weaker reactions compared to those of the first four. Barber and Yedloustchnig (1970) investigated the cultural, biochemical and pathogenic characteristics of several caprine strains. They found that M. mycoides subsp. capri, a Vom strain, a Connecticut strain, a Mexican strain and two California strains 1959 and 1967 fermented glucose, maltose, mannose, mannitol, sorbitol and salicin. All except a Texas strain fermented xylose and reduced tetrazolium. The Mexican and the two M. agalactiae California strains did not cause any changes in any of the sugars tested. The Vom and Connecticut strains were related to M. mycoides subsp. capri because they showed similar biochemical characteristics.

Al-Aubaidi, Dardiri and Fabricant, (1972) compared the biochemical characteristics of M. mycoides subsp. capri and related

strains of bovine origin. He investigated 47 strains. All the strains fermented glucose, reduced tetrazolium aerobically, produced Beta haemolysis in sheep blood and reduced methylene blue aerobically. None of the strains hydrolysed arginine or formed film and spots. Twenty five of the caprine strains were biochemically similar to the bovine strains.

Barile, Delguidice and Tully (1972) also investigated the cultural, biochemical and serological characteristics of several M. conjunctivae strains of caprine and ovine origin. They observed that all the strains fermented glucose, mannose and reduced tetrazolium. They did not hydrolyse arginine or urea and neither did they produce phosphatase nor liquefy serum. Al-Aubaidi (1975) also found that all the A. oculi strains shared the same biochemical characteristics.

MacOwan (1976b), working with Mycoplasma strain F38, observed that it fermented glucose slowly, digested serum but did not hydrolyse arginine. The strain also required cholesterol for its growth but did not produce phosphatase or form film and spots. Leach (1976) and Ernø (1976) independently confirmed the findings of MacOwan (1976b). There was however, one discrepancy with the serum digestion test. Leach (1976) found that the F38 strain he used did not digest serum. Ernø, Leach and MacOwan (1979) suggested that inability of F38

to digest serum as reported by Leach (1976) may have arisen due to differences in the type of media used.

Ojo (1976) investigated the biochemical characteristics of 30 caprine strains. From that study, the caprine strains were placed into one of three groups. In the first group (called A) were placed strains which fermented glucose and mannose, digested serum and also reduced tetrazolium. In the second group (known as B) were placed strains which reduced tetrazolium, formed film and spots and had phosphatase activity. In the third group (called C) were strains which hydrolysed arginine only. Cottew and Yeats (1978) compared the biochemical characteristics of bovine and caprine strains of M. mycoides subsp. mycoides. He found that all the strains had the same biochemical characteristics. There were however, differences based on their colonial morphology. The caprine strains produced larger colonies than the bovine strains. The caprine strains also digested serum and casein.

Caprine Mycoplasma can be placed into one of four major groups (Ernø, Al-Aubaidi, Ojo, Minga and Sikdar, 1978). This conclusion was arrived at after the above mentioned workers carried out extensive biochemical and serological tests on 13 reference strains representing caprine and ovine mycoplasma. These groups are:-

1. Glucose fermentation positive and arginine hydrolysis negative. In this group were placed M. mycoides

subsp. mycoides (PG1), M. mycoides subsp. mycoides (Y-goat), M. mycoides subsp. capri, M. ovipneumoniae, M. conjunctivae and M. putrefaciens.

2. Glucose positive and arginine positive. In this group was placed M. capricolum.

3. Arginine hydrolysis positive only. The organisms placed in this group are M. arginini, M. gallinarum and caprine/ovine serogroup 5.

4. Glucose fermentation negative and arginine hydrolysis negative. Mycoplasma agalactiae, group 7 (1343) and group II (2 - D) were placed in this group.

Other biochemical characteristics were also investigated for the strains. These tests were, the formation of film and spots,. presence/absence of phosphatase activity and reduction of tetrazolium. All the M. mycoides subgroups readily reduced tetrazolium aerobically, digested coagulated serum but did not form film and spots or produce phosphatase activity. M. capricolum digested serum, had phosphatase activity and reduced tetrazolium aerobically. However, it did not form film and spots. M. agalactiae and M. conjunctivae formed film and spots and reduced tetrazolium anaerobically.

Once Mycoplasma have been placed into one of the above mentioned groups, serological characterization can then easily be done. Tests such as the growth

inhibition, metabolic inhibition and indirect immunofluorescence can be carried out. Any two of these tests can be used to conclusively identify any strain of mycoplasma. These tests use hyperimmune sera reagents prepared against the type reference strains of already established mycoplasma species.

3. MATERIALS AND METHODS

3.1. Mycoplasma reference cultures and field strains.

Fourteeen type reference Mycoplasma strains and three Acholeplasma strains were investigated. They are shown in Table 1.

TABLE 1. THE TYPE REFERENCE STRAINS OF MYCOPLASMA AND ACHOLEPLASMA STUDIED.

Species/serogroup	Reference strain
Mycoplasma agalactiae	PG 2
Mycoplasma arginini	G 230
Mycoplasma capricolum	California kid
Mycoplasma conjunctivae	HRC 581
Mycoplasma gallinarum	PG 16
Mycoplasma mycoides subsp. mycoides	PG 1
Mycoplasma mycoides subsp. capri	PG 3
Mycoplasma mycoides subsp. mycoides	Y-goat
Mycoplasma ovipneumoniae	Y-98
Mycoplasma putrefaciens	KSI
Caprine serogroup 5	Goat 145
Caprine serogroup 6	G 189
Caprine serogroup 7	A 1343
Caprine serogroup 11	2-D
Acholeplasma laidlawii	PG 8
Acholeplasma granularum	BTS 39
Acholeplasma oculi	19 L

Thirty four unidentified field strains isolated from CCPP cases from 1978 through 1980 were investigated.

TABLE 2. FIELD STRAINS INVESTIGATED

Locality/District	Number of strains isolated
Kabete	20
Kitui	2
Kajiado	2
Nyeri	2
Baringo	2
Laikipia	1
Mombasa	1
Machakos	1
Nakuru	1
Nairobi	1
Uasin-Gishu	1

3.2. Media.

For the initial isolation of the field strains Viande Foie Goat (VFG) medium was used. Medium B (Modified Hayflick Medium), medium N and FF74 were used in the identification studies.

3.3. Culture procedure.

The clone culture technique was used in the purification of all the strains before characterisation was carried out (Freundt, Ernø and Lemcke, 1979). The technique involved the picking of single four day

old colonies using a pasteur pipette, transferring it into 2 ml liquid Medium B and mixing well. Serial tenfold dilutions were carried out up to 10^{-3} dilution for each culture. Then, each dilution of culture was inoculated onto agar plates and incubated for four days in 8% CO_2 and at 37°C in a moist chamber. The purification process was repeated three times for each strain before it was ready for characterization.

3.4. Biochemical characterization.

For each type reference and field strain, routine biochemical tests such as sensitivity to digitonin, glucose fermentation, arginine hydrolysis, phosphatase activity/production, reduction of tetrazolium, film and spot formation and digestion of serum were carried out.

(i). Sensitivity to digitonin.

Medium.

Medium B containing 20% horse serum was used. Both the liquid and solid media were required.

Reagents.

Sterile paper discs of about 6.0 mm in diameter and of the type used in antibiotic sensitivity testing were impregnated with 0.02 ml of a 1.5% (w/v) ethanolic solution of digitonin and dried overnight at 37°C. The discs were then stored at 4°C until used.

Procedure.

The test was performed on agar media free from surface moisture. A volume of 0.01 ml of the culture was allowed to run down the surface of the plate tilted at an angle of 45^{o}. The inoculum was allowed to absorb. Then, a disc containing digitonin was placed in the middle of the inoculated area. The plates were incubated at $37^{o}C$ in moist chamber containing 8% CO_2. The test was read when growth was visible under a magnification of X10 using a dissecting microscope. Zones of inhibition were measured in mm.

(ii). Glucose fermentation.

Media.

Medium b_1 and medium b_g were used (Appendix 8B). Medium b_1 was a modification of Medium B in which the basal heart infusion broth was treated with glucose oxidase, peroxidase and arginine decarboxylase respectively, to remove any traces of glucose and arginine. Medium b_g was prepared by adding 5% (w/v) glucose to medium b_1 to a final concentration of 1%. This constituted the test medium. The pH of this medium was adjusted to 7.6 with 1.0N hydrochloric acid. Uninoculated medium b_1 were included as control.

Procedure.

Test and control tubes were inoculated with single four day old colonies and incubated aerobically at 37°C. The test and control tubes were checked every 24 hours for a total period of 7 days. A positive reaction was indicated by a change in colour of the test media from red to orange or yellow. When the same colour change appeared in the control and test media the test was regarded as negative.

(iii). Arginine hydrolysis.

Medium.

It was identical to medium b_1 except that its final pH was adjusted to 7.3 with 1.0N hydrochloric acid. The test medium b_a was prepared by supplementing the Basal Medium with 1% (w/v) L-arginine.

Procedure.

The method used was similar to that used in the glucose fermentation test. Tubes with liquid b_a medium were inoculated with colonies and uninoculated control b_1 tubes were included. In this test a distinct alkaline shift in the pH of the test medium indicated arginine hydrolysis.

(iv). Test for phosphatase activity.

An agar medium Bph containing the sodium salt of phenolphthalein diphosphate as the test substrate was used. The serum and yeast extract incorporated in this medium were pre-heated at 56°C for 1 hr to

inactivate any enzymes which might act on the test substrate in the control plates and give rise to false positive reactions.

The test plates were inoculated with liquid cultures using the running drop technique. For each test, an uninoculated Bph plate was also included. All the plates were incubated at 37°C for seven days in a moist chamber containing 8% CO_2. Following incubation each plate was flooded with 2 ml of 1.0 N NaOH solution. The appearance of a red colour on the inoculated plates within 30 seconds following addition of the hydroxide and none in the control indicated a positive reaction.

(v). Test for serum liquefaction.

The substrate was horse serum coagulated as slopes in test tubes. Three slopes were required for each culture to be tested. One was inoculated with neat (undiluted) culture and the other two, with 10^{-3} and 10^{-6} culture dilutions, respectively. Uninoculated media served as controls. Incubation was carried out for 14 days aerobically at 37°C. The test was read at intervals of two days for a total period of 14 days. Liquefaction was indicated by the appearance of shallow moist depressed areas on the surface of inoculated slopes when the growth was confluent and when the growth was scanty, small pits occurred on

the surface of the slope and this also indicated liquefaction.

(vi). Tetrazolium reduction.

The test medium was BT. This was the usual medium B to which 1% (w/v) of 2, 3, 5, triphenyl-tetrazolium chloride solution was added. Tubes containing the test media were inoculated with single colonies. Medium B without the substrate was used as control. Two tubes were prepared for each Mycoplasma strain. One set of tubes were incubated aerobically at 37°C and the other anaerobically at 37°C also. The tests were checked after one day of incubation and then every other day for changes in colour. The appearance of a pink colour in the test media indicated reduction of tetrazolium.

3.5. Serological identification.

(i). Antisera.

The antisera used for the serological characterization were prepared in albino rabbits against the type reference strains for caprine and ovine Mycoplasma. The method of Ernø, Jurmanova and Leach (1973) was employed. The procedure is described in detail in the section on immunization.

(ii). The growth inhibition test.

There are two growth inhibition methods currently being used. These are the agar well and

disc methods, respectively (Clyde, 1964; Black, 1973). In this study the agar well method was applied because it is more sensitive than the disc method.

Procedure.

Agar plates were inoculated with liquid cultures of each strain to be identified using the running drop technique. A drop of the antigen, 0.01 ml in volume was let to run down the surface of the agar. The inoculum was allowed to absorb into the agar. Then, a well 4 mm in diameter was made in the centre of the inoculated area. The well was filled with 0.05 ml of the antiserum to be tested. The plates were incubated at 27°C for two days and then examined for inhibition. Where no growth was observed, the plates were transferred to a 37°C incubator and incubated for a further two to three days. Zones of inhibition were measured in mm.

(iii). Indirect epi-immunofluorescent test.

The indirect-epi-immunofluorescent test (Epi-IMF) procedure of Delgiudice, Robillard and Carski (1967) and as modified by Rosendal and Black (1971) was employed. Unfixed Mycoplasma colonies were used in the tests except for M. ovipneumoniae strains which had to be fixed before the test was performed.

Procedure for treating and fixation of M. ovipneumoniae colonies.

The agar media to be used were first kept at 4°C for one week. Following this, they were inoculated and incubated for three days at 37°C. They were then removed and kept for a further three days at 22°C. Fixation was achieved by adding 2 ml 95% ethyl alcohol and incubated at 22°C for 90 minutes. The alcohol was poured off and the plates were further incubated for five days at 22°C. They were then washed with distilled water to remove the alcohol before the test was performed. For the other species the media were inoculated and then incubated at 37°C in 8% CO_2 until the colonies were visible at a magnification of 40X. At this point they were ready for the test.

Procedure for the indirect epi-immunofluorescent test.

Agar blocks of different sizes and geometric shapes were cut and placed on glass slides with the colonies uppermost. Two sera, that is, an antimycoplasma hyperimmune serum and a commercial fluorescein conjugated antirabbit immunoglobulin were used. The blocks with colonies were incubated with a drop of undiluted antiserum for 30 minutes at 20°C in a moist chamber. Then, the blocks were washed twice for ten minutes with PBS, pH 7.2, in test-tubes on test-tube rotator. A drop of 1:20 dilution of the fluorescein

conjugated antirabbit globulin was placed on each block and the blocks incubated for another 30 min at 20°C. After washing twice in PBS pH 7.2 the blocks were mounted on slides and examined. Microscopy was performed with a Zeiss Standard (RA) microscope equipped with an Osram HBO mercury lamp and an incident ray attachment.

(iv). The growth precipitation test.

Medium B agar plates were used to grow the strains to be used as the antigens. The agar plates were heavily inoculated with the cultures and incubated for four days at 37°C in a moist chamber containing 8% CO_2. Agar blocks measuring 1 cm by 1 cm with dense growth of mycoplasma were cut and placed in a tube containing 2 ml medium B broth. The block and the medium were mixed well by shaking vigorously for 3 min and left at 20°C for 1 hour. The culture was used as the "antigen". A well 7 mm in diameter was punched out in the centre of an uninoculated agar plate using a stainless steel cylinder. The well was filled with the antigen using a pasteur pipette. Then, one to four antiserum impregnated discs were placed on the agar surface at a distance of exactly 1 cm from the rim of the central well. The plates were incubated at 37°C in a humid 8% CO_2 atmosphere.

After three days of incubation they were examined for bands of precipitation against a dark background using obliquely transmitted light. The examinations were repeated after five, seven and ten days.

3.5. Biochemical characteristics of the F38 group.

(i). Mycoplasma strains.

Seventeen mycoplasma strains recently identified and the original F38 strains were investigated.

(ii).. Media.

Basal Medium b_1 to which was added 1% solution of the test sugar substrate was used. Basal medium B to which the substrates were incorporated to a final concentration of 1% (w/v) was also included.

The substrates investigated were glucose, cellobiose, mannose, galactose, sorbitol and xylose. Medium BT was used in the reduction of tetrazolium test.

(iii).Tests performed.

The aerobic and anaerobic catabolism of glucose was investigated. Cellobiose, galactose, mannose, sorbitol and xylose were incubated aerobically only. Tetrazolium reduction was investigated aerobically and anaerobically.

Procedure.

Two systems were used for each sugar reaction. These consisted of inoculating one set of each sugar with single colonies while the other set was inoculated with a 0.1 ml drop of the liquid culture. Medium b_1 but without substrate was included as

controls. Uninoculated test media were also incubated together with the test media. The anaerobic incubation of both glucose and tetrazolium was achieved by adding sterile liquid petroleum jelly into tubes earmarked for anaerobic incubation and their appropriate controls. All the sugar tests were incubated for a period of 14 days at 37°C. The reactions were checked every two days and recorded. For the sugar reactions the appearance of a yellow or orange colour in the test media and none in the controls was considered as a positive reaction. The appearance of a violet colour in the tetrazolium reduction test was considered as a positive reaction.

3.6. Media studies.

(i). Mycoplasma strains.

Three F38-like strains and one strain of Acholeplasma oculi were investigated.

(ii). Media.

Three types of conventional mycoplasma media were prepared and studied for their ability to support the rapid growth of the strains. The media included Medium B (Modified Hayflick medium) with and without yeast extract, Newings tryptose medium and SP4 Medium. In general solid media were tested. In a few occasions attempts were made to grow the strains in liquid media.

Procedure.

Each strain in broth was serially diluted up to a dilution of 10^{-6}. Following this, two sets of plates for each medium were inoculated with each dilution of the strains. One set was incubated in 95% N_2 + 5% CO_2 and the other in a moist chamber containing 8% CO_2. All the tubes were incubated at 37°C. They were examined for growth every 24 hours for a total period of 10 days. A stereo microscope (magnification: X40) was used.

3.7. Immunization experiments.

Mycoplasma spec. Strain F38 (original strain) was used as the source of the two types of antigens. These were a whole cell and membrane antigens.

Medium. Medium B (Modified Hayflick) and Rabbit Medium (RT Medium) were used to grow the antigens.

(i). Antigen preparation.

Two single four day old colonies grown in Medium B were picked and inoculated into two tubes, each containing 3 ml liquid RT Medium. The broth cultures were incubated aerobically for four days at 37°C. Smears were made and stained with acridine orange to ascertain that the cultures were at their logarithmic phases of growth. Once the cultures were at their logarithmic phases of growth, each

was inoculated into 30 ml of RT Medium. The inoculated media were incubated and treated in the same manner as the cultures in 3 ml volumes of media. When each culture was again at its logarithmic phase of growth, it was inoculated into 600 ml RT Medium. Each culture was incubated as above and harvested at the logarithmic phase of growth. Harvesting was achieved by centrifugation for 30 minutes at 24,000 x g, using a Super Speed MSE centrifuge ($4^{o}C$). The resulting pellets were washed three times with PBS, pH 7.2 for a total period of 30 minutes. One pellet was then dissoved in 20 ml PBS, pH 7.4 and kept at $-70^{o}C$ to be used later as the whole cell antigen. The other was processed for membrane extraction.

(ii). Membrane extraction.

The membranes were extracted using the digitonin lysis method (Razin, 1979). The pellet was dissolved in 5 ml β-buffer and transferred to 200 ml of 0.5 M NaCl containing 25 μg digitonin per ml. Following mixing the suspension was incubated at $37^{o}C$ in a water bath for 15 minutes to allow extraction. The membranes were harvested by centrifugation at 33,000 xg for a period of 30 minutes using a Super Speed refrigerated centrifuge. The resulting pellet was washed three times, first with deionized water, then 0.01 M phosphate

buffer pH 7.2 and finally with deiozined water. The clean membrane extract was dissolved in 5 ml β-buffer. Its protein content was determined using the method of Lowry, Rosebrough, Farr and Randal (1951). The membrane antigens were kept at -70°C. until used.

(iii). Antiserum production (immunization).

Procedure.

Antiserum production was carried out using the method of Ernø et al. (1973). Three albino rabbits were used to produce the antisera for each antigen. Each rabbit was inoculated five times at intervals of two to three days. On the first and second occasions, 0.75 ml of a 1/100 dilution of the concentrated antigen was given intravenously (I.V) and 1.5 ml of a mixture of the 1/100 antigen with equal parts of "Alhydrogel" was given intraperitoneally (I.P). On the third occasion, 0.75 ml of a 1/10 dilution of antigen was given I.P. and 1.5 ml of a mixture of the 1/10 antigen with equal parts of "Alhydrogel" was given subcutaneously. On the fourth and fifth occasions, a mixture of concentrated antigen with equal parts of "Alhydrogel" was inoculated intramuscularly (I.M). A volume of 0.75 ml was inoculated into the thighs of the hind legs and the prescapular area of the fore legs. After a rest of five to six weeks, 1.5 ml of concentrated antigen was given (I.V) and repeated after 10 days. The rabbits were bled 10 days later.

The sera obtained were analysed using the growth inhibition and indirect epi-immunofluorescent tests. (Freundt, Ernø, Black, Krogsgaard-Jensen and Rosendal, 1973; Freundt and Ernø, 1979; Rosendal and Black 1971).

3.8. Indirect haemagglutination test.

The method of Cho, Rhunke and Langford (1975) was employed.

(i). Antigens.

Two antigens, namely, Mycoplasma bovigenitalium and Acholeplasma laidlawii A were used.

(ii). Antigen preparation.

Each organism was first grown in solid Medium B. After four days of incubation at 37°C in a moist 8% CO_2 chamber, single colonies were picked, inoculated into 3 ml liquid Medium B and incubated aerobically at 37°C. Once in their logarithmic phase of growth, the cultures were transferred into 30 ml of Medium B. They were also similarly incubated to their logarithmic phase of growth and inoculated into 600 ml broth medium. Each 600 ml log phase culture was harvested by centrifugation for 30 minutes at 14,000 x g using MSE Super Speed refrigerated centrifuge. The resulting pellets were washed twice in 0.15 M saline and resuspended in 10 ml saline to which sodium azide was added to a final concentration of 0.01%.

(iii). Glutaraldehyde Fixation.

Equal volumes of 20% sheep blood in phosphate buffered glucose (PBG) and glutaraldehyde in PBG were mixed and incubated for 15 minutes in a water

bath at 37°C. The fixed erythrocytes were washed five times in 0.9% saline. Each wash lasted seven minutes. The cells were resuspended at a concentration of 10% in saline containing 0.1% sodium azide and stored until needed in the antigen coating stage.

(iv). Coating of the glutaraldehyde fixed erythrocytes with the respective antigens.

Five ml of the fixed erythrocyte were withdrawn and placed into each of five test tubes. The tubes were centrifuged for five minutes at 3,000 x g. The supernate was discarded and the residual pellet was washed twice in PBS, pH 7.0. Each pellet was then serially diluted, starting at 1:1 dilution to 1:64 dilution in PBS. Equal amounts of the antigen and glutaraldehyde fixed erythrocytes (1:1 ratio) were mixed and then to each mixture was added a volume of 0.4 ml sodium azide to a final concentration of 1%. All the tubes were incubated for 16 hours at 37°C on a test-tube rotator. On the following day each dilution of the antigen-coated, glutaraldehyde fixed sheep erthrocytes were washed three times for 10 minutes in PBS, pH 7.0. The antigen-coated erythrocytes were then resuspended in 2 ml glycerin in PBS, pH 7.0, and stored at 4°C until used.

(v). Indirect haemagglutination test.

The Microtitre method of Sever (1962) adopted by Cho et al. (1975) was employed. After heat

inactivation at 56°C for 30 min, the test serum and normal rabbit serum were preabsorbed with fresh sheep erythrocytes. Serial twofold dilutions of the test sera starting at 1:20 were prepared in 0.025 ml amounts in PBS containing normal rabbit serum. The mycoplasma sensitized glutaraldehyde - fixed sheep erythrocytes preserved in 50% glycerine were washed three times in PBS and resuspended in PBS at a concentration of 2%. The sensitized cell suspension was used as antigen and 0.025 ml was added to each well. Controls of normal bovine serum and antigen were included in the test. The well contents were thoroughly mixed by tapping the edge of the plate gently. The test was read after incubation for two hours at 20°C.

4. RESULTS

4.1. Cultural characteristics.

The colonial morphology of all the mycoplasma identified except those of M. ovipneumoniae was characterized by the fried egg appearance typical of Mycoplasma. M. ovipneumoniae colonies were smooth, raised and lacked the central nipple found in other mycoplasma colonies. In general, growth occurred in agar after an average of three days of incubation for the field strains and one day for most reference strains. The notable exception was in F38 group strains which showed growth after four days of incubation at the earliest. These findings agree with those of MacOwan (1976b) who observed that the original F38 strain grew very slowly in media other than Viande Foie goat (VFG) medium. Initially their colonies were without a central nipple. After subsequent subculturing, the fried egg appearance became evident. Some strains of the F38 group, especially G 242/78 grew exceptionally well and attained a colonial diameter of 2 mm as compared to the rest which never exceeded 1 mm in diameter. This strain also had a double peripheral zone of growth (egg-white) surrounding the central nipple (Figures 1a and 1b). The central nipple in this group was always twice the size of the peripheral zone except for that of Strain G 242/78.

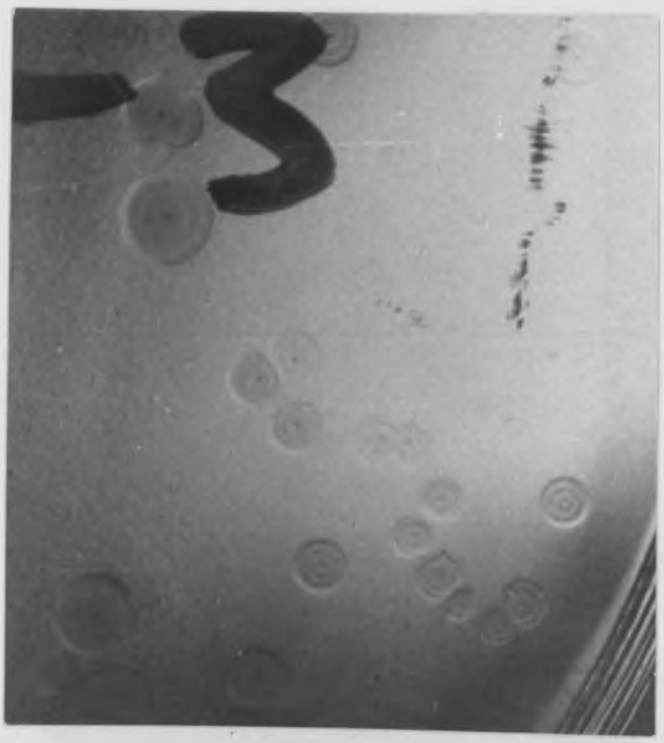

Figure 1a. Strain G 242/78 colonies showing the double peripheral zone of growth in medium B plate. Magnification: x 40.

Figure 1b. A colony of strain G 242/78 showing the double zone peripheral growth.
Magnification: x 60.

4.2. Biochemical tests.

(i). The type strains.

As shown in Table 3 the biochemical reactions of the 14 type reference strains investigated were consistent with those reported by Ernø et al. (1978). Four main groups emerged. Group 1 strains fermented glucose but did not hydrolyse arginine. In this group were Mycoplasma mycoides subsp. mycoides (PG1), M. mycoides subsp. mycoides (Y-goat), M. mycoides subsp. capri (PG3), M. conjunctivae (HRC 581), M. ovipneumoniae (Y98), M. putrefaciens (KS-1), Acholeplasma laidlawii and A. oculi (19L). The second group of mycoplasma hydrolysed arginine but did not ferment glucose. Among these were Mycoplasma arginini (G230), M. gallinarum (PG16) and Caprine/ovine serogroup 5 (Goat 145).

The third group was made up of mycoplasma which fermented glucose and hydrolysed arginine. These were Mycoplasma capricolum and Caprine/ovine serogroup 6 (Goat 189). In the fourth group were mycoplasma which did not ferment glucose or hydrolyse arginine. In this group were Mycoplasma agalactiae (PG2), Caprine/ovine serogroup 7 (A1343) and Caprine/ovine serogroup 11 (2-D). Mycoplasma agalactiae, M. capricolum, Group 6 Goat 189, Group 7, Group 11

TABLE 3. BIOCHEMICAL REACTIONS OF TYPE REFERENCE STRAINS OF CAPRINE AND OVINE MYCOPLASMA AND ACHOLEPLASMA

Species or serogroup and type reference strains		Fermentation of glucose[a]	Hydrolysis of arginine	Phosphatase activity	Digestion of serum	Formation of "film and spots"	Reduction of Tetrazolium aerobic/ anaerobic
M. mycoides subsp. *capri*	PG3	+	-	-	+	-	+/+
M. mycoides subsp *mycoides*	Y-goat	+	-	-	+	-	+/+
M. conjunctivae	HRC 581	+	-	-	-	+	-/+
M. ovipneumoniae	Y-98	+	-	-	-	-	-/+
M. putrefaciens	KSI	+	-	+	-	+	-/+
Group 6	Goat 189	+	v	+	+	-	+/+
M. capricolum	California Kid	+	+	+	+	-	+/+
M. arginini	G230	-	+	-	-	-	-/+
Group 5	Goat 145	-	+	+	-	-	-/-
M. agalactiae	PG2	-	-	+	-	+	+/+
Group 7	A1343	-	-	+	-	+	-/+
Group 11	2-D	-	-	+	-	+	-/+
M. mycoides subsp. *mycoides*	PG1	+	-	-	-	-	+/+
A. laidlawii	PG8	+	-	+	-	-	+/+
A. oculi	19L	+	-	-	-	-	+/+

Note: Except for *A. laidlawii* PG8 and *A. oculi* 19L all the rest were sensitive to digitonin.
+ Indicates a positive reaction, v indicates a variable reaction, - indicates a negative reaction.
[a] fermentation reaction indicates acid produced.

and Acholeplasma laidlawii had phosphatase activity. The rest gave negative reactions as shown in Table 3.

M. capricolum, M. mycoides subsp. mycoides (Y-goat) M. mycoides subsp. capri and Group 6 (Goat 189) digested serum. Film and spots were produced by M. conjunctivae, M. putrefaciens, M. agalactiae and Group 11 (2-D). All the species except Group 5 (Goat 145) reduced tetrazolium either aerobically or anaerobically. M. conjunctivae (HRC 581), M. ovipneumoniae (Y98), M. putrefaciens, M. arginini and Group 11 (2-D) reduced tetrazolium anaerobically only.

(ii). Field strains.

The results of the biochemical tests carried out on the field strains are shown in Table 4. Out of the 34 strains, 26 fermented glucose with acid production but did not hydrolyse arginine. The majority (17) of these strains belonged to the F38 group. Out of the remainder, five were M. ovipneumoniae strains and four were M. mycoides subsp. mycoides. All the M. ovipneumoniae and M. mycoides subsp. mycoides strains reduced tetrazolium aerobically and anaerobically. All M. arginini strains and those of the F38 group reduced tetrazolium anaerobically. One notable exception in the F38 group was Strain G 242/78 which reduced tetrazolium both aerobically and anaerobically. Although M. ovipneumoniae strains reduced tetrazolium

TABLE 4. BIOCHEMICAL TESTS FOR CAPRINE MYCOPLASMA IDENTIFIED

Species or Serogroup and Strain	Fermentation of glucose[a]	Hydrolysis of arginine	Phosphatase activity	Serum digestion	Reduction of Tetrazolium aerobic/ anaerobic
M. mycoides subsp. *mycoides* (LC) 30/76	+	-	-	+	+/+
M. mycoides subsp. *mycoides* (LC) G290/79	+	-	-	+	+/+
M. mycoides subsp. *mycoides* (LC) 1915/80	+	-	-	+	+/+
M. mycoides subsp. *mycoides* (LC) G175/76	+	-	-	+	+/+
Mycoplasma spec. F38 G75/76	+	-	-	-	+/+
Mycoplasma spec. F38 G133/79	+	-	-	-	-/+
Mycoplasma spec. F38 G176/79	+	-	-	+	-/+
Mycoplasma spec. F38 G242/78	+	-	-	+	+/+
Mycoplasma spec. F38 G272/80	+	-	-	+	-/+
Mycoplasma spec. F38 G280/80	+	-	-	+	-/+
Mycoplasma spec. F38 G282/80	+	-	-	+	-/+
Mycoplasma spec. F38 G3614/80	+	-	-	+	-/+
Mycoplasma spec. F38 G338/80	+	-	-	-	-/+
Mycoplasma spec. F38 G368/79	+	-	-	+	-/+
Mycoplasma spec. F38 612/76	+	-	-	+	-/+
Mycoplasma spec. F38 604/76	+	-	-	+	-/+
Mycoplasma spec. F38 1943	+	-	-	-	-/+

\+ indicates a positive reaction.

\- indicates a negative reaction.

spec. = species.

subsp.= subspecies.

[a]=fermentation indicates production of acid only.

TABLE 4(Cont).

Species or Serogroup and Strain	BIOCHEMICAL TESTS OF MYCOPLASMA IDENTIFIED				
	Fermentation of glucose[a]	Hydrolysis of arginine	Phosphatase activity	Digestion of serum	Reduction of Tetrazolium aerobic/ anaerobic
Mycoplasma spec. F38 1945	+	-	-	+	-/+
Mycoplasma spec. F38 1954	+	-	-	+	-/+
Mycoplasma spec. F38 2008/80	+	-	-	-	-/+
Mycoplasma ovipneumoniae G101/79	+	-	-	-	+/+
Mycoplasma ovipneumoniae G242/79	+	-	-	-	+/+
Mycoplasma ovipneumoniae G207/80	+	-	-	-	+/+
Mycoplasma ovipneumoniae 1954	+	-	-	-	+/+
Mycoplasma ovipneumoniae 2008/80	+	-	-	-	+/+
Mycoplasma arginini G45/80	-	+	-	-	-/+
Mycoplasma arginini G115/80	-	+	-	-	-/+
Mycoplasma arginini G132/78	-	+	-	-	-/+
Mycoplasma arginini G3408/80	-	+	-	-	-/+
Mycoplasma arginini 1979/80	-	+	-	-	-/+
Mycoplasma arginini 2001/80	-	+	-	-	-/+
Mycoplasma arginini 2008/80	-	+	-	-	-/+

\+ indicates a positive reaction.

\- indicates a negative reaction.

[a] fermentation indicates acid production only.

All the strains were sensitive to digitonin.

both aerobically and anaerobically, stronger reactions were observed when the cultures were incubated anaerobically.

(iii). The serum digestion test.

All the M. mycoides subsp. mycoides (Y-goat) strains and twelve strains in the F38 group digested serum. Five F38-like strains never digested serum. This did not agree with the accepted idea of Ernø et al. (1979) that the F38 group strains are serum digestion positive.

4.3. Serological tests.

(i). Growth inhibition test.

With the growth inhibition test 17 strains were inhibited by hyperimmune sera prepared against F38 and bovine serogroup 7 (PG50). As shown in Table 5, sera prepared against other caprine mycoplasma did not inhibit these strains. The serological relationship between F38 and PG50 has been observed by Ernø (1979, personal communication). The inhibition in both cases was total and in three tests serum against PG50 gave wider zones of inhibition than that against F38. M. mycoides subsp. mycoides (Y-goat) strains were inhibited by homologous antiserum and heterologous antiserum against PG1. Eight strains were inhibited by antisera against Mycoplasma arginini and five strains were inhibited by antisera against

TABLE 5. GROWTH INHIBITION TESTS FOR MYCOPLASMA MYCOIDES SUBSP. MYCOIDES AND THE F38 GROUP

Species/ serogroup	Number of strains	ANTISERA					
		PG1	Y-GOAT	PG3	F38	PG50	CALIF. KID
M. mycoides subsp. mycoides LC	4	+	+	-	-	-	-
The F38 Group	17	-	-	-	+	+	-

+ indicates inhibition.
- indicates no inhibition.

M. ovipneumoniae. Two strains identified as M. ovipneumoniae were partially inhibited by antisera against M. dispar, as shown in Table 6.

(ii). Indirect epi-immunofluorescent test.

The results of the indirect epi-immunofluorescent antibody tests were similar to those obtained with the growth inhibition test. As shown in Table 7 all the F38-like strains fluoresced in the presence of homologous antisera and also in the presence of antiserum against PG50. On some occasions slight fluorescence was observed in the presence of antiserum against M. capricolum. Four strains showed fluorescence with antiserum against M. mycoides subsp. mycoides (Y-goat) and M. mycoides subsp. mycoides (PG1). Eight strains fluoresced in the presence of antiserum against M. arginini. There was no fluorescence with any other antiserum tested with the eight strains (Table 8). Five strains fluoresced in the presence of antiserum against M. ovipneumoniae and of these, two reacted faintly with antiserum against M. dispar.

(iii). The growth precipitation.

There were extensive cross reactions observed with this test between heterologous antigens and antisera. This was particularly so between M. mycoides subsp. mycoides (Y-goat), M. mycoides subsp. capri, M. mycoides subsp. mycoides (PG1), M. capricolum,

TABLE 6. GROWTH INHIBITION TESTS FOR MYCOPLASMA ARGININI AND MYCOPLASMA OVIPNEUMONIAE

Species/ serogroup	Number of strains	ANTISERA				
		G230	GOAT 145	PG16	462/2	Y-98
M. arginini	8	+	-	-	-	-
M. ovipneumoniae	5	-	-	-	[a]-	+

\+ indicates inhibition.

\- indicates no inhibition.

[a]- two strains were partially inhibited by antiserum against M. dispar.

TABLE 7. INDIRECT IMMUNOFLUORESCENT TESTS FOR MYCOPLASMA MYCOIDES SUBSP. MYCOIDES AND THE F38 GROUP

Species/ serogroup	Number of strains	ANTISERA					
		PG1	Y-GOAT	PG3	F38	PG50	CALIF. KID
M. mycoides subsp. *mycoides* LC	4	+	+	-	-	-	-
The F38 Group	17	-	-	-	+	+	-

+ indicates fluorescence.

- indicates no fluorescence.

TABLE 8. INDIRECT EPI-IMMUNOFLUORESCENT TESTS FOR MYCOPLASMA ARGININI AND MYCOPLASMA OVIPNEUMONIAE

Species/ serogroup	Number of strains	ANTISERA				
		G230	Goat 145	FG 16	M. dispar 462/2	Y98
M. arginini	8	+	-	-	-	-
M. ovipneumoniae	5	-	-	-	(+)	+

\+ indicates fluorescence.

\- indicates no fluorescence.

Note (+) indicates some fluorescence shown by 2 of the M. ovipneumoniae strains when hyperimmune serum against M. dispar was used.

Caprine/ovine serogroup 6, the F38 group and bovine serogroup 7 (PG50). There were four common antigens shown by all these species (Figure 2 (a) and (b). More precipitin lines however, formed between homologous antigens and antisera. Many F38 strains did not show any cross reactions with heterologous antisera as shown in Table 9. Precipitin lines were visible after seven days of incubation for the F38 group as compared to the other Mycoplasma which appeared after three days of incubation. These observations agree with those of Ernø and Salih (1980) who observed that extensive cross reactions occurred between the above named Mycoplasma in their study. Homologous antiserum to M. arginini and heterologous antisera against Goat 145 formed common precipitin lines with eight strains identified as M. arginini (Figure 3). All the five strains of M. ovipneumoniae reacted with homologous antiserum as was expected. Figure 4 shows a growth precipitation reaction between homologous antigen and antisera. Two of the M. ovipneumoniae strains cross reacted with heterologous antisera prepared against M. dispar. This was in agreement with the findings of Perreau (1973).

Figure 2a. The growth precipitation test showing bands of precipitin lines formed when a F38 strain C 1673 was used as the antigen in the central well with heterologous antisera against M. mycoides subsp. mycoides PG1 (disc 1), M. mycoides subsp. capri PG3 (disc 2), Bovine serogroup 7 (disc 3) and California kid (disc 4). Notice the faint merging of the precipitin lines around the central well.

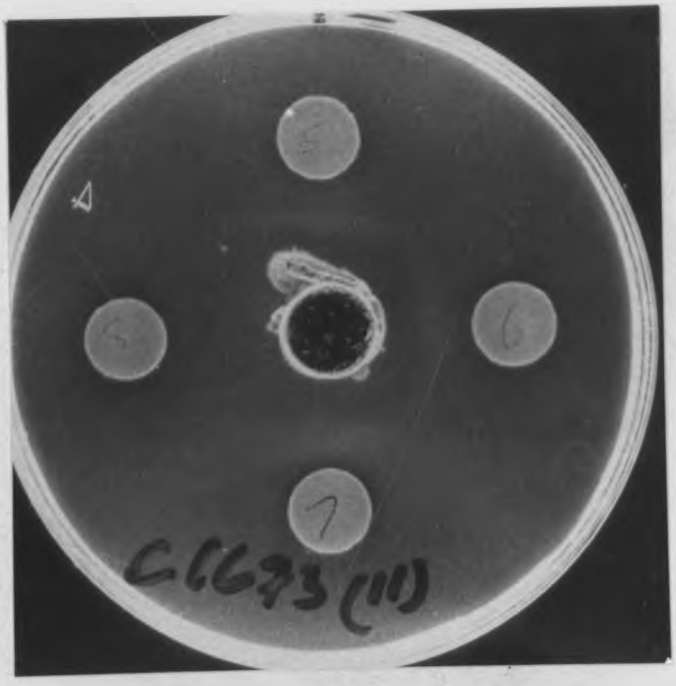

Figure 2b. The growth precipitation test showing the antigenic relationships between a F38 strain C 1673 and M. mycoides subsp. mycoides Y-goat (disc 5), caprine/ovine serogroup 6, original F38 strain and M. conjunctivae (HRC. 581).

Figure 3. Growth precipitation test showing the antigenic relationship between M. arginini (G230) and caprine/ovine serogroup 5 (G145) (top disc). The central well contains M. arginini antigen and the disc below contains homologous antisera.

Figure 4. The growth precipitation test showing a reaction between homologous antigen and antiserum of M. ovipneumoniae.

TABLE 9. THE GROWTH PRECIPITATION TEST RESULTS FOR MYCOPLASMA MYCOIDES SUBSP. MYCOIDES AND THE F38 GROUP.

Species and Strain		ANTISERUM					
		PG1	Y-GOAT	PG3	CALIF KID	PG50	F38
Mycoplasma mycoides subsp. mycoides LC	G290/79	+	+	+	+	+	+
Mycoplasma mycoides subsp. mycoides LC	1915	+	+	+	+	+	+
Mycoplasma mycoides subsp. mycoides LC	G175/78	+	+	+	+	+	+
Mycoplasma spec. F38	G242/78	+	+	+	+	+	+
Mycoplasma spec. F38	G272/80	-	-	-	-	+	+
Mycoplasma spec. F38	0280/80	-	-	-	-	+	+
Mycoplasma spec. F38	G281/80	-	-	-	-	+	+
Mycoplasma spec. F38	G316A/80	+	+	+	-	-	+
Mycoplasma spec. F38	G338/80	+	+	+	+	-	+
Mycoplasma spec. F38	G368/78	+	+	+	+	-	+
Mycoplasma spec. F38	1868/79	+	+	+	+	+	+
Mycoplasma spec. F38	1943	-	-	-	-	+	+
Mycoplasma spec. F38	1954	-	-	-	-	+	+
Mycoplasma spec. F38	2008/80	-	-	-	-	-	+
Mycoplasma spec. F38	G176/79	+	+	+	-	-	+
Mycoplasma spec. F38	G133/79	-	-	-	-	-	+

\+ Bands of precipitation lines present.

\- No bands of precipitation lines. spec. = species.

4.4. The biochemical reactions of the F38 group.

As shown in Table 10, all the F38-like strains fermented glucose with production of acid both aerobically and anaerobically. However, stronger and faster reactions occurred anaerobically. Mannose and galactose were metabolized readily by most of the strains except four which gave trace reactions with galactose and two which did not metabolize it at all. Three strains did not metabolize mannose. Thirteen strains metabolized xylose readily and of the remainder two gave trace reactions and three never metabolized this sugar. Cellobiose and sorbitol were poorly metabolized by the majority of the strains. Three strains metabolized sorbitol readily and two metabolized cellobiose. There were no differences between tests in which yeast extract was incorporated and those without. This finding was in agreement with that of Ernø and Stipkovits (1973).

Tetrazolium was reduced anaerobically by all the F38 strains investigated. A strain G242/78 however reduced this reagent aerobically also. This was not in agreement with the observations of Salih (1981, personal communication) who found that the original F38 strain he investigated reduced tetrazolium anaerobically only.

TABLE 10. SUGAR REACTIONS FOR F38 AND F38-LIKE STRAINS

Mycoplasma/Serogroup and strain.	Glucose metabolism aerobic/ anaerobic	Mannose	Galactose	Cellobiose	Xylose	Sorbitol
Mycoplasma spec. F38	+/+	+	+	Trace	+	-
Mycoplasma spec. G75/76	+/+	+	+	Trace	+	-
Mycoplasma spec. G133/79	+/+	+	+	Trace	+	-
Mycoplasma spec. G176/79	+/+	+	+	-	+	-
Mycoplasma spec. G242/78	+/+	+	+	+	+	+
Mycoplasma spec. G272/80	+/+	+	+	Trace	+	Trace
Mycoplasma spec. G280/80	+/+	+	+	Trace	+	-
Mycoplasma spec. G281/80	+/+	+	Trace	-	Trace	+
Mycoplasma spec. G316A/80	+/+	+	+	Trace	-	Trace
Mycoplasma spec. G338/80	+/+	-	+	-	+	-
Mycoplasma spec. G368/78	+/+	+	+	Trace	+	Trace
Mycoplasma spec. 630/76	+/+	+	Trace	-	+	-
Mycoplasma spec. 904/76	+/+	+	+	+	+	+
Mycoplasma spec. 1868/79	+/+	+	-	-	+	-
Mycoplasma spec. 1943/80	+/+	+	Trace	-	Trace	-
Mycoplasma spec. 1945/80	+/+	+	-	-	+	-
Mycoplasma spec. 1954/80	+/+	-	+	Trace	-	-
Mycoplasma spec. 2008/80	+/+	-	Trace	-	-	-

+ indicates a positive reaction.. - indicates a negative reaction.
Trace = slight drop in pH of the medium as compared to controls.
Note: Mannose, galactose, cellobiose, xylose and sorbitol were tested under aerobic conditions only.

4.5. Media experiment results.

Growth of the F38 strains was evident within three days of incubation in Medium B and Newings tryptose incubated either in 8% CO_2 or anaerobically. At this stage the colonial morphology was not fried egg in appearance. The colonies were very small, smooth and raised. On the fourth day of incubation only the central papillae was present and a slight peripheral growth surrounding it. On further incubation the colonies became fried egg in appearance but, the nipple was always larger than the peripheral zone. Growth on plates inoculated with 10^{-2} and 10^{-3} culture dilutions was evident on the third day of incubation. Plates inoculated with 10^{-4} and 10^{-5} culture dilutions appeared on the fourth and fifth day of incubation, respectively. No growth appeared on plates inoculated with culture dilutions of 10^{-6}. No significant differences were observed between plates incubated in 8% CO_2 and those incubated anaerobically except that, colonies on plates incubated under the latter conditions were slightly larger than those incubated in 8% CO_2. SP4 medium poorly supported the growth of the F38 strains irrespective of the gaseous conditions employed. In comparison with Medium B and Newings tryptose, in plates inoculated with 10^{-1} to 10^{-3} culture dilutions, growth appeared after five days of incubation. This indicated that

growth of F38 in Medium SP4 was much slower than in the other two media. Growth on plates seeded with culture dilutions higher than 10^{-3} occurred after seven days of incubation. The colonies of the F38 strains in the SP4 medium remained very small (less than 1 mm in diameter) even after 10 days of incubation. They did not have the fried egg appearance characteristic of Mycoplasma colonies.

Acholeplasma oculi grew well in all the media tested. Colonies appeared after 24 hours of incubation in either 8% CO_2 or anaerobically. Best growth was observed in both Medium B and Newings tryptose. However "larger" colonies measuring up to 2 mm in diameter were observed in Newings tryptose medium while those on Medium B never exceeded 1.5 mm in diameter. The fried egg type colony was evident in both media. For this reason, either Medium B or Newings tryptose can therefore be used successfully to cultivate both Mycoplasma spec. strain F38 and Acholeplasma oculi.

4.6. Results of the immunization experiments.

With the growth inhibition test, antisera to the F38 cell antigen gave a growth inhibition of 4 mm in diameter with the homologous antigen. The membrane antisera gave a growth inhibition of 3 mm in diameter. This level of inhibition was double the accepted level.

With these results the antisera can be said to be of comparable quality. With the indirect epi-immunofluorescent test, the hyperimmune serum obtained with the cell antigen gave good fluorescence even with serum dilutions of as high as 1:80. On the other hand the membrane antiserum gave fluorescence only up to a 1:10 dilution. Within the context of this test the cell antisera can be said to be superior to the membrane antisera.

4.7. The indirect haemagglutination results.

Fifty six sera were tested using two different antigens. These were Mycoplasma bovigenitalium (PG 11) and Acholeplasma laidlawii A (PG 8) suspected to be the cause of the clinical disease in the animals whose sera were tested. All the sera except S 305, S 327, S 345 and S 346 gave negative reactions. Sera S 305, S 346 and S 327 had an IHA titre of 1:40, serum S 345 had an IHA titre of 1:20 and serum S 352 a titre of 1:80. With the method of Cho et al. (1975) used in this study, serum S 352 was taken to be suspicious while the rest gave unspecific reactions. The positive control for this test had a titre of 1: 59,200.

5. DISCUSSION.

The main purpose of the present study was to identify and establish which Mycoplasma species were represented by the 34 Mycoplasma strains isolated from acute CCPP cases. Three Mycoplasma species in addition to the F38 group were identified, namely, Mycoplasma mycoides subsp. mycoides (Y-goat), Mycoplasma arginini and Mycoplasma ovipneumoniae.

The results indicated that M. mycoides subsp. capri was not responsible for any of the outbreaks from which the 34 strains were isolated. In Kenya only one strain, that is, of M. mycoides subsp. capri has been identified at the Mycoplasma Reference Laboratory in Aarhus, Denmark (Salih, personal communication). This strain was isolated from an outbreak of CCPP in a farm in Kikuyu. The role played by M. mycoides subsp. capri in CCPP in Kenya would therefore seem to be limited since, many outbreaks regularly occur throughout the country and M. mycoides subsp. capri has only been isolated once.

The reverse is true in some other countries. In Turkey, all the CCPP outbreaks are caused by M. mycoides subsp. capri (Cottew et al., 1969). Longley (1940; 1951) working in Nigeria and India found that CCPP was mainly caused by M. mycoides subsp. capri. This organism has also been isolated

in Australia from goats with pneumonia (Littlejohns and Cottew, 1977). Despite the apparent absence of M. mycoides subsp. capri in Kenya, the one strain already identified indicates that it is present in this country and may possibly have been overlooked since not all the outbreaks which occur are investigated.

A high proportion of the strains (17) isolated in the present work belonged to the F38 group. These findings agree with those of MacOwan and Minette (1976). MacOwan and Minette (1977) also reported that the F38 group caused most of the outbreaks they investigated. In that investigation 46 CCPP outbreaks were screened for Mycoplasma and it was found that the F38 group were the only Mycoplasma isolated. Their conclusion was that in Kenya CCPP is mainly caused by the F38 group of Mycoplasma. This author's view is in agreement with their conclusions since the majority of the strains isolated at the Veterinary Research Laboratory at Kabete belong to this group.

For a long time the F38 group was believed to be confined to Kenya. This belief arose from the fact that it had not been isolated in any other country. Harbi, El-Tahir, MacOwan and Nayil, (1981) however, isolated members of this group from goats in Sudan. It is

possible that the F38 strains they isolated originated from Kenya since there is free stock movement across the Kenya - Sudan border. MacMartin et al. (1981) observed that CCPP outbreaks in Eden were clinically and pathologically similar to those caused by the F38 group (Kaliner and MacOwan 1976). They suggested that the outbreaks could have been caused by the F38 group. It should be pointed out here that the agent causing CCPP in Eden has not speciated and could be any of the three species known to cause CCPP. In the present study a small proportion (4) of the strains were identified as M. mycoides subsp. mycoides (Y-goat). These findings were in agreement with those of MacOwan (1976a) who found that very few of the CCPP outbreaks in Kenya were caused by M. mycoides subsp. mycoides (Y-goat). However, in his study all the isolates were recovered from chronic CCPP cases whereas in the present study all the strains were isolated from acute CCPP cases. These results indicate that M. mycoides subsp. mycoides (Y-goat) can cause both acute and chronic CCPP. Acute pleuropneumonia has been induced experimentally with this organism (Rosendal, 1980). Ojo (1976) observed that the M. mycoides subsp. mycoides (Y-goat) he used was capable of causing severe pleuropneumonia. Bar-moshe and Rappaport (1979) also found that CCPP is caused by M. mycoides subsp. mycoides Y-goat in Israel.

Five out of the 33 strains were identified as M. ovipneumoniae. This was the first time that this mycoplasma was isolated in our laboratory. Three of the five M. ovipneumoniae strains were individually isolated from acute CCPP-like cases while the other two were simultaneously isolated from mixed infection which included the F38 group strains. M. ovipneumoniae is a well documented pathogen of sheep in which it causes interstitial pneumonia (Carmichael, St. George, Sullivan and Horsfal, 1972; Friis et al., 1976; Foggie, Johnes and Buxton, 1976). However, its pathogenicity in goats has not been documented so far. During the last few years, it has been isolated and with increasing frequency from goat pneumonia (Ali, 1977; Harbi et al., 1981; Masiga and Rurangirwa, 1979; Livingstone, 1979). The latter workers isolated it from an outbreak that resembled CCPP clinically and pathologically. In that outbreak, half of the herd involved died. There were no other mycoplasma isolated from the dead cases. This led Masiga and Rurangirwa (1979) to strongly suspect that M. ovipneumoniae was the causative agent of the outbreak. In the present study M. ovipneumoniae was isolated in pure culture from a CCPP-like disease on three different occasions. This pointed that it was the likely cause of the disease. Pathogenicity experiments with M. ovipneumoniae should however be carried out so as

to conclusively determine the exact role it plays in CCPP.

Mycoplasma arginini was also isolated for the first time in our laboratory. One third of the strains identified belonged to this species. M. arginini has been isolated from many animal species and human beings (Barile, Delgiudice, Carski, Gibbs and Morris, 1968). In goats, it has been isolated from the nose, eyes with keratoconjunctivitis, pneumonic lungs and most frequently from apparently normal mouth and oesophagus (Cottew, 1979; Alley, Quinland and Clarke, 1975). Foggie and Angus (1972) found no pathological effects when they introduced M. arginini into the nasal and tracheal cavities of specific pathogen free (SPF) lambs. In their experiment, the lambs were expected to succumb to infection since they did not have immunity to M. arginini. The results obtained indicated that M. arginini by itself was probably not pathogenic. However, experiments in which M. arginini and other Mycoplasma are concurrently inoculated into test animals to verify that M. arginini does not contribute to the severity of an infection have not been carried out. Experiments of this nature will be performed at a later date in our laboratory.

5.1. Cultural and biochemical reactions.

The cultural characteristics of the mycoplasma strains investigated were similar to those reported by MacOwan and Minette (1976), and Ernø et al. (1978). The F38 group strains grew slowly in the liquid and solid media used. For this reason it was necessary to subcultivate them several times before good growth was obtained. The F38 field strains produced small colonies which initially lacked the fried-egg appearance. However with subsequent cultivation the fried egg appearance became evident. Strain G 242/78 grew rapidly in both liquid and solid media. In agar its colonial morphology was characterized by a double peripheral zone as shown in Figure 1(a) and 1(b). This type of colonial morphology was not observed in any of the other strains investigated. A unique growth feature of the F38 group strains in agar was that the nipple was approximately twice the size of the peripheral zone.

The biochemical characteristics of all the type and field strains agreed with those reported by Ernø et al. (1978). The glucose fermentation reactions for the F38 group strains were similar to those reported by MacOwan (1976b). Stronger and faster reactions occurred in cultures incubated anaerobically. Cultures incubated anaerobically

normally showed reactions within two days of incubation whereas those incubated aerobically required four to five days before reactions could be observed. With strain G 242/78, there was no difference in reaction time between cultures incubated aerobically and anaerobically. The unique colonial morphology on agar and the rapid fermentation of glucose suggest that strain G 242/78 is possibly a biochemical variant of the F38 group.

The original F38 strain digested serum (MacOwan, 1976; Ernø *et al.*, 1979). In the present study many F38 group strains also digested serum. This finding agreed with those of MacOwan (1976) and Ernø *et al.* (1979). There were, however five F38 group strains which never digested serum. This was in agreement with the findings of Leach (1976) who observed that the F38 strain he studied did not digest serum. Ernø *et al.* (1979) suggested that the inability by the F38 strain Leach (1976) studied to digest serum could have arisen from media influences. In the present study attempts were made with different batches of media to find out whether the strains could digest serum. They were consistently unable to digest it. The results indicated that within the F38 group are some strains which do not digest serum. Caution should therefore be taken when such

strains are identified as members of this group and yet are unable to digest serum.

Tetrazolium was reduced almost exclusively under anaerobic conditions. The only exception observed was strain G242/78 which readily reduced it both aerobically and anaerobically. Except for this one strain which could reduce tetrazolium aerobically, it is reasonably safe to conclude that the F38 group reduce tetrazolium anaerobically.

Mannose, galactose and xylose were metabolized aerobically by the F38 group. Cellobiose and sorbitol were metabolized poorly by the majority of the 18 F38-like strains investigated. This author recommends that a positive xylose metabolism reaction when yeast extract is added to the medium, should be regarded as negative. This was because uninoculated control test media to which yeast extract was incorporated turned colour with time. For this test, media without yeast extract were found to be ideal since there were no changes in colour of uninoculated control media.

Mannose is widely metabolized by many Mycoplasma species. To cite but a few examples, M. pulmonis (Klieneberger, 1936), M. mycoides subsp. capri (El-Hasri, 1967; Hudson et al., 1967; Ernø and Stipkovits, 1973). There

was an indication that there is a correlation between the ability to ferment glucose and mannose. Most glucose fermenting Mycoplasma also metabolized mannose.

5.2. Serological tests.

Extensive cross reactions were observed between the F38 group and bovine serogroup 7 (PG 50) with respect to the growth inhibition and the indirect-epi-immunofluorescent tests. All the F38 group strains were inhibited by antiserum PG 50 and also fluoresced in the presence of antiserum against PG 50. This raised a problem with regard to the identification of the F38 group strains. Similar observations were noted by Ernø and Salih (unpublished observations). For this reason, additional tests were found necessary in order to conclusively identify these strains. In the present study the tetrazolium reduction test was used to differentiate the F38 group strains from PG 50. PG 50 reduces tetrazolium both aerobically and anaerobically while the F38 group almost exclusively reduced it anaerobically. Further serological tests employing the metabolic inhibition test will be attempted later to determine if this test is more specific than the growth inhibition and indirect-immunofluorescent tests.

Cross reactions between M. mycoides subsp. mycoides (Y-goat), M. mycoides subsp capri (PG3), M. capricolum bovine serogroup 7 (PG 50), the F38 group (commonly referred to as the serum digestion group) and M. mycoides subsp. mycoides (PG1) were demonstrated with the growth precipitation test. Ernø and Salih (1980) demonstrated the same cross reactions. All the above mentioned strains shared four common antigens. It was, however observed that with homologous antisera more precipitin lines formed than when heterologous antisera were used. A close relationship between the serum digestion group was therefore demonstrated with the growth precipitation test. Although these species share common antigens, serological tests such as the growth inhibition and the indirect immunofluorescent tests do not show any such cross reactions except that between F38 and PG 50 and also that between M. mycoides subsp. mycoides (PG1) and M. mycoides subsp. mycoides (Y-goat). For this reason the growth inhibition and the indirect immunofluorescent tests are used to conclusively identify most mycoplasma except where there are cross reactions.

5.3. Media studies.

It was possible to achieve good growth of the F38 group strains in medium B and Newings tryptose medium within three days of incubation. This indicated that the two media are capable of supporting luxurious growth of fastidious Mycoplasma. The F38 group have been cultivated in a medium called VFG (MacOwan, 1976b). This medium has a concentration of about 50 percent goat serum. It was MacOwan's belief that the F38 group grow well in media that have a concentration of approximately 50 per cent animal serum. However, the present study showed that Medium B and Newings tryptose which have 20 per cent concentration of animal serum are also capable of supporting growth of freshly isolated strains of the F38 group.

Exogenous deoxyribonucleic acid (DNA) was believed to be necessary for the growth of Mycoplasma in artificial media (Hayflick, 1969). Motty-Sabry and Atefa (1975) found that media to which DNA was incorporated gave better chances of isolating Mycoplasma from the genital tract of farm animals in contrast to media without DNA. In this study, Mycoplasma grew well in medium B to which exogenous DNA was incorporated and also in Newings tryptose medium but without DNA added to it. The fact that

there was no difference between growth of the strains in medium B and Newings tryptose indicated that endogenous nucleic acids provided by the serum and other ingredients used in preparing the medium provide all nucleic acid requirements for mycoplasma growing in it. This observation is also supported by the fact that MacOwan's VFG medium and medium FF 74 (Friis medium) do not have exogenous DNA and yet support good growth of many Mycoplasma species.

Medium B has been shown to support the growth of many Mycoplasma species of bovine and caprine origin (Ernø and Stipkovits, 1973; Ali, 1977; Ernø et al., 1978). Some Mycoplasma, for example, M. dispar and M. hyopneumoniae do not grow well in this medium (Ernø and Stipkovits, 1973). The two species require selective media such as G.S. medium and medium FF 74 for their growth. M. ovipneumoniae grew best under reduced oxygen tension in medium B (Ali, 1977). For this reason candle jars were recommended for incubating certain Mycoplasma when medium B is used to cultivate them. In the present study the F38 group strains investigated grew best in 8% CO_2. Some growth also occurred anaerobically but this was not as good as that obtained in 8% CO_2. This author therefore recommends that the F38 group be grown in medium B in an atmosphere of 8% CO_2.

Newings tryptose medium supported good growth of the F38 group strains studied under both anaerobic and 8% CO_2 atmospheres. There was not much difference in growth obtained in cultures incubated in the two atmospheres except that colonies from organisms incubated anaerobically were slightly larger than those incubated under 8% CO_2. MacOwan (1976b) found that the F38 strain he studied grew best under 5% CO_2. The present study confirms his findings except for the minor differences in the concentrations of CO_2 used.

It is not clear why the F38 group strains did not grow well in SP4 medium. The SP4 medium has been found to yield more isolations of M. pneumoniae from sputa specimens of patients with atypical pneumonia than when other media were used (Tully et al., 1979). The present study showed that though SP4 was satisfactory for the cultivation of M. pneumoniae, it was not ideal for growing the F38 group strains. Similar observations of media supporting good growth of only certain mycoplasma have been observed (Longley, 1951; Abdulla and Lindley, 1967; Ernø and Stipkovits, 1973; Friis, 1979). SP4 is a well balanced medium containing all the necessary growth requirements for mycoplasma. It

was therefore surprising to find that it poorly supported the growth of the F38 group strains. A possible explanation for this finding is that some Mycoplasma need to be subcultivated many times before they grow well in some laboratory media. With subsequent subcultivation of the F38 strains in the SP4 medium they may adapt to the medium and grow well. Investigations along this line are in progress in our laboratory.

The fact that medium B and Newings tryptose medium supported luxurious growth of the F38 strains opened doors to the possibility of using them for vaccine production. Since it was possible to achieve good growth within three days of incubation in solid medium, it should be possible to obtain even better growth in broth media. For this reason large volumes of culture could be grown in a short time, a condition which must be taken into consideration in vaccine production.

Based on the results of this study it is recommended that Newings tryptose medium be investigated further with the objective of using it for growing vaccine strains. Newings tryptose is a medium of very simple composition and the ingredients used in it are easily available.

5.4. Immunization.

The immunogenicity of mycoplasma membranes was investigated by Argaman and Razin (1969). They extracted protein antigens with sodium dodecyl sulphate (SDS) and tested them by immunodiffusion tests. The antigens had serological specificity similar to that of the soluble cell protein. In the same study they also found that a hydrophobic protein fraction isolated from A. laidlawii membranes obtained by detergent action and ammonium sulphate precipitation reacted with antiserum to the whole cell antigen. In rabbits it was immunogenic and induced the production of an antiserum which reacted with itself but not with the SDS solubilized protein and membranes.

In the present study, membrane extraction was achieved by digitonin lysis. The membrane proteins obtained were immunogenic in rabbits. When tested by the growth inhibition and indirect-epi-immunofluorescent tests, the membrane antisera were of lower titre than whole cell antisera. This indicated that intact cell antigens are superior to the membrane antigens for producing typing antisera. Further experiments need to be carried out to explore this point.

Studies on the specificity of the membrane antisera were not carried out in this study because it is the subject of another investigation which is in the planning stages in our laboratory. The objective of the study will be to determine if antisera to membranes of closely related Mycoplasma such as bovine serogroup 7 (PG.50) and the F38 group are monospecific. Production of monospecific antisera will solve the problem of cross reactions experienced with certain Mycoplasma species and will aid in the proper identification of closely related species.

5.6. The indirect haemagglutination test.

The indirect haemagglutination test (IHA) has been used in the serodiagnosis of Mycoplasma infections in cattle (Cho et al., 1975). The IHA test was found to detect more cases than the growth inhibition test. The above mentioned workers also found that when the IHA test was compared to the isolation of mycoplasma from the milk of infected cattle, the two procedures gave similar results. Animals which had IHA titres of 1:160 were found to be shedding mycoplasma in their milk. For this reason, Cho et al. (1975) recommended that when the IHA test is used to diagnose mycoplasma infections in cattle, titres of 1:160 and above should be regarded as positive. Titres of 1:80 should be regarded as suspicious and those lower than 1:80 be taken as unspecific or negative.

In the present study only one serum sample had a titre of 1:80 with A. laidlawii A (PG8) antigen. The rest of the sera gave lower titres and were regarded as negative. The method of Cho et al. (1975) was preferred in the present investigation because glutaraldehyde fixed erythrocytes coated with the antigens were easy to handle and the reagents required are easily available. Another advantage

with the use of this method is that the antigens required remain stable for a long time when stored at 4°C(Lam and Morton, 1974). Further, the fixation of commercially obtained defibrinated sheep blood cells eliminates the necessity for screening many sheep in order to obtain a suitable source of fresh erythrocytes. Glycerine which is added to the suspending fluid makes it possible to obtain a uniform suspension of fixed sensitized sheep erythrocytes even after repeated freezing and thawing. In contrast to the tedious process involving the use of a sonicated antigen for fixation as required when fresh tanned erythrocytes are used (Krogsgaard-Jensen, 1972), intact mycoplasma cell were used in this study. The sensitivity of the test was not affected at all.

CONCLUSIONS

1. This study established that M. mycoides subsp. capri did not cause any of the CCPP outbreaks from which the 34 Mycoplasma strains were isolated.

2. It confirmed the findings of MacOwan and Minette (1976 , 1977), that the F38 group is responsible for most CCPP outbreaks which occur in Kenya. This was verified by the fact that 17 of the field strains identified belonged to this group.

3. Other Mycoplasma namely, M. ovipneumoniae and M. arginini are present in goat pneumonia in Kenya. However, their role is not clearly understood.

4. It is not possible to distinguish the F38 group from the bovine serogroup 7 (PG50) using specific serological tests such as the growth inhibition and the indirect epi-immunofluorescence.

5. In this study the anaerobic reduction of tetrazolium was used to differentiate the two Mycoplasma, namely F38 and PG 50. The F38 generally reduced this reagent anaerobically while PG50 reduced it both aerobically and anaerobically.

6. The F38 group ferment glucose, mannose, galactose and xylose aerobically. They do not ferment cellobiose and sorbitol.

7. There are strains within the F38 group which do not digest serum.

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APPENDIX 1. GROWTH INHIBITION TESTS FOR CAPRINE MYCOPLASMA FROM KENYAN GOATS

Species/ serogroup and strain		ANTISERA						Medium	Temperature	
		PG1	Y-goat	G230	Calif. kid	Y-98	F38			
M. mycoides subsp. mycoides (LC)	30/76	3	2	-	-	-	-	B	27°C	T
M. mycoides subsp. mycoides (LC)	G290/79	4	5	-	-	-	-	B	27°C	T
M. mycoides subsp. mycoides (LC)	1915/80	10	6.5	-	-	-	-	B	27°C	T
M. arginini	G45/80	-	-	6	-	-	-	B	27+37°C	T
M. arginini	G115/80	-	-	4	-	-	-	B	27+37°C	T
M. arginini	G132/78	-	-	3	-	-	-	B	27+37°C	T
M. arginini	G268/78	-	-	6	-	-	-	B	27+37°C	T
M. arginini	G340B/80	-	-	4	-	-	-	B	27+37°C	T
M. arginini	1979/80	-	-	6	-	-	-	B	27+37°C	T
M. arginini	2001/80	-	-	4	-	-	-	B	27+37°C	T
M. arginini	2005/80	-	-	5	-	-	-	B	27+37°C	T
M. mycoides subsp. mycoides (LC)	G175/78	6	3	-	-	-	-	B	27°C	T
M. ovipneumoniae	G101/79	-	-	-	-	3	-	B	37°C	T
M. ovipneumoniae	G242/79	-	-	-	-	3.5	-	B	37°C	T
M. ovipneumoniae	G207/80	-	-	-	-	2	-	B	37°C	T

\- Indicates no inhibition.
B Indicates Modified Hayflick Medium.

Note: The figures are in mm.

APPENDIX 2. GROWTH INHIBITION TESTS FOR CAPRINE MYCOPLASMA FROM KENYAN GOATS

Species/ serogroup and strain		ANTISERA PG1	G230	Calif. kid	Y-98	F38	Medium	Temperature	
M. ovipneumoniae	1954/80	-	-	-	10	-	B	37°C	T
M. ovipneumoniae	2008/80	-	-	-	5	-	B	37°C	T
Mycoplasma Spec. F38	G75/76	-	-	-	-	5	B	37°C	T
Mycoplasma Spec. F38	G133/79	-	-	-	-	4	B	37°C	T
Mycoplasma Spec. F38	G176/79	-	-	-	-	6	B	37°C	T
Mycoplasma Spec. F38	G242/78	-	-	-	-	4	B	37°C	T
Mycoplasma Spec. F38	G272/80	-	-	-	-	8	B	37°C	T
Mycoplasma Spec. F38	G280/80	-	-	-	-	5	B	37°C	T
Mycoplasma Spec. F38	G281/80	-	-	-	-	5	B	37°C	T
Mycoplasma Spec. F38	G316A/80	-	-	-	-	6	B	37°C	T
Mycoplasma Spec. F38	G338/80	-	-	-	-	6	B	37°C	T
Mycoplasma Spec. F38	G368/78	-	-	-	-	6	B	37°C	T
Mycoplasma Spec. F38	630/76	-	-	-	-	8	B	37°C	T
Mycoplasma Spec. F38	904/76	-	-	-	-	6	B	37°C	T
Mycoplasma Spec. F38	1868/79	-	-	-	-	3	B	37°C	T
Mycoplasma Spec. F38	1943/80	-	-	-	-	10	B	37°C	T
Mycoplasma Spec. F38	1943/80	-	-	-	-	8	B	37°C	T
Mycoplasma Spec. F38	1954/80	-	-	-	-	7	B	37°C	T
Mycoplasma Spec. F38	2008/80	-	-	-	-	7	B	37°C	T

Note: The figures are in mm. - Indicates no inhibition..
T = Total inhibition.

APPENDIX 3. INDIRECT EPI-IMMUNOFLUORESCENT TESTS FOR CAPRINE MYCOPLASMA FROM KENYAN GOATS

Species/ serogroup and strain		ANTISERA						
		PG1	Y-goat	G230	Calif. kid	Y-98	F38	PG50
Mycoplasma Spec. F38	G75/76	-	-	-	-	-	+	+
Mycoplasma Spec. F38	F133/79	-	-	-	-	-	+	+
Mycoplasma Spec. F38	F176/79	-	-	-	-	-	+	+
Mycoplasma Spec. F38	G242/78	-	-	-	-	-	+	+
Mycoplasma Spec. F38	G272/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	G280/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	G281/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	G316A/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	G338/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	G368/78	-	-	-	-	-	+	+
Mycoplasma Spec. F39	630/76	-	-	-	-	-	+	+
Mycoplasma Spec. F38	904/76	-	-	-	-	-	+	+
Mycoplasma Spec. F38	1868/79	-	-	-	-	-	+	+
Mycoplasma Spec. F38	1943/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	1945/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	1954/80	-	-	-	-	-	+	+
Mycoplasma Spec. F38	2008/80	-	-	-	-	-	+	+

\+ Indicates positive reaction.
\- Indicates negative reaction.

APPENDIX 4. INDIRECT EPI-IMMUNOFLUORESCENT TESTS FOR CAPRINE MYCOPLASMA FROM KENYAN GOATS.

Species/ serogroup and strain		ANTISERA PG1	Y-goat	G230	Calif. kid	Y-98	F38	PG50
M. mycoides subsp. mycoides (LC)	30/76	+	+	-	-	-	-	-
M. mycoides subsp. mycoides (LC)	G290/79	+	+	-	-	-	-	-
M. mycoides subsp. mycoides (LC)	1915/80	+	+	-	-	-	-	-
M. arginini	G45/80	-	-	+	-	-	-	-
M. arginini	G115/80	-	-	+	-	-	-	-
M. arginini	G132/78	-	-	+	-	-	-	-
M. arginini	G268/78	-	-	+	-	-	-	-
M. arginini	G340B80	-	-	+	-	-	-	-
M. arginini	1979/80	-	-	+	-	-	-	-
M. arginini	2001/80	-	-	+	-	-	-	-
M. arginini	2005/80	-	-	+	-	-	-	-
M. mycoides subsp mycoides (LC)	G175/78	+	+	-	-	-	-	-
M. ovipneumoniae	G101/79	-	-	-	-	+	-	-
M. ovipneumoniae	G242/79	-	-	-	-	+	-	-
M. ovipneumoniae	G207/80	-	-	-	-	+	-	-
M. ovipneumoniae	1954/80	-	-	-	-	+	-	-
M. ovipneumoniae	2008/80	-	-	-	-	+	-	-

\+ Indicates positive reaction.
\- Indicates negative reaction.

APPENDIX 5. MYCOPLASMA ISOLATES STUDIED

Species/serogroup and strain		Locality specimen originated
Mycoplasma spec. F38	G75/76	Kabete
Mycoplasma spec. F38	G133/79	Kabete
Mycoplasma spec. F38	G/176/79	Kabete
Mycoplasma spec. F38	G242/78	Eldoret/Moiben
Mycoplasma spec. F38	G272/80	Nairobi
Mycoplasma spec. F38	G280/80	Rumuruti
Mycoplasma spec. F38	G281	Marigat
Mycoplasma spec. F38	G316A/80	Karatina
Mycoplasma spec. F38	G368/78	Eldama Ravine
Mycoplasma spec. F38	630/76	Kabete
Mycoplasma spec. F38	904/76	Kabete
Mycoplasma spec. F38	1868/79	Kabete
Mycoplasma spec. F38	1943/80	Kabete
Mycoplasma spec. F38	1945/80	Kabete
Mycoplasma spec. F38	1954/80	Kabete
Mycoplasma spec. F38	2008/80	Kabete
Mycoplasma arginini	G45/80	Mombasa
Mycoplasma arginini	G115/80	Kabete
Mycoplasma arginini	G132/78	Machakos
Mycoplasma arginini	G268/78	Kitui
Mycoplasma arginini	G340/80	Loitoktok
Mycoplasma arginini	1979/80	Kabete
Mycoplasma arginini	2001/80	Kabete
Mycoplasma arginini	2005/80	Kabete
Mycoplasma mycoides subsp. mycoides Y-goat	G175/78	Kajiado
Mycoplasma mycoides subsp. mycoides Y-goat	30/76	Kabete
Mycoplasma mycoides subsp. mycoides Y-goat	G290/79	Kabete
Mycoplasma mycoides subsp. mycoides Y-goat	1915/80	Kabete
Mycoplasma ovipneumoniae	G101/79	Nakuru
Mycoplasma ovipneumoniae	G242/79	Karatina
Mycoplasma ovipneumoniae	1954/80	Kabete
Mycoplasma ovipneumoniae	G207/80	Kitui
Mycoplasma ovipneumoniae	2008/80	Kabete

APPENDIX 6. NUMBER OF STRAINS FOR EACH MYCOPLASMA AND THEIR PERCENTAGE IN RELATION TO THE TOTAL NUMBER OF STRAINS IDENTIFIED.

Species	Number of strains isolated	Total number of strains identified	% of total
Mycoplasma species strain F38	17	34	50
Mycoplasma arginini	8	34	23.5
Mycoplasma ovipneumoniae	5	34	15.2
Mycoplasma mycoides var. *mycoides* (Y-goat)	4	34	11.3

APPENDIX 7. CAPRINE AND OVINE MYCOPLASMA

Species or serogroup and strain		Source.
M. agalactiae	PG2	Udder (sheep)
M. arginini	G230	Brain (Scrapie infected mouse)
M. mycoides subsp. *capri*	PG3	Pleural fluid (goat)
M. mycoides subsp. *mycoides*	Y-goat	Peritoneum (goat)
M. conjunctivae	HR5581	Eye (goat)
M. capricolum	California kid	Joint (goat)
M. ovipneumoniae	Y-98	Respiratory tract (sheep)
M. putrefaciens	KSI	Goat
Group 5	Goat 145	Joint (goat)
Group 6	Goat 189	Joint (goat)
Group 7	A1343	Lung (goat)
Group 11	2-D	Genital tract (sheep)
Acholeplasma oculi	19-L	Eye (goat).

Adapted from Ernø *et al.* (1978).

APPENDIX 8. FORMULAE OF GROWTH AND TEST MEDIA

A. GROWTH MEDIA

1. Medium B (Modified Hayflick Medium)

(a) Liquid Medium

Heart infusion broth (Difco)	90.0 ml
Horse serum (unheated)	20.0 ml
25% Yeast extract (Taylor Robinson et al., 1963)	10.0 ml
1% (w/v) Thallium acetate solution	1.0 ml
Penicillin (20,000 I.U. per ml)	0.25 ml
0.2% (w/v)[a] Deoxyribonucleic acid solution	0.2 ml

Adjust pH to 7.8 with 1.0N NaOH solution

Solid Medium

Prepare the corresponding solid medium by replacing heart infusion broth with heart infusion agar (Difco).

[a] From calf thymus type 1 catalogue No. 1501, Sigma Chemical Company, St. Louis, Missouri, U.S.A.

A2 MEDIUM N.

a) Liquid Medium

Bacto brain heart infusion (Difco)	3.7 g
Yeast extract (Difco)	0.5 g
Distilled water	100.0 ml

Steam and autoclave at 15 psi ($121^{o}C$) for 15 min

To the above add

Horse serum (unheated)	20.0 ml
[b] 25% (w/v) Yeast extract	10.0 ml
1% (w/v) Thallium acetate solution	1.0 ml
Penicillin (20,000 I.U per ml) solution	0.25 ml
[a] 0.2% (w/v) Deoxyribonucleic acid (DNA) solution	0.25 ml
50% (w/v) Glucose solution	2.0 ml

Adjust the pH to 7.8 with 1.0H NaOH

[a] From calf thymus type 1 catalogue no. 1501, Sigma Chemical Company, St. Louis, Missouri, U.S.A.

[b] Taylor-Robinson et al., 1963).

b) Solid Medium

Prepare by adding 1.4 g ion agar No. 2 (Code 2.2 Oxoid) to the Bacto-brain heart infusion (Difco) 3.7 g, yeast extract (Difco) 0.5 g in 100 ml distilled water autoclave and add the rest of ingredients listed above.

A3 NEWINGS TRYPTOSE MEDIUM

Stock

Tryptose (Difco)	2.0 g
Sodium chloride	0.5 g
Disodium hydrogen phosphate	0.25 g

Dissolve in 1000 ml distilled water and steam for 20 minutes. Adjust the pH to 8.0 with 1.0N NaOH Filter through a Seitz filter to remove excess phosphate.

Adjust the pH finally to 7.6 with 1.0N HCl.

Pour aliquots of 392 ml and add 1.5% Noble agar to be used for solid medium.

Autoclave at 15 psi (121°C) for 15 minutes.

A4 VIANDE FOIE GOAT MEDIUM (VFG).

a) Liquid Medium

Viande Foie base	95.0 ml
50% (w/v) Glucose solution	4.0 ml
10% (w/v) Yeast extract solution	2.0 ml
Glycerol	1.0 ml
Horse serum (inactivated)	20.0 ml
Penicillin (20,000 I.U per ml) solution	0.4 ml

Solid Medium

Viande Foie base	24.0 ml
50% (w/v) Glucose solution	5.0 ml
Glycerol	2.5 ml
Penicillin (20,000 I.U per ml) solution	10.0 ml

Place in water bath at 56°C

Prepare the agar component by adding 6 g Noble agar in 116 ml distilled water, autoclave at 15 PSI (121°C) for 15 minutes, remove and immediately mix with the broth. To this mixture add 150 ml of sterile heat inactivated horse serum. Prepare plates by pouring 7 ml volumes into petri dishes.

A5 RABBIT MEDIUM

Medium RT

Rabbit meat infusion	100.0 ml
Rabbit serum	10.0 ml
Yeast extract (Taylor-Robinson et al., 1963)	10.0 ml
0.2% (w/v)[a] Deoxyribonucleic acid solution	1.0 ml
Penicillin (20,000 I.U per ml) solution	0.25 ml
10% (w/v) Thallium acetate solution	1.0 ml

Adjust the pH to 7.8 with 1.0N NaOH.

[a] From calf thymus type 1 - catalogue No. 1501, Sigma Chemical Company, St. Louis, Missouri, U.S.A.

A6 MEDIUM FF74 (FRIIS MEDIUM)

a) Liquid Medium

Brain - heart infusion broth (Difco)	0.82 g
Bacto PPLO Broth MO CV (Difco)	0.75 g
Dissolve in double distilled water	75.0 ml
Then add	
Sterile Modified Hanks Balanced Salt (HSS)	50.0 ml
Fresh yeast extract (FG)	6.0 ml
Horse serum (inactivated)	15.0 ml
Swine serum (inactivated)	15.0 ml
0.06% (w/v) Phenol red solution	5.0 ml
0.5% (w/v) Bacitracin solution	1.0 ml
2.5% (w/v) Methicillin solution	1.0 ml

b) Solid Medium

Agar - agar (Merck ort 1612)	1.3 g
DEAE dextran	0.017 g
Modified Hanks Balanced Salt Solution	16.0 ml

Sterilize by autoclaving at 121°C for 5 min

Cool to 56°C solution and add one portion of liquid FF74.

c) Modified Hanks Balanced Salt Solution (HBSS)

Prepare from stock solution A and B

Solution A	NaCl	80.0 g
	KCl	4.0 g
	$MgSO_4$	1.0 g
	$(MgCl_2)$ $6H_2O$	1.0 g

Dissolve in 40.0 ml water. Add 1.4 $CaCl_2$ and add 500.0 ml distilled water.

Solution B	$Na\ HPO_4 . 12H_2O$	1.5 g

Dissolve in 400 ml distilled water

Mix with another 500 ml distilled water containing 0.6 g KH_2PO_4.

Make HBSS by adding 25 ml solution A to 400 ml distilled water and mix with 25 ml of solution B in 50 ml distilled water.

Yeast Extract (FG)

Fleishman pure dry yeast type 2040	125 g
Double distilled water	750 ml

Heat the suspension to 37°C for 20 minutes thereafter heat to 90 - 100°C for 5 minutes before using.

A7 SP4 MEDIUM

BASE

Mycoplasma broth base (BBL)	3.5 g
Tryptone (Difco)	10.0 g
Peptone (Difco)	5.3 g
Noble agar for plates	10.0 g
OS/ On Distilled water	621.0 ml

Autoclave 15 min at 15 psi (121°C)

Cool to room temperature before supplementing (56°C for agar)

Supplement.

To 621 ml base, add:

Ampicillin Na (100mg/ml)	10.0 ml
50% Glucose solution	10.0 ml
0.5% Phenol Red Solution (GIBCO)	4.0 ml
25% Yeast extract solution (GIBCO)	35.0 ml
10 x CMRL 1066, w/g W/O $NaHCO_3$	50.0 ml
2% Yeastolate solution (Difco)	
Fetal calf serum (Inactivated IHR @ 56°C)	170.0 ml
2% Thallous acetate solution	25.0 ml

Adjust to the pH to 7.4 with 1.0N NaOH.

B MEDIA FOR BIOCHEMICAL TESTS

1. a) Glucose Fermentation

Basal medium (medium b_1) heart infusion broth (Enzyme treated)	120.0 ml
PPLO Serum fraction (Difco)0.2% (w/v)	
Deoxyribonucleic acid solution	1.2 ml
0.06% (w/v) Phenol red solution	5.0 ml
1% (w/v) Thallium acetate solution	1.0 ml
Penicillin (20,000 I.U per ml) solution	0.25 ml

b) Test Medium bg

Medium b_1	128.0 ml
5% (w/v) Glucose solution	1.6 ml

Adjust the pH to 7.8 with 1.0N NaOH

2. a) Arginine Hydrolysis

Basal medium Medium b_2 is the same as b_1

Adjust the pH to 7.3 with 1.0N HCl.

b) Test Medium ba

Medium b_2	128.0 ml
30% (w/v) L-arginine solution	4.25 ml

Adjust the pH to 7.3 with 1.0H HCl.

3. Phosphatase activity

Test Medium Medium BpH.

Heart infusion agar (Difco)	74.0 ml
Sterilize by autoclaving	
Add Horse serum heated to 60°C for 60 minutes	20.0 ml
Then Add	
25% (w/v) Yeast extract (Taylor-Robinson et al., 1965)	5.0 ml
1% (w/v) Sodium phenolphthalein diphosphate solution	1.0 ml
Penicillin (20,000 I.U per ml) solution	0.2 ml
1% (w/v) Thallium acetate solution	1.0 ml

4. Proteolytic activity (Digestion of Serum)

Test Medium Medium Sd.

Heart infusion agar (Difco)	8.0 ml
Sterilize by autoclaving	
Add Horse serum	30.0 ml
25% (w/v) Yeast extract	0.8 ml
Sterile distilled water	1.2 ml

Adjust the pH to 7.8 with 1.0N NaOH

Dispense in screw cup tubes and heat in a slanted position in flowing steam for 5 minutes.

5. Tetrazolium reduction Medium RT

Heart infusion broth (Difco)	90.0 ml
Horse serum (heated)	20.0 ml
Yeast extract	10.0 ml
0.2% (w/v) DNA solution	1.2 ml
1% (w/v) 2, 3, 5-triphenyltetrazolium chloride solution	5.0 ml
1% (w/v) Thallium acetate solution	1.0 ml

Penicillin (20,000 I.U per ml) solution

Adjust the pH to 7.8 with 1.0N NaOH.

APPENDIX 9. FORMULAE FOR BUFFERES 0.01 M PHOSPHATE BUFFERED SALINE.

NaCl	8.5 g
KH_2PO_4	0.17 g
Na_2HPO_4	0.72 g

Dissolve in distilled water 1000.0 ml.

Adjust the pH to 7.2 with 1.0N HCl

REAGENTS FOR GLUTARALDEHYDE FIXATION

PHOSPHATE BUFFERED GLUCOSE (PBG)

0.15 M Na_2HPO_4 solution	76.0 ml
0.15 M $KHPO_4$ solution	24.0 ml
5.4% (w/v) Glucose in distilled water	100.0 ml

OTHER REAGENTS

0.15 NaCl solution	100.0 ml
60% Glycerin in PBS	100.0 ml
0.5% (w/v) NaCl solution	100.0 ml
0.2% (w/v) Glutaraldehyde	100.0 ml