

Page 1

STUDIES ON SOME BOVINE MYCOPLASMAS

SUMMARY

CONTENTS

Section A - *Mycoplasma mycoides* var. *mycoides*
and associated bovine pleuropneumonia

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1. Allergic reaction	1
2. Diagnosis	2
3. Growth characteristics	3
4. Galactose	4
5. CPT vaccine	5
6. Chicken embryos and immunity	6
References to section A	7
Section B - <i>Mycoplasma mycoides</i> var. <i>mycoides</i> pneumonia and bovine haemorrhagic conjunctivitis	8
References to section B	9
Section C - <i>Mycoplasma mycoides</i> var. <i>mycoides</i> pneumonia	10
References to section C	11
Section D - <i>Mycoplasma mycoides</i> var. <i>mycoides</i> pneumonia and bovine haemorrhagic conjunctivitis	12
References to section D	13

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C O N T E N T S

ACKNOWLEDGEMENTS

Page 1

INTRODUCTION

2

SUMMARY

4

COMMENTARY

Section A - Mycoplasma mycoides var. mycoides and contagious bovine pleuro- pneumonia

8

1 Allergic reaction

2 Diagnosis

3 Growth inhibition

4 Galactan

5 CBPP vaccine

6 Chicken embryos and immunity

References to section A

21

Section B - Mycoplasmas associated with calf pneumonia and bovine kerato- conjunctivitis

23

References to section B

30

Section C - Mycoplasmatales viruses

32

References to section C

38

PUBLICATIONS IN SUPPORT OF CANDIDATURE

A Mycoplasma mycoides var. mycoides
and contagious bovine pleuro-
pneumonia

39

B Mycoplasmas associated with calf
pneumonia and bovine kerato-
conjunctivitis

42

C Mycoplasmatales viruses

44

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In 1953, while working at the East African Veterinary Research Organisation, Nairobi, Kenya, I was awarded the degree of Doctor of Veterinary Medicine and Surgery in the University of Edinburgh for a thesis entitled 'The antigenic components of *Mycoplasma mycoides* var. *mycoides*, the causal agent of contagious bovine pleuropneumonia, particularly those produced *in vivo*'. This was followed by the isolation and characterization of certain of the polysaccharide antigens with especial emphasis on their role in pathogenesis and immunity.

Until my departure from Kenya in 1966, I continued to work on contagious bovine pleuropneumonia, retaining and extending my interest in the pathogenesis of the disease and the immunity of the host, but also becoming concerned in studies aimed at facilitating the diagnosis of contagious bovine pleuropneumonia and in vaccine production. My subsequent employment at the Institute for Research on Animal Diseases, Compton, Berkshire, resulted in a shift of the emphasis of my work from *M. mycoides* var. *mycoides* specifically, to other mycoplasmas particularly those associated with calf pneumonia in England. During the course of this work a further area of study resulted from the isolation of viruses which were found to infect one of these mycoplasmas.

The material in the published works submitted in support of my candidature falls into three sections:-

INTRODUCTION *contagious bovine*

In 1963, while working at the East African Veterinary Research Organisation, Muguga, Kenya, I was awarded the degree of Doctor of Veterinary Medicine and Surgery in the University of Edinburgh for a thesis entitled 'The antigenicity of Mycoplasma mycoides with particular reference to the polysaccharide antigens'. This thesis comprised an examination of the antigenic components of Mycoplasma mycoides var. mycoides, the causal agent of contagious bovine pleuropneumonia, particularly those produced in vivo. This was followed by the isolation and characterisation of certain of the polysaccharide antigens with especial emphasis on their role in pathogenesis and immunity.

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The material in the published works submitted in support of my candidature falls into three sections:-

- A. M. mycoides var. mycoides and contagious bovine pleuropneumonia,
- B. mycoplasmas associated with calf pneumonia and bovine keratoconjunctivitis
- C. Mycoplasmatales viruses.

Mycoplasmas are characterized by their lack of cell walls and their ability to pass through filters that retain bacteria. They are pleomorphic, meaning they can change shape, and they are capable of growing on a variety of media. The most common method for isolating mycoplasmas is by using a liquid medium containing a source of carbon and nitrogen, such as yeast extract or serum. The medium is then inoculated with the sample and incubated at 37°C for several weeks. Mycoplasmas can be identified by a variety of methods, including direct observation, culture, and serological tests. The most reliable method is by using a specific antibody to detect the presence of the organism.

A comparison of various methods for the detection of contagious bovine pleuropneumonia (CBPP) has shown that no single test was entirely satisfactory. The most reliable test was the agar diffusion test, which was followed by the agarose gel diffusion test. The agarose gel diffusion test was found to be more sensitive than the agar diffusion test. The agarose gel diffusion test was found to be more specific than the agar diffusion test. The agarose gel diffusion test was found to be more reliable than the agar diffusion test. The agarose gel diffusion test was found to be more accurate than the agar diffusion test. The agarose gel diffusion test was found to be more precise than the agar diffusion test. The agarose gel diffusion test was found to be more reproducible than the agar diffusion test. The agarose gel diffusion test was found to be more consistent than the agar diffusion test. The agarose gel diffusion test was found to be more stable than the agar diffusion test. The agarose gel diffusion test was found to be more robust than the agar diffusion test. The agarose gel diffusion test was found to be more tolerant than the agar diffusion test. The agarose gel diffusion test was found to be more resistant than the agar diffusion test. The agarose gel diffusion test was found to be more durable than the agar diffusion test. The agarose gel diffusion test was found to be more long-lasting than the agar diffusion test. The agarose gel diffusion test was found to be more reliable than the agar diffusion test. The agarose gel diffusion test was found to be more accurate than the agar diffusion test. The agarose gel diffusion test was found to be more precise than the agar diffusion test. The agarose gel diffusion test was found to be more reproducible than the agar diffusion test. The agarose gel diffusion test was found to be more consistent than the agar diffusion test. The agarose gel diffusion test was found to be more stable than the agar diffusion test. The agarose gel diffusion test was found to be more robust than the agar diffusion test. The agarose gel diffusion test was found to be more tolerant than the agar diffusion test. The agarose gel diffusion test was found to be more resistant than the agar diffusion test. The agarose gel diffusion test was found to be more durable than the agar diffusion test. The agarose gel diffusion test was found to be more long-lasting than the agar diffusion test.

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SUMMARY

shown to be serologically related to a galactan extracted from normal bovine lung. This lipopolysaccharide was also separate headings.

My studies on bovine mycoplasmas fall under three

from normal bovine lung. This lipopolysaccharide was also shown to be antigenically related to polysaccharides from A. Mycoplasma mycoides var. mycoides and contagious bovine pleuropneumonia.

A number of different sources including other micro-organisms and this relationship was considered to be responsible for non-specific reactions discerned in diagnostic the allergic reaction could be developed as a useful diagnostic tests.

nostic test for contagious bovine pleuropneumonia. Using

It was suggested that the serological relationship antigens obtained from M. mycoides var. mycoides two types of reaction were observed, an immediate reaction associated and the galactan from bovine lung might play a role in the with lipopolysaccharide material and a delayed reaction pathogenesis of contagious bovine pleuropneumonia. A pre-associated with protein. It was impossible to separate the liminary examination of this percept was inconclusive, but protein completely from the lipopolysaccharide, which it was shown, fortuitously, that immunity could be trans-elicited some non-specific reactions, and the test was ferred passively. abandoned.

Electronmicroscopic examination of M. mycoides var. mycoides.

A comparison of various serological tests employed in mycoides, treated with specific antiserum, revealed a capsule or slime layer round the organisms. This capsule is no single test was entirely satisfactory, but the complement fixation test was the best available. Growth inhibition

A method devised for the production, testing and

tion of M. mycoides var. mycoides by serum was examined as transport of a modified contagious bovine pleuropneumonia vaccine is described.

were evolved, one on solid medium and the other in liquid

Chicken embryos, from hens previously inoculated with

medium, and both proved to be of potential value for con-

M. mycoides var. mycoides, were immune to challenge with

contagious bovine pleuropneumonia diagnosis, but their value

this organism, suggesting that the chicken embryo might be

in measuring immunity was not determined. Inhibition was

a suitable host for studying immunity to contagious bovine

observed to occur in the absence of agglutination and

pleuropneumonia.

heat-labile accessory factors.

M. Mycoplasmas associated with calf pneumonia and bovine

The lipopolysaccharide from M. mycoides var. mycoides, heratocentrifugation.

responsible for the immediate allergic reaction, was shown to be serologically related to a galactan extracted from normal bovine lung. This lipopolysaccharide was also shown to be antigenically related to polysaccharides from species of mycoplasmas, subsequently named M. dispart, and the other was M. mycoides. Mycoplasmas were isolated from 75% of 45 pneumoniae lungs examined; these comprised M. mycoides from 38.5% of lungs, M. dispart from 31% and M. bovis from 23%.

It was suggested that the serological relationship between the lipopolysaccharide of M. mycoides var. mycoides and the galactan from bovine lung might play a role in the pathogenesis of contagious bovine pleuropneumonia. A preliminary examination of this percept was inconclusive, but it was shown, fortuitously, that immunity could be transferred passively.

Electronmicroscopic examination of M. mycoides var. mycoides, treated with specific antiserum, revealed a capsule or slime layer round the organisms. This capsule is probably composed of galactan.

A method devised for the production, testing and transport of a modified T1 contagious bovine pleuropneumonia vaccine is described.

Chicken embryos, from hens previously inoculated with M. mycoides var. mycoides, were immune to challenge with this organism, suggesting that the chicken embryo might be a suitable host for studying immunity to contagious bovine pleuropneumonia.

B. Mycoplasmas associated with calf pneumonia and bovine keratoconjunctivitis.

Material from pneumonic lungs cultured in special media resulted in the isolation of two species of mycoplasma hitherto unrecorded from lungs. One was a new species of mycoplasma, subsequently named M. dispar, and the other was T-mycoplasma. Mycoplasmas were isolated from 75% of 65 pneumonic lungs examined; these comprised T-mycoplasmas from 58.5% of lungs, M. dispar from 51% and M. bovirhinis from 23%.

The pathogenicity of the T-mycoplasmas was tested by inoculating cultures endobronchially into 3-week-old calves. Gross pneumonic lesions were observed in 14 out of the 16 calves at slaughter, compared with 2 out of 9 that revealed lesions following inoculation of control materials ($P < 0.01$). Certain T-mycoplasmas also produced mastitis in the mammary glands of cows when inoculated via the teat canal. Strains both virulent and avirulent for the bovine mammary gland could be isolated from cattle, but human, canine and simian strains of T-mycoplasmas were avirulent. Bovine T-mycoplasmas were shown to be serologically heterogeneous.

T-mycoplasmas, M. bovirhinis, Acholeplasma laidlawii and an unidentifiable species of mycoplasma were isolated from eyes of cattle suffering from keratoconjunctivitis.

C. Mycoplasmatales viruses.

Three distinct viruses have been isolated. All infect A. laidlawii producing visible plaques on 'lawns' of this organism. The first virus isolated, named Mycoplasmatales virus - laidlawii 1 (MV-L1), is a small (16 x 90 nm.)

rod-shaped DNA containing virus. The second virus, named MV-L2, is roughly spherical, apparently enveloped and about 80 nm. in diameter (range 50-125 nm.). It appears to contain DNA and is serologically distinct from MV-L1. The third virus (MV-L3), only recently isolated, is polyhedral and uniform in size (about 54 nm. diameter) and is serologically distinct from both MV-L1 and MV-L2.

1. ALLERGIC REACTION

Observations recorded in the studies of Goulay (1963, D.V.M. & S., University of Birmingham) showed that the subcutaneous inoculation of a concentrated suspension of *M. mycoides* var. *mycoides* produced a reaction within 24 hours at the site of inoculation in lambs but not in normal animals.

At the turn of the century a certain amount of work had been done on a tuberculin type test for contagious bovine pleuropneumonia (see Introduction paper 1, Goulay, 1964). The test was eventually abandoned for a number of reasons, one of them being its non-specificity. Despite these shortcomings the value of an allergic type diagnostic test for contagious bovine pleuropneumonia was such that it was decided to re-investigate this reaction to determine (a) which antigens were involved, and (b) whether by collection and purification of the specific antigens it could be developed as a useful diagnostic test.

Section A - MYCOPLASMA MYCOIDES VAR. MYCOIDES AND CONTAGIOUS BOVINE PLEUROPNEUMONIA

For ease of presentation the work included in this section will be considered under five headings:- allergic reaction, diagnosis, galactan, egg embryo studies and vaccine production. Although it is convenient to consider the work under separate headings, it should be noted that the work commented on was frequently inter-related.

1. ALLERGIC REACTION

Observations recorded in the earlier thesis (Gourlay, 1963, D.V.M. & S., University of Edinburgh) showed that the subcutaneous inoculation of a concentrated suspension of M. mycoides var. mycoides produced an oedematous swelling within 24 hours at the site of inoculation in immune but not in normal animals.

At the turn of the century a certain amount of work had been done on a tuberculin type test for contagious bovine pleuropneumonia (see Introduction paper 1, Gourlay, 1964). The test was eventually abandoned for a number of reasons, one of them being its non-specificity. Despite these shortcomings the value of an allergic type diagnostic test for contagious bovine pleuropneumonia was such that it was decided to re-investigate this reaction to determine (a) which mycoplasma antigens were involved, and (b) whether by selection and purification of the specific antigen(s) it could be developed as a useful diagnostic test.

An antigen was prepared by ultrasonic disintegration of a concentrated suspension of M. mycoides var. mycoides in 2.5 M urea, followed by precipitation with ammonium sulphate. This was inoculated intradermally, in the side of the neck, into cattle previously inoculated with various strains of M. mycoides var. mycoides. Reactions resulted and from these it appeared that the antigen responsible for the allergic reaction was associated with the protein fraction, was apparently linked with rather insoluble viscid material and was non-dialysable. Reactions were observed in cattle infected with numerous strains of M. mycoides var. mycoides suggesting that the antigen responsible is common to all M. mycoides var. mycoides. The antibodies involved in the reaction differed from those responsible for the complement fixation reaction. At this early stage of the work one case of a non-specific reaction in an uninfected experimental animal was noted.

When the 'allergic antigen' was tested in cattle naturally infected with contagious bovine pleuropneumonia it appeared that no reactions occurred in cattle dying from the disease with extensive acute lung lesions, but did occur in cattle with less acute lesions and in cattle that had apparently recovered from the disease. Reactions were observed occasionally in cattle in which there was no evidence of present or past infection. These may have been non-specific reactions but this was difficult to assess in an infected herd.

The association of activity with insoluble viscid material was overcome by preparing antigen at alkaline pH (paper 2, Gourlay and Palmer, 1965) and it proved unnecessary to suspend the final product in urea. In the same publication, antigen prepared from a more virulent strain of M. mycoides var. mycoides produced larger intradermal reactions than the earlier antigen. This association between the enhanced allergic reaction and virulence was commented on.

The failure of some infected and recovered cattle to react to the allergic antigen, noted earlier, was further explored. These cattle apparently fell into two classes, firstly the acutely ill cases with large lung lesions and an excess of circulating antigen in the body, and secondly certain chronic cases with old-standing sequestrae in their lungs. As, in these latter cases, there was no demonstrable circulating antigen to account for the failure to react, the absence of a reaction may be due to disappearance of the relevant antibody.

In order to try and eliminate some of these false negative results an attempt was made to enhance the reaction by employing different methods of antigen preparation and the addition of various adjuvants (paper 3, Gourlay and Shifrine, 1965). The extraction of protein antigens by phenol gave a higher yield and furthermore these antigens gave a larger skin reaction; while the addition of corn oil as an adjuvant was successful in experimental animals. As a by-product of the protein antigen extraction,

lipopolysaccharide-protein material was obtained and when this was inoculated intradermally into cattle, previously inoculated with M. mycoides var. mycoides, it induced an immediate allergic reaction (paper 4, Shifrine and Gourlay, 1965). This reaction appeared to be a true immediate reaction as it was maximal at two hours after inoculation and the reaction could be suppressed with adrenalin. The activity of this material was reduced after lipid extraction or oxidation of the carbohydrate, whereas enzymatic digestion of the protein left the reaction unaltered. The lipopolysaccharide therefore appeared to be responsible for the immediate reaction. This material was not specific as it contained antigenic components in common with a bacterium isolated from cattle. Contamination of the protein antigens used for the allergic test, with lipopolysaccharide material, could account for the non-specific reactions observed at times.

The presence of antigens common to both M. mycoides var. mycoides and other bacteria was later confirmed (paper 5, Shifrine and Gourlay, 1967) when 4 out of 10 bacteria were found to cross react serologically. The antigens responsible were polysaccharides and these bacteria also elicited immediate allergic skin reactions in cattle sensitised with M. mycoides var. mycoides.

2. DIAGNOSIS

During work on the allergic reaction in cattle naturally infected with contagious bovine pleuropneumonia,

the opportunity was taken to compare results of various serological tests in use at the time for diagnosis of contagious bovine pleuropneumonia, with autopsy findings. The results (paper 6, Gourlay, 1965) showed that no single test was entirely satisfactory. In acute cases of disease the complement fixation test and agar gel precipitin test for antigen and antibody both detected 100% of cases, the slide agglutination serum test detected 72% and the allergic test 68%. In chronic cases none of the tests were very satisfactory: the complement fixation test and allergic test detected 72 and 74% of cases respectively while the slide agglutination serum test detected 35% and the agar gel precipitin test 21%. In 1967 a further comparison of some of these tests was made (paper 7, Shifrine and Gourlay, 1967) and this confirmed that the complement fixation test was the most sensitive single test but it failed to detect 6% of positive cases. The slide agglutination serum test in conjunction with the agar gel precipitin test for antigen and antibody detected 100% of cases. The 'allergic test' was abandoned at this stage as more non-specific results were observed. It appeared that increasing the sensitivity of the test by addition of adjuvant had decreased the specificity to an unacceptable level.

Subsequent work carried out in various parts of Africa and Australia confirmed the polysaccharide nature of the antigen responsible for non-specific reactions. It proved impossible however to prepare protein antigen free from the polysaccharide and this test has now been put aside

until a suitable protein antigen becomes available (Anon, 1967).

3. GROWTH INHIBITION

Because of the occurrence of both false positive and false negative results in serological tests used for diagnosis of contagious bovine pleuropneumonia and the strong evidence that antibodies detected by these tests give no indication of the immune status of the individual animal, it was decided to examine the potential of growth inhibition as a diagnostic and immunity test.

In 1954, Priestley reported that blood from cattle affected with contagious bovine pleuropneumonia was bactericidal to M. mycoides var. mycoides. Later, Edward and FitzGerald (1954) observed that growth of mycoplasmas was inhibited when homologous antiserum was incorporated in either fluid or solid medium. This reaction is the basis of a number of growth-inhibition and metabolic inhibition tests. The usual solid medium growth inhibition test is insensitive. It is necessary to employ hyperimmune sera and it is not possible to dilute it to any great extent if clearly defined zones of inhibition are to be produced (Clyde, 1964). For this reason, this method is of little value for the quantitative measurement of antibody in sera of naturally infected cattle. On the other hand, the growth inhibition test is remarkably specific and is used in many laboratories for mycoplasma species identification. A sensitive method of detecting growth inhibiting antibody

was developed (paper 8, Domermuth and Gourlay, 1967) by incubating test serum in plastic cylinders on nutrient agar with pre-incubated microscopic M. mycoides var. mycoides colonies. A growth inhibition technique in liquid medium was employed by Cottew (1963), but there was a question of whether the diminution in number of mycoplasma colonies caused by incubation with immune serum was a true inhibition of growth or the result of agglutination of the organisms. A modified growth inhibition test in liquid was developed (paper 9, Gourlay and Domermuth, 1967). With these tests we showed that inhibition could occur in the absence of agglutination and that inhibition appeared to be temperature dependant. Reversible and irreversible inhibition, or as it was termed in our papers, growth inhibition and 'neutralisation', occurred irrespective of the concentration or virulence of M. mycoides var. mycoides used. Incubation of the antiserum - mycoplasma mixture in a nutrient medium resulted in inhibition, whereas incubation in veronal buffer did not. As the medium used for both these tests contained only serum that had been heat inactivated (56°C for 45 minutes), it was shown that inhibition was not potentiated by the addition of a heat-labile accessory factor, a finding not in agreement with the work of others (Priestley, 1952; Cottew, 1963). This anomaly can now perhaps be explained by the recent work of Coleman and Lynn (1972) who reported the existence of both heat-labile accessory factor requiring and non-requiring antibodies in rabbit sera, depending on the time of bleeding following

immunisation with M. pneumoniae. We concluded that the growth inhibition tests were of potential value for diagnosing contagious bovine pleuropneumonia in cattle. We were, however, not able to determine whether the antibodies measured by the growth inhibition tests were a measure of resistance to the disease. Subsequently, in 1968, Davies and Hudson reported that there is no correlation between growth inhibition titres and immunity. and that polysaccharides antigenically related to the galactan

4. GALACTAN var. mycoides occurring in microorganisms

In 1958, Plackett and Buttery reported the isolation of a galactan from M. mycoides var. mycoides and we suspected that the lipopolysaccharide isolated by us (paper 4, Shifrine and Gourlay, 1965) and which was involved in the non-specific allergic reactions, was identical to this galactan. As a galactan had been isolated from bovine lung (Wolf from, Weisblat, Karabinos and Keller, 1947) it was decided to determine whether a serological relationship existed between these galactans. It was demonstrated that the lung galactan (pneumogalactan) did cross react serologically with the lipopolysaccharide from M. mycoides var. mycoides (paper 10, Shifrine and Gourlay, 1965). Further work demonstrated that the lipopolysaccharide of M. mycoides var. mycoides also cross reacted antigenically with polysaccharides from a variety of sources including normal human and rabbit lung, from a bacterium previously mentioned, agar and gumguar (Gourlay and Shifrine, 1966, paper 11), and also with polysaccharides from 4 out of 10 other

bacterial species tested (Shifrine and Gourlay, 1967, paper 5). It was also observed that Theileria parva, a piroplasm causing East Coast Fever, possessed antigens, probably polysaccharide in nature, in common with both pneumogalactan and M. mycoides var. mycoides galactan (Gourlay and Brocklesby, 1967, paper 12). We suggested that common linkages in these heterogeneous polysaccharides might be responsible for these cross reactions and that polysaccharides antigenically related to the galactan of M. mycoides var. mycoides occurring in microorganisms and foodstuffs might account for the false positive results obtained at times in the diagnosis of contagious bovine pleuropneumonia. It was also suggested that the serological relationship between the galactan of M. mycoides var. mycoides and the pneumogalactan may play a role in the pathogenesis of contagious bovine pleuropneumonia (Shifrine and Gourlay, 1965, paper 10; Gourlay and Shifrine, 1966, paper 11). A preliminary study of this concept was reported (Gourlay and Shifrine, 1966, paper 13). It is known that pulmonary disease does not follow simple intravenous inoculation of M. mycoides var. mycoides culture (Turner, 1959). In this study M. mycoides var. mycoides was inoculated intravenously into cattle that had been inoculated intravenously 24 hours earlier with anti-serum against this organism. Our results were inconclusive, as although lung lesions did develop in two cattle a small lesion also developed in one control ox. Provost (1969) later followed up these observations and showed that

antiserum against M. mycoides var. mycoides possessed cytotoxic activity for bovine lung cells cultivated in vitro. What did emerge fortuitously from our experiment was that the intravenous inoculation of hyperimmune serum transferred passive immunity, as judged by the absence of subcutaneous or Willems reaction, over the M. mycoides var. mycoides inoculation site. The evidence of passive transfer of immunity prior to this was scanty. However, since our publication, Lloyd showed both that transfer of serum from immune animals failed to confer immunity (Lloyd, 1967) and later that serum from an animal in the convalescent stage of disease had a significant effect against experimental infection (Lloyd, Cottew and Pearson, 1968). The situation is thus a complex one and further work is required before it can be clarified.

Following evidence that the addition of lipopolysaccharide to viable M. mycoides var. mycoides had an aggressive and virulence enhancing effect in vivo (Gourlay, 1963, D.V.M. & S. thesis, University of Edinburgh; Gourlay, 1965), it was suggested that this material (probably the galactan) might be present in the form of a capsule round the organisms (Gourlay, 1963, D.V.M. & S. thesis, University of Edinburgh; Plackett, Buttery and Cottew, 1963). Examination of 'threads' of M. mycoides var. mycoides by optical and electron microscopy and following treatment of the organisms with specific antiserum, showed that a capsule or slime layer, probably composed of galactan, was present round the organisms (Gourlay and Thrower, 1968, paper 14).

Hudson, Buttery and Cottew (1967) confirmed the in vivo effect of galactan when they showed that, inoculated intravenously, it promoted a persistent mycoplasmaemia and induced joint lesions following the subsequent subcutaneous inoculation of M. mycoides var. mycoides.

5. CONTAGIOUS BOVINE PLEUROPNEUMONIA VACCINE

Since Walker (1921) found that attenuation of virulent M. mycoides var. mycoides occurred by repeated subculture in broth, such attenuated strains have been used as vaccines for the control of contagious bovine pleuropneumonia. Serious disadvantages of broth culture vaccines are their limited shelf life, and transportation and storage difficulties. These short-comings were apparently overcome when Sheriff and Piercy (1952) attenuated M. mycoides var. mycoides by subculture in embryonated hen's eggs and used the homogenised infected embryos as a vaccine which could be freeze dried. However, avianised contagious bovine pleuropneumonia vaccine was subsequently found to cause lung lesions in some cattle. This dangerous defect is apparently associated with the presence of egg materials in the vaccine (Hudson and Leaver, 1965). Because of this danger we decided to use an egg embryo attenuated strain of M. mycoides var. mycoides to prepare a broth culture vaccine, thereby eliminating the egg material from the vaccine. The methods of production, testing and transport of this vaccine were published (Brown, Gourlay and MacLeod, 1965, paper 15). This vaccine has been prepared since 1962 but on a large

scale only since 1964 and is the vaccine currently in use in East Africa for control of contagious bovine pleuropneumonia. Production of this vaccine reached 3 3/4 million doses in 1970 (Anon, 1971).

The disadvantages of broth culture vaccines mentioned above remain to be solved. In 1964 we showed (Palmer and Gourlay, 1964) that on a small scale culture vaccines could be freeze-dried with little loss of titre, but when this procedure was applied on a large scale the results were poor (Gourlay and MacLeod, 1965, unpublished observations).

During 1964 a few batches of broth culture vaccines were prepared from the T2 avianised strain of M. mycoides var. mycoides (Piercy and Knight, 1956). These batches did not maintain viability as well as the T1 vaccine. Investigations showed that this loss of viability was associated with an excessive lowering of the pH, which was also associated with the glucose content of the medium; furthermore, different strains of M. mycoides var. mycoides were shown to vary in their ability to ferment glucose (Gourlay and MacLeod, 1966, paper 16).

6. CHICKEN EMBRYO AND IMMUNITY

M. mycoides var. mycoides has not yet been shown to induce recognisable disease in laboratory animals.

However, subcutaneous lesions have been produced by means of subcutaneous inoculation of agar plugs containing virulent organisms (see Whittlestone, 1972). The chicken

embryo has been used for many years as a medium for growing and attenuating mycoplasmas. We decided, therefore, to see whether the embryo could be used for studies on immunity to M. mycoides var. mycoides infection. This was done by inoculating the hens that lay the eggs with M. mycoides var. mycoides and determining whether embryos produced subsequently were immune to challenge with this organism. This proved to be the case (Gourlay and Shifrine, 1966, paper 17) and later studies (Gourlay and Shifrine, 1968, paper 18) showed that this immunity persisted for at least 17 weeks and that there was a decrease in the number of organisms in immune embryos from the 4th day after inoculation compared with normal embryos.

In the latter paper it was also demonstrated that the KH3J strain of M. mycoides var. mycoides, which is avirulent for cattle, was also avirulent for egg embryos. This is in contrast to the other 4 strains tested, all of which were virulent for cattle, which proved virulent for egg embryos. However, there was no quantitative correlation between chicken embryo and cattle virulence.

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Section B - MYCOPLASMAS ASSOCIATED WITH CALF PNEUMONIA
AND BOVINE KERATOCONJUNCTIVITIS

In the last few years as a result of the intensification of beef production, calf pneumonia has become a serious problem in this country, and is one of the major causes of calf mortality. Mortality, however, is not the only cause of economic loss from this disease: morbidity, and the resulting lower weight gains, is probably equally or more important. The aetiology of pneumonia in calves under intensive husbandry conditions is complex and numerous infectious agents have been recovered from the lungs and other organs and tissues of calves suffering from respiratory infections. Comprehensive reviews of agents isolated from the bovine respiratory tract in diseased conditions are those by Omar (1966) and Darbyshire and Roberts (1968). Certain mycoplasma species have been isolated on a number of occasions from the respiratory tract of calves in health and disease, but their role in respiratory disease, while not clear, is generally considered insignificant.

The mycoplasma species referred to above can be cultivated by the use of conventional mycoplasma media. But such media are not suitable for the cultivation of all types of mycoplasmas: for example, M. hyopneumoniae (Mare and Switzer, 1965), a mycoplasma shown to cause enzootic pneumonia in pigs, requires a special acellular medium modified from tissue culture fluids. This

observation suggested that there might be as yet unrecognised mycoplasma species in calves, associated with pneumonia, that might be detected by the use of special media. With this in mind a modification of the medium of Goodwin, Pomeroy and Whittlestone (1965), which had been used for cultivating M. hyopneumoniae, was prepared as well as two other media, that had not previously been used for attempted isolation of mycoplasmas from calf lungs, namely urea and arginine containing media.

Culture of material from pneumonic calf lungs in these media resulted in the isolation of two species of mycoplasma, hitherto unreported from calf lungs. One was shown to be a new species with some unusual features including the production of atypical colonies on solid medium (Gourlay, 1968, paper 19). It could be isolated in medium free of known bacterial inhibitors and when subcultured in this medium, showed no evidence of reversion to bacterial forms. This organism was subsequently examined in detail and named M. dispar (Gourlay and Leach, 1970, paper 20). The general characteristics of this species were those of mycoplasmas; these included cultural and metabolic characters, morphological appearance in stained films and electron micrographs, growth inhibition by specific antiserum and a DNA base composition of 28.5% GC. However, the species showed some unusual features, including failure to pass through 450 nm. pore diameter filters without considerable loss in titre, very poor growth on conventional media and the production of atypical colonies,

mentioned above, on the special medium. M. dispar was shown to be serologically distinct from all other recognised bovine mycoplasmas and also a wide range of mycoplasmas from other sources.

The other species of mycoplasma isolated was T-mycoplasma (Gourlay, 1968, paper 21). This mycoplasma had previously been isolated from the human urogenital tract (Shepard, 1954) and oropharynx (Taylor-Robinson and Purcell, 1966) and the urogenital tract of cattle (Taylor-Robinson, Haig and Williams, 1967). Since 1968 T-mycoplasmas have also been isolated from eyes of cattle (Gourlay and Thomas, 1969, paper 22), from the throats of cats (Tan and Markham, 1971), from the genital tract of dogs and the throats of squirrel monkeys (Taylor-Robinson, Martin-Bourgon, Watanabe and Addey, 1971) and the urogenital tract of goats (Gourlay, Brownlie and Howard, 1973).

This might be an appropriate point to digress momentarily from the subject of calf pneumonia to refer in more detail to the paper mentioned above (Gourlay and Thomas, 1969, paper 22) in which we reported the isolation of a number of mycoplasma species from bovine eyes infected with keratoconjunctivitis. These mycoplasma species comprised T-mycoplasmas, M. bovirhinis, M. laidlawii (now named Acholeplasma laidlawii) and a mycoplasma which appeared not to be one of the recognised strains of bovine origin. Shortly after our report, Langford and Dorward (1969) in Canada reported the isolation of a mycoplasma from the eyes of cattle with the same infection. Further

13 of the 16 lungs and in 8 of these lungs there was a rise in titre of T-mycoplasmas of between 3 and 6 \log_{10} compared with the inoculum. In 9 control calves inoculated with sterile medium, clinical signs of pneumonia were observed in only one and a slight pneumonic lesion was found in its lung at autopsy; the lung of one other calf also possessed pneumonic lesions at autopsy. No T-mycoplasmas were isolated from either of these two calves. A serological response to T-mycoplasmas was observed in all but 2 of the calves inoculated with T-mycoplasmas but in only one of the control calves. (1972, paper 25).

The results of the calf inoculations were encouraging but, as we stated in the paper, the calves we used were not gnotobiotic and frequently possessed mycoplasmas in their upper respiratory tract prior to inoculation and we hoped to carry out further work in gnotobiotic calves carrying no mycoplasmas. In view, however, of the great difficulty of obtaining calves of a suitable gnotobiotic specification and the difficulty of monitoring the progress of pneumonia for anatomical reasons, we decided to investigate whether the bovine mammary gland could be an alternate site for studies of T-mycoplasma infection. Advantages of the mammary gland are that it comprises four separate quarters, all of which are accessible for clinical examination, it is easy to sample and has a limited and readily determined bacterial flora. Six milking cows were, therefore, inoculated into the mammary gland via the teat canal with bovine and human T-mycoplasmas and control materials (Gourlay, Howard & Brownlie, 1972, paper 25). Control materials

work by Langford and Leach (1973) indicated that our unidentified strains and the Canadian strains were serologically similar and hitherto undescribed. They named the new species M. oculi. The role of this mycoplasma, and the others we isolated, in the pathogenesis of bovine keratoconjunctivitis is unknown.

The isolations of M. dispar and T-mycoplasmas were made during the course of a survey undertaken to study the microbiology and pathology of 65 pneumonic calf lungs (Gourlay, Mackenzie and Cooper, 1970, paper 23). Mycoplasmas were isolated from 75% of all lungs and comprised T-mycoplasmas from 58%, M. dispar from 51% and M. bovirhinis from 23%. The main bacteria isolated were pasteur-ella from 31% of lungs. No viruses nor chlamydiae were isolated. Apart from pulmonary collapse, the main pathological lesions observed were peribronchial lymphoid hyperplasia, bronchiolitis, purulent bronchopneumonia, fibrinous alveolitis, alveolar giant cell reaction and interstitial pneumonitis.

The high incidence of T-mycoplasmas isolated during the survey raised the question of their role in calf pneumonia. We therefore inoculated, by the endobronchial route, T-mycoplasmas that we had isolated from calf lungs into calves 3 weeks of age and examined their lungs at slaughter 4 weeks later (Gourlay and Thomas, 1970, paper 24). Clinical signs of pneumonia were observed in 6 out of 16 calves before slaughter and pneumonic lesions were found in 14 of them. T-mycoplasmas were reisolated from

produced only a transient cell response in the milk, whereas bovine T-mycoplasmas produced mastitis in 9 out of 10 quarters inoculated. In 3 of these quarters milk secretion ceased completely. Two quarters inoculated with human T-mycoplasmas produced no signs of mastitis perhaps indicative of a species specificity. Infection of the mammary gland did not stimulate high serum antibody levels, but two cows that were able to resolve the infection possessed high titres of antibody in their whey. We concluded that the bovine mammary gland is a suitable model for studying the pathogenesis of bovine T-mycoplasma infections and the immune response of the host.

Further work with this model system has shown that both virulent and avirulent strains of T-mycoplasmas can be isolated from cattle (Howard, Gourlay and Brownlie, 1973, paper 27). In addition a further 4 strains of human T-mycoplasmas were tested and again all proved to be avirulent, as did a strain of canine and simian origin.

Strains of T-mycoplasma isolated from pneumonic calf lungs were compared serologically and were found to be heterogeneous but with antigenic cross reactions between strains (Howard and Gourlay, 1972, paper 26). This is analogous to the results obtained by Purcell, Chanock and Taylor-Robinson (1969) with the T-mycoplasmas of human origin.

More work still needs to be done to ascertain the role of mycoplasmas in calf pneumonia. While the final definitive experiments need to be performed in calves, much of the basic pathological and immunological investigational

work with the T-mycoplasma and with M. dispar, which is also capable of producing mastitis, can be carried out in the bovine udder.

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Section C - MYCOPLASMATALES VIRUSES

That the Mycoplasmatales, the smallest known free-living microorganisms, might be parasitized by even smaller microorganisms has been a topic of conjecture for a number of years. In their study of the ultrastructure of mycoplasmas, published in 1960, G. A. Edwards and Fogh speculated as to whether certain dense bodies seen either attached to the plasma membrane or free within the cytoplasm could be bacterial viruses. Swartzendruber, Clark and Murphy (1967) reported small electron-dense bodies about 25-30 nm. in size resembling intracellular bacteriophage in electron-micrographs of an unidentified mycoplasma of human origin, but as far as is known, no virus was isolated by them, then or subsequently.

It was therefore decided to investigate the susceptibility to virus attack of mycoplasmas isolated from the bovine nasal passages. Screening for virus was performed by adding filtrates of mycoplasma broth cultures to solid medium plates seeded with other mycoplasmas. A report (Gourlay, 1970, paper 28) describes for the first time the isolation and some properties of a virus which infects a member of the Order Mycoplasmatales, namely Acholeplasma laidlawii. This virus produced plaques on a lawn of the acholeplasma, was completely inactivated on exposure to a temperature of 100°C for 30 minutes and ultraviolet light. It was sensitive to chloroform but not to ether, and passed readily through the smallest millipore membrane

filter (designated 10 nm. pore size) and produced turbid plaques. This virus, subsequently named Mycoplasmatales Virus - laidlawii 1 (MV-L1) was purified and further characterised (Gourlay, Bruce and Garwes, 1971, paper 29) and by electron microscopy was shown to be rod shaped with rounded ends and a mean diameter of about 14.5 nm. and a mean length of about 90 nm. Its nucleic acid appeared to be DNA. The anomaly caused by the earlier filtration studies and the electron microscopic measurements was resolved when the manufacturers of the filters redesignated the relevant filter as being 25 nm. instead of 10 nm. pore size.

More detailed ultrastructural studies of MV-L1 showed that the virus particle was rounded at one end and the other end was either rounded, flat or visibly degraded to give one or two short protuberances. The average length of the complete virion was 90 nm. and of the degraded virion 80 nm. Its diameter was more accurately estimated as 16 nm. Some virus particles had hollow centres and aberrant forms of the virus were occasionally observed (Gourlay, 1972, paper 30; Bruce, Gourlay, Hull and Garwes, 1972, paper 31). The virus particles appeared to attach to the host by one end only and as the unattached end was invariably rounded, degradation would seem to occur at the end normally involved in attachment.

The biological characteristics of MV-L1 were studied in more detail (Gourlay and Wyld, 1972, paper 32) and it was shown that the virus was apparently host specific as

it produced plaques on lawns prepared from many strains of A. laidlawii but not on lawns prepared from 12 other species of mycoplasma examined. Clones of A. laidlawii resistant to MV-L1 could readily be obtained and these resistant acholeplasmas were found to carry virus which was serologically similar to MV-L1. An interesting fact to emerge during this work was that the strain of A. laidlawii used as host (BN1), itself carried virus identical to MV-L1 while still being susceptible to virus attack. This situation is most interesting but an explanation will be obtained only after further study.

About a year after the isolation of MV-L1, a second virus, which also infects A. laidlawii, was isolated (Gourlay, 1971, paper 33). This virus was roughly spherical and apparently enveloped, about 50-120 nm. in diameter, serologically dissimilar from MV-L1, more sensitive to heat than MV-L1 and sensitive to detergent. Individual plaques on lawns of A. laidlawii were smaller and, unlike MV-L1, plaques were not formed at 22°C. This second virus was clearly fundamentally different from MV-L1 and represents a new group of Mycoplasmatales viruses. It was designated Mycoplasmatales Virus - laidlawii 2 (MV-L2).

At the International Mycoplasma Symposium in Mainz in 1971, I reported (Gourlay, 1972, paper 30) the isolation of viruses serologically similar to MV-L1 from 4 different strains of A. laidlawii and viruses serologically similar to MV-L2 from 2 different strains of A. laidlawii. At about this time Liss and Maniloff (1971) in America

reported the isolation of 9 viruses from A. laidlawii (which they designated MV-L52 to MV-L60) and also a virus (MV-G51) from a mycoplasma species other than A. laidlawii, namely *Mycoplasma* species strain 14 (goat). At the Mainz Symposium Maniloff and Liss (1972) - see paper 30, page 172 - reported that the viruses isolated by them then numbered 15, which included a virus from M. pneumoniae and from various other sources. Three viruses, including MV-L1, had been characterised and were different from each other. Electron microscopical examination of 5 of their viruses showed them to be 'bullet-shaped'.

In a paper presented at the Ciba Foundation Symposium on Pathogenic Mycoplasmas in London in 1972 (Gourlay, 1972, paper 34), 26 virus isolates obtained at Compton from 14 different strains of A. laidlawii and one strain of A. granularum were compared by various means including those that had proved of value in distinguishing between MV-L1 and MV-L2. All but one of these isolates appeared to be similar to MV-L1 and the remaining one resembled MV-L2.

The relationship between the viruses isolated by Liss and Maniloff (1971) and Maniloff and Liss (1972) and MV-L1 or MV-L2 is not clear, but the situation was clarified to some extent when 17 of the viruses isolated by Maniloff and Liss were acquired at Compton from Maniloff and were examined by the criteria mentioned above and in addition by filtration through a 25 nm. Millipore filter. All 17 viruses formed plaques on A. laidlawii and by all the

criteria used, resemble MV-L1 (Gourlay, 1973b) and are presumably different strains of MV-L1.

MV-L2 was purified and examined in more detail (Gourlay, Garwes, Bruce and Wyld, 1973, paper 35). Electronmicrographs of virus particles revealed predominantly spherical enveloped particles with a mean diameter of about 80 nm. (range 52-125 nm.). The envelope was 'unit membrane' in structure and was apparently serologically dissimilar to the 'unit membrane' of the host A. laidlawii. No obvious isometric or helical capsid was observed within the envelope and the nucleic acid appeared to be DNA.

Recently a third mycoplasma virus has been isolated (Gourlay and Wyld, 1973, paper 36). This virus is intrinsically dissimilar to both MV-L1 and MV-L2, being polyhedral in outline and uniform in size (about 54 nm. diameter), and represents a new group of mycoplasma viruses for which the designation Mycoplasmatales virus - laidlawii 3 (MV-L3) is proposed.

The Mycoplasmatales viruses are of interest not only to mycoplasmologists but also to virologists. The viruses themselves are of interest, possessing certain unique characteristics. The 3 isolated so far differ one from the other to an extent that within a general virus classification, they are likely to be separated not only within different genera but within different families. From the mycoplasmologist's point of view, the isolation of three distinct viruses infecting A. laidlawii indicates that virus infection may be commonplace amongst the

Mycoplasmatales and it is interesting to speculate on the role that these viruses might play in antigenic variation and pathogenicity (Gourlay, 1973a).

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THE ALLERGIC REACTION IN CONTAGIOUS
BOVINE PLEUROPNEUMONIA

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INTRODUCTION

Serological diagnosis of Contagious Bovine Pleuropneumonia (CBPP) has been primarily by means of the complement fixation test (CFT), but also to a lesser extent by the slide agglutination serum test (SAST), the slide agglutination blood test (SABT) and the agar gel double diffusion precipitin test (AGT). All these tests, except possibly the slide agglutination tests, require some laboratory facilities and in the case of the CFT quite elaborate ones. In addition, all these tests, except possibly the SABT, necessitate the numbering of each animal for subsequent identification. These factors form a serious obstacle to the diagnosis of CBPP in some countries of Africa due to the vast distances between the laboratory and outbreaks of the disease and the lack of communications and transport. The development, therefore, of a simple diagnostic test, along the lines of the tuberculin test, has had a high priority in research programmes on CBPP in East Africa.

At the end of the last century and beginning of this, work was done by various people on a tuberculin type test for CBPP. Arloing (1888, cited by Hutyra, Marek and Manninger, 1949), Siedamgrotzky and Noack 1892, Walter 1892, and Bietzen 1919, (all cited by Titze, Giese and Wedemann, 1923) carried out diagnostic inoculations with sterilized pulmonary exudate, and Titze, *et al.* (1923) with concentrated sterilized culture. They obtained temperature reactions in a percentage of infected as well as non-infected cattle following subcutaneous inoculation. Since the results depended on temperature reactions, it was considered that the test would be of little value in tropical countries owing to the variations in temperature which occur in healthy non-housed cattle. The intrapalpebral and ophthalmic routes of inoculation were of no assistance. Ono (1925, cited by Curasson, 1935) provoked a cutaneous reaction using fluid heated at 60°C. and Curasson himself (1935) obtained an oedematous reaction in the eyelid using an antigen obtained by precipitating lung fluid with acetone. He concluded, however, that the reaction was not very specific. Turner (1959) reported an unpublished observation of his that infected animals gave allergic oedematous swellings in the caudal fold after injection of a concentrated suspension of organisms. Observations recorded during experiments which entailed the subcutaneous inoculation of a very concentrated suspension of washed *M. mycoides* into both normal and immune cattle (Gourlay, 1964a) showed that an oedematous swelling occurred within 24 hours at the site of inoculation in immune animals. Following these observations it was decided to investigate this reaction to determine whether it could be developed as a useful diagnostic test for CBPP and also to study the antigens responsible for the reaction. A brief preliminary report on the work has been published (Gourlay, 1963).

MATERIALS AND METHODS

Cattle. Experimental cattle were grades i.e. crosses between East African Zebu and breeds of European origin in which the latter dominated. They were obtained from farms in areas free from CBPP. All were inoculated subcutaneously with 2 ml. of virulent culture of *M. mycoides* (T3 strain) in the thread phase and were then examined every second day for swellings and tested weekly for CF antibodies. After two months or when the swellings had regressed, whichever time was the longer, they were used for testing the various fractions. In addition cattle inoculated endobronchially (Brown, 1964) were also used.

Cattle infected naturally with CBPP were Zebu animals in the Karamoja District of Uganda.

Strains of *M. mycoides*. Details of the strains used are given in a previous publication (Gourlay, 1964b), except for the following. The Mara strain was obtained as lung material from a natural case of the disease from the Mara area of Southern Province, Kenya, and the Gladysdale strain was obtained from Australia as a 10 per cent., lung suspension which was inoculated subcutaneously into cattle from which lymph was obtained and stored at -25°C .

Centrifugation. All centrifugation operations, unless otherwise stated, were performed at 2,000 G and at 3 to 4°C .

Ultrasonic disintegration. Disintegration of bacterial cells was carried out at 4°C . using a M.S.E. ultrasonic disintegrator Cat. No. 3000 at maximum output.

Preparation of the concentrated suspension of *M. mycoides*. The T3 strain of *M. mycoides* was grown and harvested as previously described for the preparation of *M. mycoides* antigen (Gourlay, 1964b), but the organisms were washed in, and the final suspension made up in phosphate buffer, pH. 7.4, to an opacity of 9 times tube 10 using Wellcome opacity tubes.*

Assay method. Antigenic materials were inoculated by the intradermal route in cattle with a dose of 0.1 ml. in the side of the neck. Up to 6 different materials were injected in the same animal at 3 sites on each side of the neck. Where possible each sample was inoculated at different sites to counteract site variations and 2 or 3 animals were used for each experiment. Twenty-five negative control animals were also used at various stages of the work. Details of serological and other methods used are given in a previous publication (Gourlay, 1964b).

Examination of naturally infected cattle. Blood for serum was collected from each animal before autopsy, whilst at autopsy specimens of lung lesions and/or pieces of mediastinal lymph gland were collected from any case where there was any doubt of the diagnosis. The sera were examined by the CFT and SAST for antibody and the AGT for both antigen and antibody while the lung and gland specimens were examined by the AGT for antigen and in many cases cultured for viable *M. mycoides*.

RESULTS

Preparation of Antigen

In preliminary experiments it was shown that ultrasonic treatment of the concentrated suspension of organisms for periods up to 240

*Burroughs Wellcome & Co. Ltd., London.

minutes produced very little evidence of disintegration macroscopically, but under dark ground illumination microscopically the particles of organisms appeared to be broken into fairly uniform minute particles after only 60 minutes and thereafter very little change was seen. A suspension of organisms that had received ultrasonic treatment for 60 minutes was passed through a millipore HA cellulose acetate membrane filter (pore size $0.45\mu \pm 0.02\mu$)[†] and the filtrate and deposit collected. Considerable deposit was obtained and was washed 3 times by carefully mixing it with 5 ml. of buffer and passing the mixture through the HA filter again each time. Finally the washing fluids and the deposit were mixed together and concentrated with carbowax* to the original volume (5 ml.). Two ml. of HA filtrate was then passed through a millipore VM filter (pore size $50m\mu \pm 3m\mu$)[‡]. Only a very little deposit was obtained this time and it was washed 3 times as before and the washing fluids added and concentrated to 2 ml. The filtrate was also retained. The various fractions were then assayed in cattle and the reaction recorded. The results showed that the activity was primarily in the HA deposit with a little in the HA washings; the other fractions possessed very little if any activity. The HA deposit, however, was dark grey in colour and very thick and viscid, and would not go into solution at or near neutral pH which would be necessary if animals were to be inoculated with the material.

Urea possesses the property of making proteins soluble (Jenkins and Rowley, 1959), so solid urea was added to the suspension of *M. mycoides* to give a concentration of 2.5 molar. This suspension was then subjected to ultrasonic treatment for varying periods of time. The suspension slowly cleared until after 60 minutes it became translucent and had a greenish brown colour. Under darkground microscopy the material was seen to consist of similar uniform minute particles as seen in the ultrasonic treated buffer suspension, but they were far fewer in number. Ultrasonic treatment for a further 60 minutes did not alter the macro- or microscopic appearance of the suspension. The material that had been so treated for 60 minutes was then processed in an identical manner to the earlier buffer suspension, i.e., millipore HA and VM filtration. It was, however, noticeable that very little HA and VM deposits were produced. The various fractions were then assayed in cattle as before.

The effect of varying lengths of time of ultrasonic treatment on the reaction produced in cattle showed that 60 minutes was the most suitable time. Table 1 shows the reactions produced by the various fractions obtained by ultrasonic treatment and filtration. Some activity was lost on filtration, as was to be expected when each fraction was made up to the original volume before inoculation, but the activity appeared to be primarily in the VM deposit with only a little in the VM filtrate. Urea 2.5 M. alone was inoculated into 6

[†]Millipore Filter Corporation, Bedford Mass. U.S.A.

*George T. Gurr, Ltd., London.

TABLE 1

COMPARISON BETWEEN REACTIONS PRODUCED BY SUSPENSION OF ORGANISMS BEFORE AND AFTER ULTRASONICATION FOR 60 MINUTES AND FRACTIONS PRODUCED BY FILTRATION

Animal No.	Max. increase in skin thickness (mm.)							
	Susp. of Orgs.	Orgs. u-s. 60 mins.	HA deposit	HA wash.	HA filt.	VM deposit	VM wash.	VM filt.
8274	5	7.5			3.5			
8499			0.5		7	4		0.5
8522			0.5	1	14	11	4.5	5
8597			0.5		9.5	11		3.5
8883	8	15			11.5			4
8896	11.5	10			6			3.5
8993	7	11			6			5

cattle and the maximum increase in skin thickness noted was 1.5 mm.

The ultrasonic treated suspension of organism was diluted with buffer to 3 times opacity tube 10 and then 10 ml. was taken and passed through a millipore HA filter. The filtrate which was an almost clear, straw-coloured fluid was treated with ammonium sulphate. Aliquots of 2 ml. were taken and made up to 10, 20, 33.3, 50 and 66.7 per cent. saturation with an ammonium sulphate solution saturated at 4°C. The mixtures were left overnight at 4°C. and then centrifuged for 30 minutes. Considerable precipitate was evident in the 66.7 and 50 per cent. tubes, less in the 33.3 per cent. tube and only a trace in the 20 and 10 per cent. tubes. Similarly, the supernatant fluids varied in opacity. The fluids produced by 66.7 and 50 per cent. saturation were completely clear whilst that of the 33.3 per cent. saturation was slightly opaque and the 20 and 10 per cent. supernatant fluids were very opaque. Each precipitate was made up to 4 ml. with 2.5 M. urea in buffer and reprecipitated twice more with the same percentage of saturated ammonium sulphate and the final precipitate made up to 2 ml. with 2.5 M. urea in buffer. Assays were performed in cattle with the various precipitates. The results showed that the activity was proportional to the amount of ammonium sulphate used, but even the precipitate produced by 20 per cent. saturated ammonium sulphate contained a considerable proportion (79 per cent.) of the activity.

Seven ml. of HA filtrate was then treated with saturated ammonium sulphate to 67 per cent. saturation and the precipitate which formed was made up to 33 ml. with 2.5 M. urea in buffer and reprecipitated with ammonium sulphate to 67 per cent. saturation. This process was repeated 6 times and the final precipitate

(ppt. 6) was made up to the original volume (7 ml.) with 2.5 M. urea in buffer. The original HA filtrate and the final precipitate, (ppt. 6) were compared by animal assay and the precipitin bands produced in the AGT were also compared, using sheep \times ' serum absorbed with pig and ox antisera (Gourlay, 1946b).

The results, Table 2, indicated that only a little activity was lost on repeated precipitation, whilst the AGT results showed that a considerable amount of the precipitating antigenic material had been removed.

The final solution contained a certain amount of insoluble material which deposited on standing. After centrifugation the deposit was washed twice in 2.5 M. urea and then made up to the original volume with 2.5 M. urea. The supernatant, washed deposit and original material were then compared by animal assay. The results (Table 3) showed that the bulk of the activity was in the supernatant but a certain amount of activity remained in the deposit.

TABLE 2

COMPARISON BETWEEN THE REACTIONS PRODUCED BY THE HA FILTRATE AND THE PRECIPITATE PRODUCED BY 6 PRECIPITATIONS WITH 67 PER CENT. AMMONIUM SULPHATE

Animal no.	Max. increase in skin thickness (mm.)	
	HA filtrate	ppt. 6
8883	11.5	7
8896	6	8.5
8993	6	6
8658	11	8

TABLE 3

COMPARISON BETWEEN THE REACTIONS PRODUCED BY THE SUPERNATANT AND DEPOSIT OF THE ALLERGIC ANTIGEN

Animal no.	Max. increase in skin thickness (mm.)		
	Antigen	Supernatant	Deposit
8990	6	7	3
8990	5	5	3
8978	3.5	3.5	0
8970	6.5	4	1.5
9253	14.5	11	10

In the light of these experiments the following procedure was adopted for the routine preparation of the antigen. Solid urea was added to 25 ml. concentrated suspension of *M. mycoides* (9 times opacity tube 10) to give 2.5 M. strength. This was then subjected to ultrasonic treatment for 60 minutes at maximum output until the suspension became opaque and then passed through a millipore HA filter. The filtrate was made up to 100 ml. with 2.5 M. urea and precipitated 6 times with saturated ammonium sulphate to 50 per cent. saturation. The precipitate was resuspended in 100 ml. of 2.5 M. urea each time. Following the final precipitation the antigen was made up to 25 ml. with 2.5 M. urea, dialysed against distilled water to remove the remaining ammonium sulphate, finally measured and urea added to 2.5 M. strength. It was stored at -25°C .

Increase in Skin Thickness in Relation to Time after Inoculation

In the early stages of the work, skin thickness measurements were recorded daily after inoculation with antigenic fractions. The maximum reaction occurred in the great majority of cases at 24 hours and slowly regressed over a period of 1 to 2 weeks. In the occasional animal, however, the maximum increase occurred at 48 hours, but never later than this. Subsequently it became routine to examine experimental cattle at 24 and 48 hours after inoculation; natural cases, however, were examined only at 24 hours.

Activity of Antigen in Cattle Inoculated or Infected with Different Strains of M. mycoides.

The antigen was inoculated into cattle that had been inoculated subcutaneously with T₃, Mara, KH₃J, Somaliland, and the Gladysdale strains of *M. mycoides*, cattle inoculated endobronchially with the Mara, Gladysdale and KH₃J strains and also into natural cases of the disease obtained from Ndeiya and Karamoja. Allergic reactions were obtained with all strains including KH₃J, but in the case of this strain the reactions were only observed when the organisms had been inoculated endobronchially together with a simultaneous subcutaneous inoculation of KAG rinderpest vaccine as a form of stress (Brown, 1964). More details of this are given under the section dealing with the action of the antigen in cattle infected by the endobronchial method.

Correlation between Allergic Response and the Complement Fixation Response

In the experimental animals, excluding those inoculated endobronchially, there were 10 instances in which complement fixation antibodies were no longer detectable, but the animals gave allergic reactions.

Non-specific Reactions

During the investigations an instance of non-specific reaction occurred. In this case a negative control animal developed a swelling of 9 mm. increase. Serum from this animal gave a weak positive

reaction ($++$ reaction at 1/20 dilution) in the CFT and a fairly strong positive reaction in the SAST. The animal was isolated and its sera checked weekly. It was found that the CFT and SAST reactions had disappeared a week later and did not recur. After a further month the animal was destroyed, but no obvious lesions of CBPP were seen in the lungs.

Cattle Tested more than Once with Allergic Antigen

There were 6 instances when cattle were used twice to test antigen. These animals were not, however, inoculated with the same fractions both times so a direct comparison cannot be made. The time that elapsed between the two tests varied from 1 to 2½ months and as far as could be judged there was little if any loss of activity (Table 4).

TABLE 4
COMPARISON BETWEEN THE REACTIONS PRODUCED WHEN
CATTLE WERE INOCULATED TWICE WITH ANTIGEN

Animal no.	Antigen	Increase (mm.)	Time elapsed (days)	Antigen	Increase (mm.)
8265	3 precip. \bar{c} ammon. sulph.	11	71	1 precip. \bar{c} ammon. sulph.	6
8267	1 precip. \bar{c} ammon. sulph.	12.5	78	Buffer HA deposit	16.5
8499	3 precip. \bar{c} ammon. sulph.	9	40	Urea HA filtrate	7
8522	11 precip. \bar{c} ammon. sulph.	14	38	Urea HA filtrate	14
8597	11 precip. \bar{c} ammon. sulph.	12	36	Buffer HA filtrate	18
8568	3 precip. \bar{c} ammon. sulph.	14.5	36	6 precip. \bar{c} ammon. sulph.	11

Action of the Antigen in Natural Cases of CBPP

During the course of the work an opportunity occurred to test the antigen in naturally infected cattle in the Karamoja District of Uganda where an acute outbreak was occurring in an area that had apparently been clear of CBPP since 1948.

In January, 1963 a group of 39 cattle were tested at the Iriri Quarantine Station and the following day, after reading the test, they were killed and the lungs examined. Of the 18 cattle positive to the test, 14 had unmistakable lesions of CBPP, whilst 2 (7 & 23) had evidence of old lesions, 1 of the remainder had severe pleurisy and the other small abscesses and adhesions. The 2 doubtful cases (11 & 33) showed no macroscopic or serological evidence of CBPP.

Of the 19 animals negative to the test all except 3 showed no macroscopic or serological evidence of CBPP, but the 3 remaining ones (18, 21 & 31) had large acute lesions of CBPP. Details are given in Table 5. In April, 1963 a further 121 cattle were tested; 110 were negative, 7 gave doubtful swellings and 4 were positive. Fourteen animals were killed and autopsied (Table 6), including the 4 that

TABLE 5

REACTIONS PRODUCED IN CATTLE FROM AN OUTBREAK OF CONTAGIOUS BOVINE PLEUROPNEUMONIA AT IRIRI (JANUARY 1963)

Animal no.	Allergic test* mm. increase if positive	Lung lesions at autopsy
1	+ 8.5	Encapsulated CBPP lesion $4 \times 2 \times 3''$
2	—	Nil
3	—	Nil
4	—	Nil
5	—	Nil
6	—	Nil
7	+ 6.5	Adhesions and scars on lungs
8	+ 7	Acute CBPP lesion of whole diaphragmatic lobe
9	—	Numerous calcified hydatid cysts
10	—	Nil
11	±	Nil
12	—	TB both lungs
13	+ 11.5	Encapsulated CBPP lesion, whole diaphragmatic lobe
14	+ 11	Encapsulated CBPP lesion, whole lung
15	—	Nil
16	—	Nil
17	—	Nil
18	—	Acute CBPP lesion of diaphragmatic and cardiac lobes
19	+ 7.5	Encapsulated CBPP lesion in both lungs
20	—	Hydatid cysts
21	—	Acute CBPP lesion whole diaphragmatic and cardiac lobes
22	+ 8.5	Small abscesses and adhesions (Probable CBPP)
23	+ 7	Scars on lung and adhesions to pleura
24	+ 5	Acute CBPP lesion whole diaphragmatic lobe
25	—	Nil
26	+ 6.5	Encapsulated lesion whole diaphragmatic lobe
27	+ 5.5	Encapsulated CBPP lesion diaphragmatic lobe $7 \times 5 \times 5''$
28	+ 5	Acute CBPP lesion whole lung
29	+ 6.5	Encapsulated CBPP lesion whole diaphragmatic lobe
30	+ 5.5	Acute CBPP lesion whole diaphragmatic lobe
31	—	Acute CBPP lesion whole diaphragmatic lobe, TB other lung
32	—	Abscesses in both lungs with yellow pus (not CBPP)
33	±	Nil
34	+ 5	Encapsulated CBPP lesion whole diaphragmatic lobe
35	+ 5	Severe pleurisy both thoracic cavities, lungs normal
36	—	Nil
37	—	Nil
38	+ 4.5	Acute CBPP lesion $3 \times 3 \times 3''$
39	+ 6	Encapsulated CBPP lesions in apical and cardiac lobes each $5 \times 3 \times 3''$

*Any reaction less than 3 mm. increase was termed negative. Reaction 3 to 4 mm. were termed doubtful

TABLE 6

REACTIONS PRODUCED IN CATTLE FROM AN OUTBREAK OF CONTAGIOUS BOVINE PLEUROPNEUMONIA AT IRIRI (APRIL, 1963)

Animal no.	Allergic test* mm. increase if positive	Lung lesions at autopsy
1	±	Nil
2	+ 12	Adhesions to diaphragm and 3 small CBPP lesions in lung
3	+ 9	Adhesions and small CBPP lesion 2 × 2"
4	±	Adhesions
5	+ 6.5	Nil
6	±	Nil
7	±	Nil
8	±	Nil
9	+ 6	Whole diaphragmatic lobe solid CBPP
10	—	Nil
11	±	Adhesions to pericardial sac from lung, also large calcified lesion on inner surface of rib opposite the adhesions
12	±	Adhesions
13	—	Generalized TB
14	—	Nil

*Any reaction less than 3 mm. increase was termed negative. Reactions 3 to 4 mm. were termed doubtful.

TABLE 7

REACTIONS PRODUCED IN CATTLE FROM AN ACUTE OUTBREAK OF CONTAGIOUS BOVINE PLEUROPNEUMONIA AT APOPOA

Animal no.	Allergic test* mm. increase if positive	Lung lesions at autopsy
H.18	—	Whole of one lung acute CBPP
H.32	—	Whole of one lung acute CBPP plus excess pleural fluid
H.44	+ 12	Large encapsulated lesion CBPP 12 × 5 × 7"
H.45	+ 6.5	Large encapsulated lesion CBPP 12 × 5 × 5"
H.46	—	Half diaphragmatic lobe acute CBPP
H.67	+ 4.5	Whole lung acute CBPP
H.73	—	Both lungs acute and chronic CBPP lesions 14 × 10 × 7" and 8 × 8 × 5"
I.41	±	Whole lung acute CBPP
I.68	—	Whole lung acute CBPP
I.71	—	Whole lung acute CBPP

*Any reaction less than 3 mm. increase was termed negative. Reactions 3 to 4 mm. were termed doubtful.

TABLE 8
REACTIONS PRODUCED IN CATTLE INOCULATED ENDOBRONCHIALY

<i>Animal no.</i>	<i>Strain</i>	<i>Days after inoc.</i>	<i>Max. increase in skin thickness (mm.)</i>	<i>CFT*</i>	<i>Lung lesions at autopsy</i>
8883	Glad	27	7	++++	Nil
8896	"	"	8.5	++++	2 encapsulated CBPP lesions $2 \times 2 \times 2''$ $\frac{1}{2} \times \frac{1}{2} \times \frac{1}{2}''$
8898	"	"	5	++++	Half diaphragmatic lobe acute and chronic CBPP
8950	"	"	7	++++	CBPP lesion $4 \times 2 \times 10''$ acute becoming encapsulated
8952	"	"	7	++++	Half diaphragmatic lobe sequestrum surrounded by $\frac{1}{2}''$ area acute CBPP
8956	"	"	20	++++	2 CBPP lesions $3 \times 4 \times 4''$ necrotic and acute becoming encapsulated
8993	"	"	6	++++	Nil
8887	"	221	5	++++	Fibrosis and adhesions
8888	"	"	1	o	Nil
8958	Glad + KAG	"	5	o	Acute CBPP lesion $2 \times 2 \times 1''$
8999	Glad + KAG	218	8	++++	Nil
9251	T ₃ + KAG	96	8	++++	Encapsulated CBPP lesion $1 \times 1 \times 1''$, adhesions
9253	T ₃	100	14.5	o	Nil
8895	KH ₃ J	90	0.5	o	Nil
9009	"	"	1.5	o	Nil
9152	"	"	0.5	o	Nil
9252	"	"	3	o	Nil
8894	KH ₃ J + KAG	"	10.5	o	Nil
9052	KH ₃ J + KAG	"	22	o	Nil
9054	KH ₃ J + KAG	"	9.2	o	Nil
9188	KH ₃ J + KAG	"	6.5	o	Nil
9248	KH ₃ J + KAG	"	6.5	++	Nil

*Campbell & Turner (1953), fixation at 1:10 dilution

were positive, the 7 doubtfuls and 3 others which were sick and in very poor condition. Three of the 4 animals positive to the test had unmistakable lesions of CBPP while the other one had no macroscopical evidence of infection. Four of the doubtful cases had no macroscopic or serological evidence of infection whilst the remaining 3 had some evidence of past infection. All 3 cattle negative to the test had no evidence of CBPP infection. The other 107 cattle negative to the test were killed a few days later at Kampala abattoir, the lungs were examined and 106 of them had no lesions of CBPP but the remaining one had an acute CBPP lesion.

The opportunity was taken to test a herd of African-owned cattle at Apopoa, that were in the course of a very acute outbreak in which many deaths were occurring daily. One hundred and twenty-six cattle were tested, but it was only possible to autopsy 10 of them as the owners were very reluctant to sell any but severely sick animals that were likely to die. However, after great difficulty we were able to purchase 2 cattle positive to the test that were in good condition (H.44 & H.45). Three cattle were positive to the test and all had unmistakable lesions of CBPP. This included the 2 in good condition which had large encapsulated lesions. The 6 cattle negative and the 1 doubtful to the test all had very large acute lesions of CBPP and all were very sick. Details are given in Table 7.

Action of the Antigen in Cattle Inoculated Endobronchially with M. mycoides

Cattle inoculated by the endobronchial method (Brown, 1964) with various strains of *M. mycoides* were tested at various lengths of time after inoculation. Seven cattle inoculated with Gladysdale strain and tested 27 days after inoculation and 3 out of 4 tested 218 and 221 days after inoculation were positive. Two animals tested 96 and 100 days after inoculation with T3 strain were also positive. Cattle inoculated with KH₃J strain (an avirulent strain) and tested 90 days after inoculation produced interesting results. Five cattle that had received an inoculation of KAG rinderpest vaccine at the same time as they were inoculated endobronchially as a form of stress (Brown 1964) produced reactions, whereas 4 cattle that had not received the vaccine produced no reactions. No lung lesions were seen in any of these animals at autopsy, and CF antibodies were demonstrable in only one of the sera at the time of testing. Details of the reactions produced by the endobronchially inoculated cattle are given in Table 8.

DISCUSSION

Ultrasonic disintegration of concentrated suspensions of organisms in 2.5 M. urea produced a larger reaction than the untreated whole organisms when inoculated into immune cattle and also had the essential advantage of killing the organisms. A considerable amount of thick, viscid material was present in the ultrasonic treated organisms and the fraction active in the allergic reaction appeared to be closely linked with this viscid material which made manipulations difficult. The addition of urea helped considerably by making the material more soluble so that it passed through the HA filter. The active fraction would not, however, pass readily through a VM filter, although some activity was present in the filtrate. The precipitations with ammonium sulphate indicated that the active fraction was either protein in nature or was closely linked with protein material, and the precipitations removed some of the polysaccharide precipitating antigenic material. The final antigen contained a certain amount of insoluble material. Unfortunately, the activity

appeared to be in both the soluble and insoluble fractions so, for the present, the insoluble material was retained.

Reactions were observed in cattle that had been inoculated or infected with numerous strains of *M. mycoides*, including strains from Kenya, Uganda, Tanganyika, Somaliland and Australia, indicating that the antigen responsible is probably common to all strains of *M. mycoides*. No reactions were observed, however, in cattle inoculated endobronchially with the KH₃J strain unless the cattle had been stressed at the time of inoculation, suggesting that insufficient multiplication of the organisms occurred without stress, and also that multiplication was necessary before cattle would react. The reason for be that the antigen responsible was a product of metabolism or more this might likely, that it was simply a question of antigenic mass.

It was apparent that the antibodies responsible for the allergic reaction differed from those responsible for the CF reaction. Evidence obtained from later work (Gourlay, unpublished) suggests that they also differ from the antibodies responsible for the agglutination and precipitation reactions.

There appeared to be little if any loss of activity when experimental cattle were inoculated twice with antigen at an interval of 1 to 2½ months. It is not known, however, how many times this could be repeated without loss of activity, as presumably desensitisation would occur. The specific antigen is probably not one of the polysaccharide antigens already described (Gourlay, 1964a), as those isolated from urine did not produce an allergic response in immune animals: moreover the removal of a considerable amount of the polysaccharide antigenic material by precipitation with ammonium sulphate did not significantly alter the activity. It seems probable because of its precipitation with ammonium sulphate that the antigen responsible for the reaction is protein in nature or closely linked with protein.

Despite the relatively small number of tests performed on naturally infected cattle, certain limitations of the test, all to some extent inherent in allergic type tests, became obvious. The most important was the failure to detect acute cases which were likely to die, and this can probably be explained by neutralisation of the antibody responsible by the influx of antigenic material into the circulation from the lung lesions. Retesting after an interval might be indicated, as by then the great majority of these acute cases would have died, and any survivors would probably react to the test. It was also evident that this reaction detected cases that had apparently recovered from the disease, possibly for a considerable time, when the only evidence of past infection, which could not always be confirmed, was scars on the lungs and fibrous adhesions. Occasionally positive reactions were observed in cattle in which no macroscopic lesions, scars or adhesions were evident in the lungs and examination of sera and autopsy specimens showed no evidence of present or past infection with *M. mycoides*. These may have been instances of non-specific reactions

but in an infected herd it is probable that all stages of the disease exist, including very early cases where no macroscopical lesions are present and possibly no antibodies are detectable by the usual serological tests, as well as very old or very mild cases where again no lesions are seen and where antibodies have disappeared. It was obvious that reactions to this antigen did not occur in cases of tuberculosis or hydatid cysts in the lungs, but the demonstration of one instance of non-specific reaction in experimental cattle which was also detected by the CF and agglutination tests showed that further work on the specificity of the reaction is required.

The main advantages of this test are its ability to detect "lungers", its simplicity and the elimination of the necessity for numbering the animals. The last two points are of special importance under East African conditions.

The development of an allergic test for the diagnosis of CBPP is in the very early stages and obviously much work can be done on purification of the antigen, the first priority being to obtain the antigen in solution. Work on alternate sites of injection and the addition of dyes or various other materials to the antigen to enhance the reaction might also be profitable. A study of reactions produced following vaccination is required and although it does not seem likely that the inoculation of allergic antigen will interfere with the CF and other diagnostic tests this has to be confirmed.

CONCLUSIONS

An antigen was prepared by ultrasonic disintegration of a concentrated suspension of *Mycoplasma mycoides* in 2.5 M. urea, followed by precipitation with ammonium sulphate. It was inoculated intradermally, in the side of the neck, into cattle previously inoculated subcutaneously or endobronchially with various strains of *M. mycoides* and also into cattle naturally infected with Contagious Bovine Pleuropneumonia. From tests in experimentally infected cattle it appeared that the antigen responsible for the allergic reaction was probably associated with the protein fraction, was apparently linked with rather insoluble viscid material and was non-dialysable. Reactions were observed in cattle infected or inoculated with numerous strains suggesting that the antigen responsible is common to all strains of *M. mycoides*. Maximal reactions generally occurred 24 hours after inoculation, rarely later. The antibodies involved in the reaction differed from those responsible for the CF reaction, and there appeared to be little if any loss of activity when experimental cattle were inoculated twice with antigen at an interval of 1 to 2½ months. From tests performed on naturally infected cattle it appeared that reactions did not occur in cattle dying with large acute lesions. Reactions did occur in cattle with less acute lesions and in cattle that had apparently recovered from the disease with only scars on the lungs and adhesions. Non-specific reactions did not occur in cases of tuberculosis or hydatid cysts but were seen occasionally in

cattle, in which there was no evidence of present or past infection. One case of a non-specific reaction in an experimental animal is described.

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FURTHER STUDIES ON THE ALLERGIC REACTION IN CONTAGIOUS BOVINE PLEUROPNEUMONIA

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INTRODUCTION

An earlier paper (Gourlay, 1964) recorded the preparation of an allergic antigen and its use, both experimentally and in the field, for the diagnosis of Contagious Bovine Pleuropneumonia (CBPP). This antigen contained a certain amount of insoluble material that possessed activity. The present paper describes further work done in an effort to produce a more soluble antigen, the use of a more virulent strain of *Mycoplasma mycoides* for antigen production and its use experimentally and in the field.

MATERIALS AND METHODS

The materials and methods used, except for those described below, have already been published (Gourlay, 1964).

Preparation of antigen from the Gladysdale strain of M. mycoides. A batch of antigen was prepared in a manner identical to that already described, but the Gladysdale strain was used instead of T3. The suspension of organisms was less concentrated, being 7 times as opaque as tube 10 in the Wellcome opacity series.

Preparation of antigen at alkaline pH. A batch of antigen was prepared with the Gladysdale strain of *M. mycoides*, as above, but before ultrasonic treatment the concentrated suspension of organisms was brought to pH 9.5 with 10N-NaOH. Although during treatment it was noticed that the suspension cleared after only about 15 minutes, the process was continued for a further 15 minutes. After each precipitation the precipitate was suspended in 2.5 M urea at pH 9.5. Following the last precipitation, the deposit was made up in 2.5 M urea at pH 9.5 giving a clear solution, stored at +4°C. for 48 hours and then dialysed against distilled water. Following dialysis the material became only slightly cloudy. Urea was not added to the final material. The final antigen (dry wt. 6.25 mg/ml.) contained 100 per cent. protein (Folin-Ciocalten), following removal of the small amount of insoluble material.

Preparation of double strength antigen. Antigen was prepared in an identical manner to that described above, at alkaline pH, but following the final dialysis the material was concentrated by means of hair driers to half the volume.

Determination of serological response following inoculation of allergic antigen. Sera from 4 cattle that had been inoculated 3 months earlier with T3 culture subcutaneously, had developed a Willem's reactions at the inoculation site and had recovered, and sera from 3 control negative cattle were examined by the CFT and AT immediately before, 24 hours after and then at weekly intervals for 6 weeks following inoculation with 0.3 ml. of the single strength antigen.

Challenge of cattle inoculated with allergic antigen. The 3 negative cattle that had been inoculated with 0.3 ml. of single strength allergic antigen were challenged by the subcutaneous inoculation of 2 ml. of the Gladysdale culture of *M. mycoides* 6 weeks after inoculation with the allergic antigen. Three control negative cattle were also challenged.

RESULTS

Comparison between Reactions produced by Antigens prepared from the Gladysdale and T3 strains of M. mycoides

The results of assays using these antigens, one inoculated into one side and the other into the other side of the neck of the same animals, indicated that the antigen prepared from the Gladysdale strain produced larger reactions, even though the opacity of the suspension of organisms used was less dense than that used for the preparation of the T3 antigen (Table 1).

TABLE 1
COMPARISON BETWEEN REACTIONS PRODUCED BY ANTIGENS PREPARED FROM THE T3 AND GLADYSDALE STRAINS OF *M. mycoides*

Animal No.	Max. increase in skin thickness (m.m.)	
	T3 antigen	Gladysdale antigen
8894	6	10.5
9052	12.5	22
9054	6	9.5
9188	4.5	6.5
9248	5	6.5

Comparison between Reactions produced by Antigens prepared at Acid and Alkaline pH

The results of assays showed that the antigen prepared at alkaline pH gave slightly larger reactions (Table 2).

TABLE 2
COMPARISON BETWEEN REACTIONS PRODUCED BY ANTIGENS PREPARED AT NORMAL AND ALKALINE PH

Animal No.	Max. increase in skin thickness (m.m.)	
	Normal pH	Alkaline pH
8887	5	5
8957	8	9.5
8958	2.5	5
8964	4.5	5
8998	6	8.5

Comparison between Reactions produced by Single and Double Strength Antigen

Earlier, using a very crude antigen, it appeared that doubling the dose of inoculum by concentrating the antigen to half its original volume produced a

larger reaction, and conversely the reactions became smaller with dilution of the antigen. This crude antigen had undergone no precipitation with ammonium sulphate and in 3 negative control animals there was a slight increase in skin thickness with increase in concentration. On this evidence it was decided to use double-strength antigen prepared from the Gladysdale strain at alkaline pH. Table 3 gives the results of assays performed using single and double strength antigen from which it appeared that the double-strength antigen, whilst possibly giving slightly larger reactions, was not sufficiently superior to warrant its use in the field, as it would mean halving the number of animals that could be tested.

TABLE 3
COMPARISON BETWEEN REACTIONS PRODUCED BY SINGLE AND DOUBLE STRENGTH ANTIGEN

<i>Animal No.</i>	<i>Max. increase in skin thickness (m.m.)</i>	
	<i>Single strength</i>	<i>Double strength</i>
8964	5	5.5
8998	5.5	5.5
9071	4	6
9072	3.5	5
9117	6	4
9113	4	5.5
9067	4	3.5
<i>Controls</i>		
9249	0.5	0.5
9351	2	1.5
9352	0.5	0.5

Determination of Serological Response following Inoculation of Allergic Antigen

It appears that negative control cattle do not develop any CF or agglutinating antibodies following inoculation of 3 times the dose of allergic antigen. Two out of the 4 positive cattle, however, did appear to develop a slight boost to the serological response.

Challenge of Cattle Inoculated with Allergic Antigen

Two of the 3 control cattle developed extensive Willems reactions (over 25×25 cm.) and one had to be destroyed in extremis, while the third animal developed a 30×15 cm. lesion. One of the 3 cattle that had been inoculated with allergic antigen developed an extensive swelling and had to be destroyed, 1 developed a 15×10 cm. swelling and the third developed only a small nodule 1×1 cm. at the inoculation site which disappeared within 14 days.

TABLE 4

REACTIONS PRODUCED IN NATURALLY INFECTED CATTLE INOCULATED WITH DOUBLE STRENGTH ANTIGEN

<i>Animal No.</i>	<i>Allergic reaction* mm. increase if positive</i>	<i>Lung lesions at autopsy</i>
1	+ 14.5	CBPP sequestrum $2 \times 2 \times 1''$
2	+ 8	Pleurisy and possible old sequestrum
3	+ 6	CBPP sequestrum $1 \times 1 \times \frac{1}{2}''$ and adhesions
4	+ 5.5	" " $4 \times 4 \times 3''$
5	—	Few small sequestra, fibrosis, adhesions and pleurisy both lungs
6	+ 6.5	CBPP sequestrum, fibrosis and adhesions
7	—	Normal
8	+ 8.5	CBPP sequestrum, fibrosis, adhesions and pleurisy both lungs
9	+ 8	" " fibrosis and adhesions
10	+ 8	" " $6 \times 6 \times 5''$
11	+ 4.5	" " fibrosis and pleurisy
12	+ 11	" " "
13	+ 5	" " fibrosis and pleurisy
14	+ 7.5	" " $4 \times 3 \times 2''$ and pleurisy
15	+ 7.5	Normal
16	+ 6	Pleurisy and large hydatid cyst
17	+ 5	CBPP sequestrum $3 \times 2 \times 2''$ and fibrosis
18	+ 7	" " $4 \times 5 \times 5''$ (shelled out)
19	±	" " $4 \times 4 \times 3''$
20	+ 6	Normal lung, pus in lymph gland
21	+ 10	CBPP sequestrum $3 \times 2 \times 2''$
22	+ 8	" " fibrosis and pleurisy
24	±	" " "
25	—	" " $2 \times 2 \times 2''$
26	—	" " $4 \times 4 \times 3''$
27	+ 9	" " (shelled out)
28	+ 5	Severe pleurisy
29	+ 6	CBPP sequestrum, fibrosis and pleurisy
30	—	Small necrotic nodule, pleurisy
31	+ 5	CBPP sequestrum $2 \times 2 \times 3''$
32	±	" " $3 \times 3 \times 2''$
33	+ 4.5	" " $4 \times 4 \times 2''$
34	+ 6	Possible old sequestrum $2 \times 1 \times 1''$ and pleurisy
35	—	Possible old sequestrum, severe pleurisy
36	+ 9	CBPP sequestrum $6 \times 5 \times 5''$
37	+ 5	" " and pleurisy
38	+ 4.5	" " (shelled out) and pleurisy
39	+ 7	CBPP sequestrum $5 \times 4 \times 4''$ (shelled out) and pleurisy
40	+ 6	" " $6 \times 6 \times 6''$
41	+ 5	" " $2 \times 1 \times 2''$ and pleurisy
43	+ 6	Old sequestrum, adhesions and slight pleurisy
44	+ 6.5	CBPP sequestrum $4 \times 2 \times 2''$ and pleurisy
45	—	Very old CBPP sequestrum $4 \times 2 \times 2''$
47	±	CBPP sequestrum $3 \times 3 \times 2''$
48	+ 6.5	" " $3 \times 3 \times 3''$
49	—	" " $2 \times 1 \times 1''$ and $1 \times 1 \times 1''$
50	+ 4.5	" " fibrosis and pleurisy
51	—	Normal
52	+ 6	CBPP sequestrum $6 \times 6 \times 5''$
53	+ 26	" " $6 \times 5 \times 5''$
54	+ 8	" " $8 \times 6 \times 4''$
55	±	" " $1 \times 1 \times 1''$
56	+ 6	" " $5 \times 3 \times 3''$
57	±	Emphysema and possible fibrosis
58	+ 5.5	CBPP sequestrum $2 \times 2 \times 1''$
59	—	" " $1\frac{1}{2} \times 1 \times 1''$, pleurisy and adhesions
60	+ 7	" " $5 \times 5 \times 5''$
61	±	" " $3 \times 2 \times 3''$

Table 4—continued

Animal No.	Allergic reaction* mm. increase if positive	Lung lesions at autopsy
62	+ 12	CBPP sequestrum $4 \times 4 \times 3''$
63	+ 6	" " $3 \times 2 \times 2''$
64	+ 6	" " $4 \times 4 \times 5''$
65	+ 6	" " $4 \times 3 \times 3''$
66	+ 7	" " $5 \times 3 \times 3''$ and pleurisy
67	+ 7	" " $5 \times 5 \times 5''$
68	—	" " $3 \times 3 \times 3''$, adhesions and emphysema
69	±	Small CBPP sequestrum, fibrosis and pleurisy
70	—	CBPP sequestrum $3 \times 3 \times 2''$
71	+ 6.5	" " $4 \times 4 \times 4''$
72	+ 9	" " $5 \times 4 \times 4''$
73	+ 5.5	" " $6 \times 5 \times 5''$
74	—	Two CBPP sequestra $4 \times 4 \times 4''$ and pleurisy
75	+ 7	Emphysema
76	+ 5.5	CBPP sequestrum $3 \times 2 \times 2''$
77	—	Small abscess, yellow pus, scar on edge of lung
78	—	Normal
79	+ 12	CBPP sequestrum $4 \times 3 \times 3''$
80	+ 4.5	" " $1 \times 1 \times 1''$
81	—	" " $2 \times 1 \times 1''$
82	±	" " $1\frac{1}{2} \times 1 \times 1''$
83	+ 5	Pleurisy, adhesions and fibrosis
84	+ 13	Small CBPP sequestra both lungs and adhesions
85	+ 12	CBPP sequestrum whole apical lobe, pleurisy and adhesions
86	+ 8	" " $6 \times 4 \times 4''$
87	+ 5	Scar on lung, adhesions and pleurisy
88	+ 8	Lungs normal, adhesions
89	+ 5.5	CBPP sequestrum, fibrosis and pleurisy
90	+ 11	" " $5 \times 5 \times 4''$, fibrosis and pleurisy
91	+ 8	" " and pleurisy
92	+ 7	" " $3 \times 3 \times 2''$
93	+ 5	Pleurisy and fibrosis of whole lung
94	+ 5	Lungs normal, adhesions
95	±	Fibrosis of almost whole lung
96	+ 11.5	CBPP sequestrum $4 \times 4 \times 3''$
97	—	Normal
98	—	" "
99	+ 5.5	Large CBPP sequestrum, fibrosis and pleurisy
100	+ 6	Normal
101	+ 8	Pleurisy and adhesions
102	±	" " " "
103	+ 5	" " " "
104	±	Normal
105	+ 11.5	Small nodule and adhesions
106	+ 7.5	CBPP sequestrum $3 \times 2 \times 2''$
107	+ 7	Old CBPP sequestrum and adhesions
108	+ 5.5	CBPP sequestrum whole lung and fibrosis
109	±	Slight pleurisy
110	+ 6	Large CBPP sequestrum, fibrosis and pleurisy
111	+ 6	Small nodule and adhesions
112	—	CBPP sequestrum, fibrosis and pleurisy
113	±	" " " "
114	+ 4.5	" " " "
115	—	" " $1\frac{1}{2} \times 1 \times 1''$, fibrosis and pleurisy
116	—	" " $3 \times 3 \times 2''$, pleurisy both lungs

* Any reaction less than 3 mm. increase was termed negative. Reactions 3-4 mm. were termed doubtful.

Action of Double Strength Antigen in Natural Cases of CBPP

During the course of the work the opportunity arose to test the antigen in naturally infected cattle in the Karamoja District of Uganda. These cattle, which had been quarantined at Apopoa about 9 months before, had been exposed to a very acute outbreak in which many deaths occurred daily. At the time the test was carried out, however, the disease had reached a chronic stage with very few deaths occurring from CBPP. Double-strength antigen was used.

Of one hundred and thirteen cattle tested, 78 were positive, 14 were doubtful and 21 were negative. Of the 78 positive to the test, 61 had unmistakable lesions of CBPP at autopsy and 14 of the remaining 17 were probable CBPP cases as they had either one or more of the following: lung lesions, lung fibrosis, pleurisy, scars on the lung and adhesions, and/or were positive or doubtful to one or more of the serological tests performed on their sera collected at autopsy. The tests used were the complement fixation test (CFT), the slide agglutination serum test (SAST) and the agar gel precipitin test (AGT) for antigen and antibody. Two (15 and 20) of the remaining 3 animals were normal although 20 had pus in the mediastinal lymph gland and another (75) showed emphysema of the lung.

Of the 14 animals doubtful to the test, 9 had unmistakable lesions of CBPP and the remaining 5 were probably CBPP. One of the latter was normal macroscopically but gave a ++ CFT and a \pm SAST reaction.

Of the 21 animals negative to the test, only 4 showed no evidence of present or past infection and their sera were negative to all tests. Four were doubtful CBPP cases. One of these was normal macroscopically and was serologically negative, but precipitating antigen was present in a mediastinal lymph gland. The remaining 13 cases had unmistakable lesions of CBPP, and of these 3 were missed by all tests (5, 25 and 49). Details of all the tests and autopsy findings are given in Table 4.

DISCUSSION

The antigen prepared from the Gladysdale strain of *M. mycoides* produced larger reactions than the antigen prepared from the T3 strain. This is of interest as it is known that the Gladysdale strain is more virulent than the T3 strain as judged by subcutaneous challenge in cattle; the former strain killed 69 per cent. whereas the T3 strain killed only 23 per cent. of cattle (Gourlay, unpublished). This suggests that the antigen responsible for the allergic reaction may in some way be connected with virulence and further it is interesting to speculate on the role that the allergic response may play in the disease process.

The results obtained with the antigen in the chronic natural cases of CBPP showed that cases positive or doubtful to the test were almost certainly CBPP or "probable CBPP" cases, but cases negative to the test might be old CBPP cases; in other words, false positive results were rare but false negative results were not uncommon. It is important in this context to mention that a comparison of tests performed on the sera of these cattle collected at autopsy (Gourlay, unpublished) showed that of the "definitely CBPP" cases, the allergic reaction detected 74 per cent., the complement fixation test detected 72 per cent., and the slide agglutination test only 35 per cent. Although the allergic reaction was not, therefore, completely satisfactory it detected a promising proportion of these chronic cases

when compared with the other common tests. It is unfortunate that the opportunity has not occurred to test this new antigen in less chronic cases.

From the results presented in this and the previous paper (Gourlay, 1964) it seems that infected or previously infected cattle which fail to react to the allergic antigen fall into two classes, firstly the acutely ill cases with large lung lesions and an excess of circulating antigen in the body, and secondly certain old cases with chronic sequestra. As in these latter cases there is no demonstrable circulating antigen to account for the failure to react, the absence of a reaction may be due to the disappearance of the relevant antibodies.

CONCLUSIONS

The antigen prepared from the more virulent Gladysdale strain of *M. mycoides* produced larger reactions than the antigen prepared from the T3 strain.

Antigen prepared at alkaline pH was more soluble and it was unnecessary to suspend the final product in urea as before; furthermore this antigen produced slightly larger reactions.

The use of double-strength antigen was not warranted as the reactions produced in experimental cattle were not significantly larger than those produced by the single-strength antigen.

The inoculation of 3 times the dose of allergic antigen did not cause a serological response in negative cattle, but did produce a slight boost to the response in 2 out of 4 positive cattle. The inoculation of 3 times the dose of allergic antigen did not appear to produce any significant immunity to subcutaneous challenge.

In chronic cases of contagious bovine pleuropneumonia false positive reactions were rare, but false negative reactions were not uncommon.

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COMPARISON BETWEEN METHODS OF ANTIGEN PREPARATION AND THE USE OF ADJUVANT IN THE DELAYED ALLERGIC SKIN REACTION IN CONTAGIOUS BOVINE PLEUROPNEUMONIA

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INTRODUCTION

Earlier papers (Gourlay, 1962, 1964; Gourlay and Palmer, 1964) described the preparation of an antigen from *Mycoplasma mycoides* and its use both experimentally and in the field as an allergic skin test for the diagnosis of contagious bovine pleuropneumonia (CBPP). It was shown that reactions often did not occur in infected cattle dying with large acute lesions and in certain old cases with chronic sequestra. This paper compares four methods of antigen production and describes the effect of the addition of adjuvants to one of these antigens, prepared by hot phenol extraction, inoculated intradermally or subcutaneously into experimental cattle in an effort to enhance the reaction. In this way it is hoped ultimately to produce an antigen able to detect those infected cattle, which previously gave negative or doubtful reactions to the test.

MATERIALS AND METHODS

Cattle. Experimental cattle were either East African Zebu or crosses between them and breeds of European origin. They were inoculated subcutaneously with *M. mycoides* (T3 or Gladysdale strains) and used at least two months later for testing.

Strains of *M. mycoides*. The Gladysdale strain was used for antigen production.

Extraction of *M. mycoides* antigen. The organisms were grown and harvested as previously described (Gourlay, 1964). Organisms were washed twice in cold barbitol buffer, pH 7.2 (0.2 M) and resuspended in distilled water. The suspension was subjected to 3 cycles of freezing and thawing followed by disintegration, for 5 minutes, at 4°C., at maximum output with an M.S.E. ultrasonic disintegrator.† The suspension was centrifuged at 37,000 G in the cold for 30 minutes. The deposit (cell-residue) was resuspended in distilled water and lyophilized. Extraction with hot or cold phenol was done by the procedures of Westphal, Luderitz and Bister (1952). Both aqueous and phenol phases were dialysed against water until all phenol was removed, and then lyophilized. The proteins thus obtained were resuspended in carbonate/bicarbonate buffer at pH 10 (0.05 M) treated with the ultrasonic disintegrator and stored at 4°C. During the extraction some of the proteins were denatured and formed a solid mass. This material was removed, cut into small pieces and extracted 5 times with 95 per cent. ethyl alcohol to remove the phenol. The residue was lyophilized, resuspended in 0.1M NaOH and kept at 4°C. for one week. After this it was then

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treated by ultrasonic disintegration to form a fine suspension, neutralised with 2N.HCl and dialysed against running water for 2 days. The dialysate was lyophilized and resuspended in carbonate/bicarbonate buffer. Proteins were also obtained as the residue following removal of Boivin antigen prepared by the trichloroacetic acid (TCA) method (Kabat and Mayer, 1961).

Assay method. Antigenic materials (1 mg.) were inoculated in the side of the neck of cattle in doses of 0.1 ml. intradermally, or 0.1 to 2.0 ml. subcutaneously. After intradermal inoculation the increase of skin thickness at time intervals was measured; after subcutaneous inoculation skin and subcutaneous thickness and the area of the lesion were measured. Results are recorded for measurements after one day, unless otherwise stated.

Adjuvants. The paraffin-lanolin emulsion for subcutaneous inoculation was prepared as described by James and Pepys (1956), namely 8 parts light liquid paraffin BP, 1 part anhydrous lanolin BP and 1 part suspension of the protein in buffer. The corn oil* for intradermal inoculation was used in the proportions corn oil 4.5 parts, arlacel A† 0.5 parts, and buffer containing protein 5 parts.

RESULTS

Comparison of Antigens

The proteins prepared by ammonium sulphate precipitation (Gourlay and Palmer, 1965) and by hot phenol extraction were compared, weight for weight, for their ability to induce a delayed hypersensitivity reaction. The results show that protein extracted by hot phenol gave as good or better reaction than the protein prepared with ammonium sulphate (Table 1). A comparison between the proteins prepared by hot and cold phenol extractions showed that the hot phenol protein gave better results (Table 2). The coagulated proteins obtained during the hot phenol extraction induced as good an allergic reaction as the non-coagulated proteins in the hot phenol extract.

The delayed reaction produced by the proteins prepared by TCA precipitation induced larger reactions than the hot phenol extracted proteins (Table 3). As the carbohydrate material was required for further experimentation, and could be obtained only by the phenol extraction method, all further work was done with proteins obtained by this method.

TABLE 1
COMPARISON BETWEEN REACTIONS PRODUCED BY PROTEINS (1 mg.)
PREPARED BY AMMONIUM SULPHATE PRECIPITATION AND HOT PHENOL
EXTRACTION, INOCULATED INTRADERMALLY

Animal No.	Max. increase in skin thickness (mm.)	
	Ammonium sulphate precipitated proteins	Phenol extracted proteins
9120	6.5	10.5
9348	4.5	4.5
9349	3.5	7.0
9525	5.5	7.0

* "Mazola", Brown & Polson, The Strand, London.

† Atlas Chem. Ind., Willmington Delaware, U.S.A.

TABLE 2

COMPARISON BETWEEN REACTIONS PRODUCED BY PROTEINS,
PREPARED BY HOT AND COLD PHENOL EXTRACTIONS,
INOCULATED INTRADERMALLY

Animal No.	Max. increase in skin thickness (mm.)	
	Hot phenol extracted proteins	Cold phenol extracted proteins
9237	9.5	4.5
9374	7	2
9475	9	6
9845	4.5	7.5
9853	4.5	4.5

TABLE 3

COMPARISON BETWEEN REACTIONS PRODUCED BY PROTEINS
PREPARED BY THE TRICHLORACETIC ACID AND HOT PHENOL
METHODS, INOCULATED INTRADERMALLY

Animal No.	Max. increase in skin thickness (mm.)	
	Hot phenol extracted proteins	Trichloroacetic acid precipitated proteins
9721	1.5	3.5
9730	8.5	8.5
9827	3.5	6.5
9837	5	7.5
9840	7.5	9.5

Use of Adjuvants

We tested a variety of materials to determine whether an enhanced allergic reaction could be obtained when injected intradermally or subcutaneously. These materials were dextran (6 per cent.), Shell ondina oil 17, alhydrogel, paraffin-lanolin, DEAE-cellulose, medicinal white oil + 0.4 per cent. lanolin, calcium phosphate gel, glycerol, mucin, adrenalin, carbowax, agar, complete Freund adjuvant and corn oil. Only 2 of these materials appreciably enhanced the allergic reaction and did not give unacceptably large reactions in negative cattle. These were paraffin-lanolin inoculated subcutaneously and corn oil inoculated intradermally.

The results obtained when protein, with and without paraffin-lanolin adjuvant, was injected subcutaneously and intradermally respectively show the increase in size of the reaction due to the adjuvant (Table 4). Unfortunately the adjuvant itself caused moderately large reactions in some cattle. When this adjuvant was

TABLE 4

COMPARISON BETWEEN REACTIONS PRODUCED BY HOT PHENOL EXTRACTED PROTEINS INOCULATED INTRADERMALLY, PROTEINS WITH PARAFFIN-LANOLIN ADJUVANT INOCULATED SUBCUTANEOUSLY AND ADJUVANT ALONE INOCULATED SUBCUTANEOUSLY

Animal No.	Side of neck	Max. increase in skin thickness (mm.)		
		Proteins	Proteins + adjuvant	Adjuvant
7898	L	2	24	8
	R	2	15	—
8719	L	—	22.5	0
	R	3.5	—	0
8531	L	5	11	3.5
	R	0	7	2
9237	L	—	33	4
	R	—	10	7
9497	L	4.5	27.5	0
	R	4.5	0	0
9687	L	—	16.5	5
	R	—	19	14
9854	L	—	13.5	—
	R	—	11	2

TABLE 5

COMPARISON BETWEEN REACTIONS PRODUCED BY THE INTRADERMAL INOCULATION OF PROTEINS IN BUFFER, PROTEINS IN CORN OIL AND CORN OIL ALONE

Animal No.	Increase in skin thickness (mm.)		
	Proteins in buffer	Proteins in corn oil	Corn oil
8859	1	5	0
9343	3.5	8	0.5
9526	3	7	1.5
9656	1.5	7	0.5
9665	3.5	7.5	1.0
9823	1.5	5	1.0
9827	3.5	9	1.0
9837	5	8.5	0
9840	7.5	10.5	1.5

inoculated intradermally it produced very large reactions. The effect of the volume of adjuvant inoculated subcutaneously was examined, the concentration of protein per injection being standardised at 1 mg. The results showed that 1.0 ml. gave the largest reaction, better even than the 2 ml. amount, while the adjuvant itself induced a reaction which was smaller and flatter in appearance than the reaction produced by the adjuvant and protein.

Corn oil gave the better results, when inoculated intradermally, as it enhanced the reaction and, by itself, produced only a small reaction (Table 5). A total of 70 cattle was inoculated with corn oil alone. The largest reaction observed was 3.5 mm. increase in skin thickness in only 1 animal; the average increase was 1.4 mm.

When the proteins in corn oil adjuvant were inoculated subcutaneously in immune cattle they produced a soft diffuse reaction which was difficult to distinguish: in negative cattle they gave no reaction by this route.

Certain dyes were used and were abandoned as they either gave large reactions in negative cattle or were difficult to work with and did not help in distinguishing a positive reaction due to the variation in colour of the cattle.

Size of Reaction in Relation to Time after Inoculation

Immune cattle inoculated with antigen in corn oil intradermally and in paraffin-lanolin subcutaneously, were examined at intervals and measurements of reactions recorded. The maximum reactions for corn oil antigen occurred on the first or second day, usually the latter, and then slowly regressed. With the paraffin-lanolin adjuvant the reactions were large and soft after 1 day and then became harder and smaller in area but more defined on the second day, after which they regressed slowly becoming smaller and harder with time.

DISCUSSION

The preparation of proteins by phenol extraction had certain advantages over the previous method of ammonium sulphate precipitation. A higher yield could be obtained (unpublished data) and the end product gave larger skin reactions than an equal weight of the ammonium sulphate precipitated protein. A further advantage of this method was that the carbohydrate from the extraction could be retained and utilized to give an immediate allergic reaction (Shifrine and Gourlay, 1965). It was noticed incidentally that the protein also gave, in addition to the delayed reaction, a slight immediate reaction 1 to 2 hours after inoculation, which disappeared in about 4 hours. This probably was due to contaminating carbohydrate in the preparation.

Denaturation of some of the protein by phenol did not affect its ability to elicit an allergic response. Denaturation of the protein by heat (Gourlay, unpublished data) also did not affect its reactivity. Our results are in agreement with those of Gell and Benacerraf (1959) who found that heat denatured proteins were as effective as native proteins in provoking delayed hypersensitivity in guinea pigs.

The paraffin-lanolin adjuvant when used subcutaneously enhanced the reaction, but it also produced a moderately severe tissue reaction in some negative cattle. It could only be used, therefore, if control paraffin-lanolin adjuvant without protein was inoculated at the same time and the reactions compared. Corn oil gave only small reactions in negative cattle possibly because of its vegetable nature and its rapid absorption. It did however enhance the reaction in infected cattle giving a larger swelling. If the opportunity arises it is hoped to try this antigen in the field on a number of naturally infected cattle to see whether its use will help in detecting those infected cattle which with the previous test gave negative results.

The use of skin tests, with antigens as described here, prepared from the appropriate organisms may be of value in detecting mycoplasma infections in other animals, birds and man.

CONCLUSIONS

Proteins extracted from *M. mycoides* by hot phenol or trichloroacetic acid gave larger delayed allergic reactions in immune cattle than proteins extracted by cold phenol or ammonium sulphate. The addition of paraffin-lanolin adjuvant to the proteins extracted by hot phenol, resulted in an enhanced delayed reaction in immune cattle when inoculated subcutaneously or intradermally. Unfortunately this material produced large reactions in some negative cattle when inoculated intradermally. When inoculated subcutaneously it still produced reactions in some negative cattle, but they were less severe.

The addition of corn oil as an adjuvant was more satisfactory. When inoculated intradermally it gave only small reaction in a proportion of negative cattle, and appreciably enhanced the reaction in immune cattle.

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THE IMMEDIATE TYPE ALLERGIC SKIN REACTION IN
CONTAGIOUS BOVINE PLEUROPNEUMONIA

By

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INTRODUCTION

During a recent study (Gourlay and Shifrine, 1965) of the delayed allergic skin reaction in contagious bovine pleuropneumonia (CBPP) an immediate type allergic skin reaction was observed. This paper describes work carried out on this immediate type reaction.

MATERIALS AND METHODS

Cattle. Experimental cattle were crosses between East African Zebu and breeds of European origin. They were inoculated subcutaneously with *M. mycoides* broth culture and 2 months or more later were used for skin testing.

Strain of M. mycoides. The Gladysdale strain was used throughout this work.

Extraction procedures. *M. mycoides* was grown, harvested, and extracted by Westphal's hot and cold phenol methods, as previously described (Gourlay and Shifrine, 1965).

Analytical methods. Lipid was extracted with chloroform/methanol (2:1) at 60°C. under reflux for 1 hour. Protein was estimated by the Folin-Ciocalteu method and carbohydrate by the Anthrone method (Kabat and Mayer, 1961). The protein and carbohydrate determinations were done on defatted material. Periodate oxidation was done in an excess of 0.01 M sodium metaperiodate for 14 days at room temperature in the dark. Papain proteolysis was carried out as described by Kimmel and Smith (1957); Trypsin digestion was estimated by the procedure described in Kabat and Mayer (1961).

Assay method. Antigenic materials prepared by phenol extraction, and further treated as described in "Results", were inoculated intradermally in the side of the neck of cattle. The dose used was 1.0 mg. in 0.1 ml. of carbonate/bicarbonate buffer (0.05 M) pH 10. Skin thickness measurements were recorded at intervals after inoculation.

Serological tests. These were carried out as described previously (Gourlay, 1964).

RESULTS

The lipopolysaccharides (LPS) extracted by the hot and cold phenol procedures were compared for their ability to elicit an immediate reaction. The hot phenol preparation induced as large, or larger immediate reactions, estimated by the increase in skin thickness, than the cold phenol preparation (Table 1); only the latter produced a delayed allergic reaction (Table 2). In view of these results all further studies were done with material extracted with hot phenol. The composition of the antigenic materials obtained by the hot phenol extraction is shown in Table 3. The immediate reaction was frequently visible within 10 minutes of

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TABLE 1
COMPARISON BETWEEN LIPOPOLYSACCHARIDE EXTRACTED WITH
HOT AND COLD PHENOL IN ELICITING AN IMMEDIATE REACTION

Animal No.	Max. increase in skin thickness (mm.) read after 1 and 2 hours	
	Hot phenol extracted LPS	Cold phenol extracted LPS
8429	3	3.5
8475	6	7
8510	6.5	3
9237	3.5	3
9504	3	2.5
9726	4	2.5
9845	4.5	4
9853	2.5	2

TABLE 2
THE SIZE OF REACTIONS PRODUCED BY LIPOPOLYSACCHARIDE
EXTRACTED BY HOT AND COLD PHENOL, IN RELATION TO TIME
AFTER INOCULATION

Animal No.		Increase in skin thickness (mm.) Hours after inoculation					
		1	2	3	24	48	72
9237	Hot phenol	3	3.5	2.5	1	1	1
	Cold phenol	1.5	3	2.5	9.5	4.5	2
8429	Hot phenol	3	3	2.5	2	2	1
	Cold phenol	3	3.5	3.5	12.5	5.5	2.5

inoculation and reached a peak between 1 and 2 hours. The swellings were soft and oedematous and were larger than the skin thickness increase would indicate. When adrenalin (0.1 mg.) was injected into 3 cattle with the LPS it completely suppressed the immediate reaction.

In the agar gel precipitin test (AGT) the hot phenol extracted LPS gave identical precipitin lines to the polysaccharide isolated from urine of infected cattle (Gourlay and Palmer, 1965), using hyperimmune serum prepared in sheep with *M. mycoides* (Gourlay, 1964).

Further studies on the LPS were done in order to determine the effects of different chemical and enzymatic treatments on its activity. Materials before and after treatment were checked for activity in inducing an immediate skin reaction and also compared in the AGT. The LPS was extracted with chloroform, methanol and the residue and original material were inoculated into cattle. The

residue had 62.5 per cent. of the activity (measured in terms of increase in skin thickness in 2 animals) of the original LPS and gave precipitin bands in the AGT identical to the original LPS. The activity of the LPS after periodate oxidation was 49.5 per cent. of the untreated LPS (average in 4 animals). The periodate treated material gave no precipitin lines in the AGT whereas the untreated LPS gave strong lines. The activity of the LPS, in the skin test, after treatment with either papain or trypsin was compared in 3 animals with untreated LPS and was found to be unaltered. Controls of proteins, extracted from *M. mycoides*, were shown to be digested with both enzymes. In the AGT and LPS treated with either papain or trypsin gave precipitin lines identical to the untreated LPS. A solution of LPS in water was placed in a boiling water bath for 30 minutes. Its activity in 3 animals was unaltered in both the skin test and in the AGT. The effects of the treatments of the LPS on its activity in the skin test and AGT are summarized in Table 4.

Two preparations of LPS were further purified by the method of Burton and Carter (1964) and 5 fractions obtained. From both preparations fractions I, II and III reacted in the skin test whereas fractions V and VI gave little or no reactions. The active fractions gave precipitin lines identical to the original LPS.

During this work a number of normal cattle were inoculated with the LPS from *M. mycoides*. Some of these cattle gave strong immediate skin reactions. Sera from these cattle were positive to the complement fixation test, and also gave precipitin bands against hyperimmune *M. mycoides* sheep serum. Two of the cattle were killed and their organs cultured. A gram positive bacillus was

TABLE 3
CHEMICAL COMPOSITION OF ANTIGENS PREPARED BY
HOT PHENOL EXTRACTION OF *M. mycoides*

Antigen	Protein	Per cent carbohydrate	Lipid
Protein	74	0.9	20
Lipopolysaccharide	18	32	25

TABLE 4
THE EFFECTS OF VARIOUS TREATMENTS OF THE LIPOPOLYSACCHARIDE
ON ITS ACTIVITY IN THE IMMEDIATE ALLERGIC REACTION AND THE
AGAR GEL TEST

Treatment	Activity after treatment	
	Skin test	Agar gel test
Chloroform-methanol	Reduced	Not changed
Periodate	Reduced	Abolished
Papain	Not changed	Not changed
Trypsin	Not changed	Not changed
Heat	Not changed	Not changed

isolated from them and antigen prepared from this organism cross reacted in the AGT with the hyperimmune sheep serum against *M. mycoides*.

DISCUSSION

As the skin reaction commences within minutes of inoculation of the antigen and is maximal within 2 hours and as the reaction can be suppressed with adrenalin it seems to be a true type of an immediate hypersensitive reaction.

The cell residue of *M. mycoides* contains antigens which can induce both delayed (Gourlay and Shifrine, 1965) and immediate allergic reactions in immune cattle. It seems probable that the proteins extracted with hot phenol are responsible for the delayed reaction as this antigen contains 74 per cent. protein and only about 1 per cent. carbohydrate. The results presented in this paper indicate that the LPS extracted by the hot phenol method is responsible for the immediate allergic reaction. Extraction of the lipid or oxidation of the carbohydrate moiety of the LPS reduces its activity whereas enzymatic digestion of the proteins of the LPS and boiling of the LPS have no effect on its activity indicating that the proteins of the LPS are not concerned with the immediate reaction. The effects of the treatments of the LPS on its activity in the AGT and skin test seem to indicate that the antigens responsible for the skin reaction are also precipitogens.

When a LPS from *E. coli* was purified by Burton and Carter (1964) they found lipopolysaccharides in fractions III and VI while the other fractions contained peptides and nucleic acids. Following purification of the LPS from *M. mycoides* by their method the skin test activity resided in fractions I, II and III. This indicates that the purification was incomplete and that some LPS remained in fractions I and II. It is possible that the LPS of *M. mycoides* is sufficiently different from that of *E. coli* to account for the differences in results.

The LPS from *M. mycoides* reacted in animals infected with a gram positive organism indicating that the LPS is not specific, having antigenic components in common with at least one other bacterial species. This is being currently studied by us.

CONCLUSIONS

Lipopolysaccharide-protein, extracted with hot phenol from cells of *M. mycoides*, induced an immediate allergic reaction in cattle previously inoculated with *M. mycoides*. The activity of this material was reduced after extraction with chloroform/methanol, or after periodate oxidation, but was unaltered after treatment with trypsin and papain, or after boiling. The lipopolysaccharide, therefore, appears to be responsible for the immediate allergic reaction. This material was not specific as it contains antigenic components in common with another bacterium isolated from cattle.

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SEROLOGICAL RELATIONSHIPS BETWEEN
MYCOPLASMA MYCOIDES AND OTHER BACTERIA

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Summary

Ten bacterial species were tested for serological relationships with *Mycoplasma mycoides* var. *mycoides*. *Corynebacterium xerosis*, *Aeromonas hydrophila* and *Escherichia coli* had certain antigenic components in common with *M. mycoides* using the agar gel precipitin technique. A *Streptococcus* of Lancfield group K cross-reacted with *M. mycoides* in the complement fixation test. These bacteria also elicited immediate allergic skin reaction in cattle sensitized with *M. mycoides*. *Pneumococcal* S XIV antiserum reacted in the complement fixation test with *M. mycoides* antigen.

The antigens responsible for the observed cross-reactions were polysaccharides.

Introduction

There are reports in the literature on the serological relationship between *M. mycoides* var. *mycoides* and other microorganisms (Heslop 1922, Turner 1956, Provost 1958, Heidelberger 1960, Shifrine and Gourlay 1965, Gourlay and Shifrine 1966, Gourlay and Brockelsby 1966) including other species of mycoplasma (Villemot and Provost 1959, Lemcke 1965, Lemcke *et al.*, 1965).

Previous work (Gourlay and Shifrine, 1966) indicated that the substances in bacteria which cross-react with *M. mycoides* are polysaccharides. In this paper, we confirm this and also report on a limited survey in which a number of bacteria were examined for their serological relationship with *M. mycoides*.

Materials and Methods

Bacterial Cultures. The cultures which were used in this study are listed in TABLE 1.

Culture Media. All bacteria were grown in Nutrient Broth (Oxoid) with 1% glucose, for one or two days at 37°. *A. hydrophila* and *E. coli* were grown in the following medium: NH_4Cl 1g, Na_2HPO_4 7.2g, KH_2PO_4 1g, $\text{MgSO}_4 \cdot \text{H}_2\text{O}$.0.2g, glucose 1g, Tween 80 0.01g, in 1 liter distilled water.

M. mycoides was grown in modified Newings tryptose broth (Gourlay, 1964) for three days. Cells were collected by centrifugation and lyophilized.

Antigens. Antigens were prepared by suspending 40 mg of lyophilized bacteria in 1.0 ml of water, alternately freezing (in acetone and dry ice) and thawing (in warm water) ten times, followed by ultrasonic disintegration for ten minutes. The supernatant fluid after centrifugation was used for agar gel diffusion test (AGT) or for skin tests.

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TABLE 1
BACTERIAL CULTURES USED IN THIS STUDY

Bacteria	Number or designation	Source
<i>Micrococcus varians</i>	2565	Dept. Bacteriology,
<i>Corynebacterium xerosis</i>	2671	University of California,
<i>Bacillus megatherium</i>	2007	Davis, Cal.
<i>Sarcina lutea</i>	2053	" " "
<i>Pseudomonas aeruginosa</i>	2603	" " "
<i>Proteus vulgaris</i>	2017	" " "
<i>Aeromonas hydrophila</i>	2329	" " "
<i>Escherichia coli</i>	2125	" " "
<i>Escherichia coli</i>	08:K87(B)K88,a,b(L):H19	W. J. Sojka, Central Vet.
<i>Escherichia coli</i>	08:K87(B)K88,a,c(L):H19	Lab. Weybridge, Surrey,
<i>Escherichia coli</i>	0141:K85,a,b(B):H19	England.*
<i>Escherichia coli</i>	0138:K81(B):NM	" " "
<i>Escherichia coli</i>	0139:K82(B)	" " "
<i>Escherichia coli</i>	0141:K89(B)K88,a,c(L):H19	" " "
<i>Escherichia coli</i>	078:K80(B)	" " "
<i>Streptococcus sp.</i>	Lancfield "K" 12399	American Type Culture Collection.

* These cultures were received from Dr. Kramer, Veterinary College, Colorado State University.

"Polysaccharide" antigens from *M. mycoides* and other bacteria were prepared by extracting cells with hot phenol by the method of Westphal, Luderitz and Bister as previously described (Gourlay and Shifrine, 1965), dialyzing the water phase to remove any residual phenol, and lyophilizing. The product obtained by hot phenol extraction is referred to as "polysaccharide," although it is not pure. The partial chemical composition of *M. mycoides* "polysaccharide" is given elsewhere (Shifrine and Gourlay, 1965).

Pneumococcal antigen S XIV, prepared by Dr. Heidelberger, was received from Dr. M. J. How, Department of Chemistry, University of Birmingham.

Antisera. Hyperimmune *M. mycoides* antisera (x') was prepared in sheep by intravenous inoculations as described previously (Gourlay, 1964).

Rabbits were immunized with *M. mycoides* "polysaccharide" and pneumogalactan (PG)* (Shifrine and Gourlay, 1965) over a period of a year, using both intramuscular (with Freund's adjuvant) and intravenous routes. Rabbits were also inoculated with streptococcal culture (Lancfield group K) with Freund's adjuvant intramuscularly three times at biweekly intervals, followed by three intravenous inoculations, and bled one week after the last injection. Each inoculum contained 20–30mg (dry weight) of antigen.

Antisera against streptococci of the different Lancfield groups were purchased from Burrows Wellcome & Co. (London).

Pneumococcal S XIV antiserum, prepared by Dr. Heidelberger, was received from Dr. How.

Serological Tests. The agar gel test (AGT) was performed as described by Gourlay, (1964). Precipitin bands were stained for protein with Amidoblack (Grabar and Burtin, 1964), and for carbohydrate by the method of Stewart-Tull (1965). The Cambell and Turner complement fixation test (CFT) was carried out as described previously (Gourlay, 1965).

Skin Tests. For skin testing *M. mycoides*-sensitized cattle, we used cattle

* PG was obtained from Hoffmann-La Roche Inc., Nutley, N. J.

previously vaccinated with *M. mycoides* (T_1 or T_2) culture vaccine (Brown, Gourlay and MacLeod, 1965) and challenged subcutaneously six weeks later with *M. mycoides* (T_3) culture. Another group of cattle was inoculated intramuscularly twice at a 4-weekly interval with T_3 *M. mycoides* dried culture (inactivated at 56° for 1 hr) and challenged subcutaneously six weeks later with *M. mycoides* (T_3) culture.

Normal cattle at Muguga were skin-tested with *M. mycoides* "polysaccharide." The cattle which were negative for the skin test were used as normal controls.

For skin testing, antigenic materials (1 mg) in a dose of 0.1 ml were inoculated intradermally in the side of the neck of experimental or control cattle. The increase in skin thickness was measured after two to three and after 24 hours. Results are reported as the net increase in skin thickness.

Results

Of the ten species studied, *Escherichia coli*, *Aeromonas hydrophila* and *Corynebacterium xerosis* gave precipitin lines with *M. mycoides* antisera. Control nutrient broth medium (at four times concentration) did not form any lines with this serum. *M. mycoides* "polysaccharide" formed two strong lines against homo-

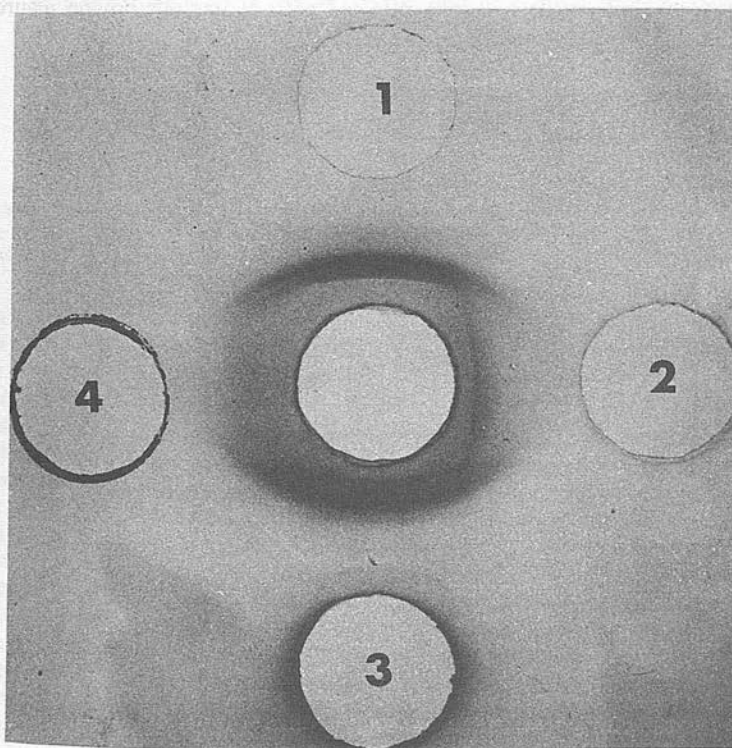


FIGURE 1. Comparison between precipitin bands produced by: (1) *M. mycoides* "polysaccharide" (1 mg/ml); (2) *E. coli* grown in nutrient broth with glucose (40 mg/ml); (3) *M. mycoides* whole cells (5 mg/ml) and (4) *E. coli* grown in nutrient broth without glucose (40 mg/ml). Center well contains *M. mycoides* antiserum (x').

logous antiserum (x') (Gourlay, 1965). *E. coli* (2125) formed a line of partial identity with one of the precipitin lines of the "polysaccharide" from *M. mycoides*. (FIGURE 1). *A. hydrophila* formed a line of partial identity with the second precipitin line (FIGURE 2), and *C. xerosis* formed lines of identity with both lines of *M. mycoides* extract. (FIGURE 3).

Precipitin lines were formed also after these bacterial antigens had been placed in a boiling water bath for 30 minutes. "Polysaccharide" (10 mg/ml) from *E. coli* (2125) and *C. xerosis* formed lines similar to the ones produced with whole cells and *M. mycoides* antisera. "Polysaccharide" (10 mg/ml) from *A. hydrophila* did not form a precipitin line with *M. mycoides* antiserum. When the broth in which this organism was grown was precipitated with twice its volume with cold ethanol (95%), the product obtained formed a precipitin line against *M. mycoides* antiserum. The product obtained when control medium was precipitated with alcohol did not form precipitin lines with *M. mycoides* antiserum.

Precipitin lines produced by these three bacteria with *M. mycoides* antiserum gave a positive carbohydrate stain.

To avoid possible, though unlikely, cross-reaction between media components and *M. mycoides* immune sera, we used both immune sera from cattle naturally infected with contagious bovine pleuropneumonia, and *A. hydrophila* and *E. coli* (2125) grown in a defined medium.

Sera from three cattle naturally infected with contagious bovine pleuropneu-

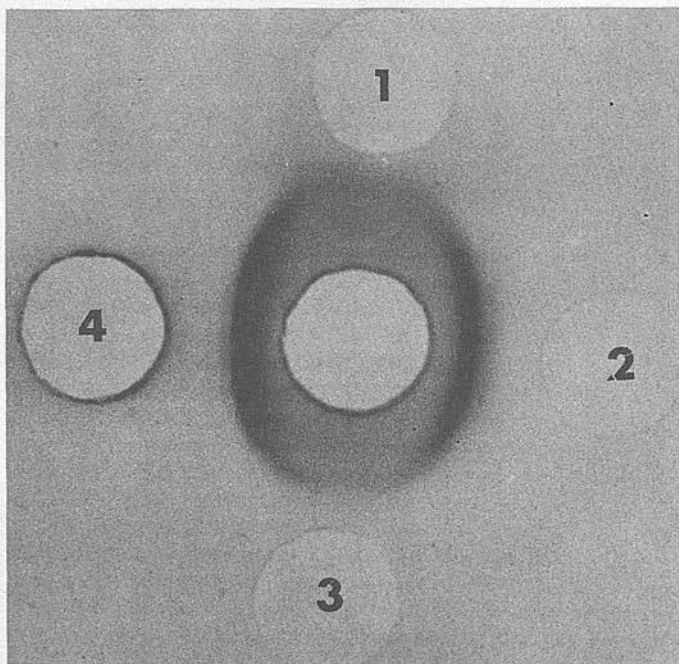


FIGURE 2. Comparison between precipitin bands produced by: (1) *A. hydrophila* grown in nutrient broth (40 mg/ml); (2) *M. mycoides* "polysaccharide" (1 mg/ml); (3) *A. hydrophila* grown in nutrient broth with glucose (40 mg/ml) and (4) *M. mycoides* whole cells (5 mg/ml). Center well contains *M. mycoides* antiserum (x').

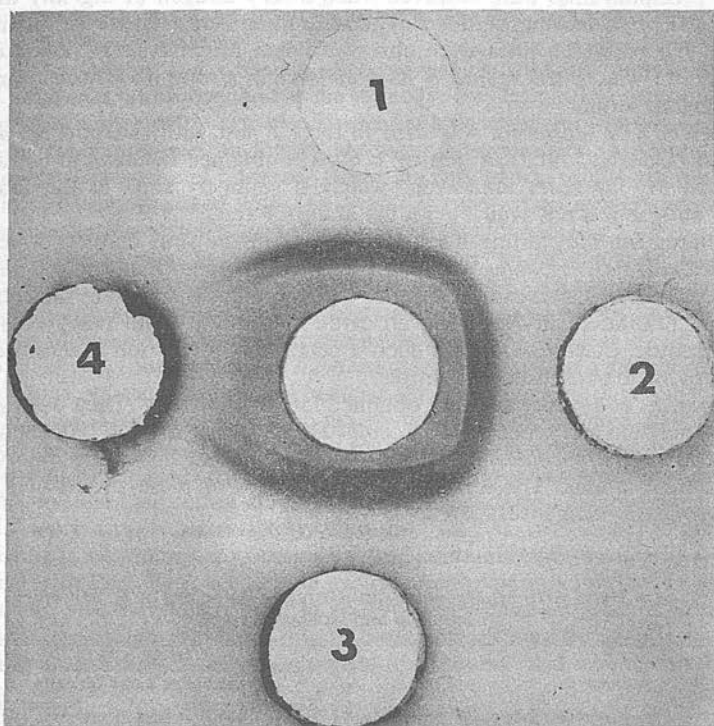


FIGURE 3. Comparison between precipitin bands produced by: (1) *M. mycoides* "polysaccharide" (1 mg/ml); (2) *C. xerosis* "polysaccharide" (20 mg/ml); (3) *M. mycoides* (5 mg/ml) and (4) *Strep.* group K (40 mg/ml). Center well contains *M. mycoides* antiserum (x').

monia gave precipitin lines when tested with *C. xerosis*, *A. hydrophila* and *E. coli* (2125); no lines were formed with nutrient broth (Oxoid) (4x) or bovine serum. These sera gave the same number of bands as the sheep antisera.

A. hydrophila and *E. coli* (2125) were grown each in a liter of defined medium for one day at 37°. The culture broths were lyophilized, resuspended each in 50 ml of water, dialyzed overnight to remove the salts, and lyophilized. The products thus obtained were each resuspended in water and frozen and thawed ten times. When tested in AGT, the antigens gave a line against *M. mycoides* antiserum (x') identical to that obtained with *A. hydrophila* or *E. coli* (2125), respectively, which had been grown in nutrient broth. These antigens also gave precipitin lines against PG and hot phenol "polysaccharide" antisera.

When *M. mycoides* antigen was tested in the CFT against *Streptococcus* grouping sera (Lancfield) a positive test was obtained with group K. Sera from two rabbits immunized with group K *Streptococcus* culture gave a CF serum titer of 1/20 with *M. mycoides* antigen. Unfortunately, the two rabbits used for hyperimmunization died at this point. When this culture was used as antigen and titrated in the CFT against *M. mycoides* antiserum at a concentration of 1 mg/ml, it gave a serum titer of 1/160. *Streptococcus* group K "polysaccharide" did not form any precipitin lines in AGT, even when concentration as high as 120 mg/ml was used with *M. mycoides* antiserum.

No precipitin lines were observed when S XIV antigen (1 mg/ml) was tested against *M. mycoides* antiserum (x'); strong lines were formed with homologous serum. No precipitin lines were observed when *M. mycoides* "polysaccharide" (1 mg/ml) was tested against S XIV antiserum; strong lines were formed with homologous serum.

When S XIV antiserum was used in the CF test with standard *M. mycoides* antigen (Gourlay, 1965), a titer of 1/60 was obtained identical to the titer with standard *M. mycoides* antiserum, which is routinely used in this laboratory. S XIV antigen was not available for use in the CF test.

Antigens for skin testing were prepared from the four cultures which cross-reacted with *M. mycoides* antiserum and tested in normal and *M. mycoides*-sensitized cattle. Three of the bacterial antigens elicited a positive immediate skin reaction (TABLE 2) in the sensitized cattle and only a slight reaction in normal control cattle. *C. xerosis* did not elicit a positive skin reaction in sensitized cattle as compared to nonsensitized controls.

Known *E. coli* serotypes were obtained late in our study. They were grown in defined medium and processed as *E. coli* (2125) for use in AGT. All cultures gave lines against *M. mycoides* antisera. However, different lines were observed with the different strains. The serological relationships of these strains of *E. coli* to *M. mycoides* is being investigated.

TABLE 2
SKIN TESTS WITH *M. Mycoides* AND BACTERIAL ANTIGENS IN *M. Mycoides*
SENSITIZED AND NORMAL CATTLE

Antigen	Max. increase in skin thickness (mm) read after 2 and 24 hours			
	Sensitized cattle		Normal cattle	
	2 hours	24	2 hours	24
<i>M. mycoides</i> "polysaccharide" (1 mg)	12.5	8.5	4.5	3.5
<i>A. hydrophila</i> (40 mg)	11.0	10.5	3.5	1.0
<i>E. coli</i> (40 mg)	8.0	5.5	4.5	3.5
<i>C. xerosis</i> (40 mg)	4.5	4.0	4.5	2.5
<i>Strep.</i> group K (40 mg)	10.0	4.0	—	—

Discussion

Agar gel diffusion and complement fixation tests have demonstrated that *M. mycoides* cross-reacts with four out of ten bacterial species and Pn.S XIV antiserum, studied. These results, together with other reports (Introduction) of serological relation of *M. mycoides* with a variety of microorganisms, indicate the prevalence of such antigenic relationship.

In view of these observations, it is not surprising that positive skin tests and CF tests were obtained with cattle which were observed to be free of contagious bovine pleuropneumonia on autopsy (Gourlay and Shifrine, 1966). However, results obtained in Africa (Provost *et al.*, 1964, Gourlay and Shifrine, 1966) are at variance with reports from Australia (Hudson, 1965, Ladd, 1965), where the incident of so called "false positive" CF reactions from normal cattle is less than 0.1%. At this time, no explanation can be offered for this discrepancy unless it is associated with the sensitivity of the CFT.

The cross-reacting antigens were found to be polysaccharides by virtue of their presence in the aqueous phase of hot phenol extracts of both *M. mycoplasma* and

the other bacteria, heat stability, positive reactions with a carbohydrate stain in AGT, and ability to elicit an immediate hypersensitive skin reaction in cattle sensitized with *M. mycoides*.

Previously (Shifrine and Gourlay, 1965) it was shown that "polysaccharide" from *M. mycoides* is responsible for the immediate allergic skin reaction. It also has been shown that cattle sensitized with bacteria antigenically cross-reactive with *M. mycoides* produce an immediate allergic response on skin-testing with *M. mycoides* (Gourlay and Shifrine, 1966). In this paper, results are presented that indicate cattle sensitized with *M. mycoides* produce an immediate skin reaction on inoculation with bacteria that are serologically related.

The above results point to the inadvisability of using an antigen of *M. mycoides* which contains "polysaccharide" for skin-testing and other serological tests. Studies are in progress with a purified protein antigen from *M. mycoides* for use in diagnosis of contagious bovine pleuropneumonia.

It is of interest to note that *M. mycoides* antisera reacts with a variety of galactose containing polysaccharides and bacteria (Shifrine and Gourlay, 1965, Gourlay and Shifrine, 1966) similar to the reactivity of pneumococcal S XIV antisera (Heidelberger, 1960); S XIV antiserum reacts with *E. coli* among other bacteria (Kabat and Meyer, 1961). In fact, the polysaccharides which were tested to date against *M. mycoides* antiserum (Gourlay and Shifrine, 1966) also cross-react with Pn.S XIV antiserum. It seems likely, therefore, that *M. mycoides* has antigenic determinants in common with other galactose-containing polysaccharides which cross-react with Pn.S XIV, e.g., blood groups. As the polysaccharides of *M. mycoides* and *M. pneumoniae* (Lemcke *et al.*, 1965) also have antigenic determinants in common, the antigenic relationship to blood groups may be of importance in the pathogenesis of both human atypical pneumonia and contagious bovine pleuropneumonia.

Provost, Perreau and Queval (1964) showed that *M. mycoides* contains a Forssman antigen. Thus, it cannot be excluded that the antigens studied for cross-reactivity with *M. mycoides* contain Forssman antigens.

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COMPARISON BETWEEN SOME DIAGNOSTIC TESTS FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA

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INTRODUCTION

Serological diagnosis of Contagious bovine pleuropneumonia (CBPP) has been mainly by the Complement fixation test (CFT), but also to a lesser extent by the Slide agglutination serum test (SAST) and the Slide agglutination blood test (SABT). Recently the Agar gel double diffusion precipitin test (AGT) and the Allergic reaction (Gourlay, 1964a; Gourlay and Palmer, 1964), still in its experimental stages, have been added to this list. During work on the allergic reaction the opportunity was taken to compare the results of these serological tests with the autopsy findings of the animals tested. The SABT, however, was not performed after the first series of tests as it was found to be considerably less reliable than the SAST.

MATERIALS AND METHODS

Cattle. All the cattle were Zebu animals in the Karamoja district of Uganda and were in herds infected with Contagious bovine pleuropneumonia. In January, 1963 tests were performed and sera collected from local Government-owned cattle, held at Iriri Quarantine, which had become infected with the disease. At this date the disease was in the acute stages and numerous deaths had occurred and were occurring daily. In April, 1963 the Iriri Quarantine was again visited and the last few remaining cattle from the outbreak were tested. At this time a visit was also made to Apopoa only a few miles from Iriri where a group of recently infected African-owned cattle were quarantined. These cattle were in the course of a very acute outbreak and many deaths were occurring amongst them daily. This same group of cattle was revisited in December, 1963 by which time the disease had reached a chronic stage and very few deaths were occurring from it.

Experimental procedure. All cattle were inoculated intradermally with allergic antigen and examined for reactions 24 hours later. The animals were then shot and blood collected for serum, followed by autopsy examination of the lungs. Serum samples were taken to the laboratory where all other tests were performed. Autopsy specimens were pieces of lung lesion if any, and if not, a mediastinal lymph node. At autopsy all cattle were classified according to the condition of the lungs as "definite CBPP cases", "doubtful CBPP cases" and "negative". Cases were only classed as "definite CBPP cases" if the lesions were unmistakably CBPP, e.g. typical acute CBPP lesions, typical sequestra. "Doubtful CBPP cases" included all cases that did not fall into the previous group and in which any one or any combination of the following lesions were present: lung lesions, pleurisy, lung fibrosis, scars on the lungs and adhesions. Only cases with macroscopically normal lungs and no signs of previous infection were termed "negative" and they were only classified as "definitely not CBPP" following the results of all tests.

In a preliminary experiment 7 cattle (3 previously inoculated endobronchially and 4 subcutaneously with *M. mycoides*) and 3 negative control cattle were bled immediately before inoculation with 0.3 ml. of allergic antigen (single strength) and again

TABLE I
COMPARISON BETWEEN SOME DIAGNOSTIC TESTS FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA, JANUARY 1963, IRIRI.

"Definite" Cases.

Animal No.	CFT*	SAST*	Allergic reaction* mm. increase if + ve	AGT* for AG	AGT* for AB	Autopsy specimens AGT for AG	Lung at autopsy
1	+	+	8.5	-	+	++	Encapsulated CBPP lesion $4'' \times 3'' \times 2''$.
8	+	+	7	+	+	++	Acute CBPP lesion, whole diaphragmatic lobe.
13	+	+	11.5	+	+	++	Encapsulated CBPP lesion, whole diaphragmatic lobe.
14	+	+	11	+	+	++	Encapsulated CBPP lesion, whole lung.
18	+	+	-	+	+	++	Acute CBPP lesions in diaphragmatic and cardiac lobes.
19	+	+	7.5	-	+	++	Encapsulated CBPP lesion in both lungs.
21	+	+	-	+	+	++	Acute CBPP lesion, whole diaphragmatic and cardiac lobes.
24	+	+	5	+	+	++	Acute CBPP lesion, whole diaphragmatic lobe.
26	+	+	6.5	+	+	++	Encapsulated CBPP lesion, whole diaphragmatic lobe.
27	+	+	5.5	+	+	++	Encapsulated CBPP lesion $7'' \times 5'' \times 5''$.
28	+	+	5	-	+	++	Acute CBPP lesion, whole lung.
29	+	+	6.5	+	+	++	Encapsulated CBPP lesion, whole diaphragmatic lobe.
30	+	+	5.5	+	+	++	Acute CBPP lesion, whole lung.
31	+	+	-	+	+	++	Acute CBPP lesion, whole diaphragmatic lobe.
34	+	+	5	+	+	++	Encapsulated CBPP lesion, whole diaphragmatic lobe.
38	+	+	4.5	+	+	++	Acute CBPP lesion $3'' \times 3'' \times 3''$.
39	+	+	6	+	+	++	Encapsulated CBPP lesion, apical and cardiac lobes, each $5'' \times 3'' \times 3''$.
Total tests	17	17	17	17	17	17	
+	17	16	14	13	7	17	
±	0	0	0	0	2	0	
-	0	1	3	4	8	0	

Table 1—continued
"Doubtful" Cases.

Animal No.	CFT*	SAST*	Allergic reaction† mm. increase if +ve	AGT* for AG	AGT* for AB	Autopsy specimens AGT for AG	Lung at autopsy
7	—	—	+	—	—	—	Scars on lung and adhesions.
9	—	—	+	—	—	—	Numerous calcified hydatid cysts.
11	—	—	±	—	—	—	Normal.
22	—	—	±	—	—	—	A few small abscesses.
23	—	—	+	—	—	—	Scars on lung and adhesions.
32	—	—	+	—	—	—	Abscesses in both lungs with yellow pus.
33	—	—	±	—	—	—	Normal.
35	—	—	±	—	—	—	Severe pleurisy, both thoracic cavities.
37	—	—	—	—	—	—	Normal.
Total tests	9	9	9	9	9	9	
+	1	2	4	2	2	2	
±	0	0	2	0	0	0	
—	8	7	3	7	7	7	

† Any reaction less than 3 mm. increase was termed negative.

Reactions 3-4 mm. were termed doubtful.

* CFT Complement fixation test.
SAST Slide agglutination serum test.
AGT Agar gel precipitation test for antigen (AG) or antibody (AB)

24 hours later, and the two serum samples from each animal compared by the CFT, the SAST and the AGT for antibody and antigen. In the CFT the end-point was taken as the highest dilution of serum giving 50 per cent. lysis. No significant differences were observed between the two samples from each animal by any of the tests.

Complement fixation test. This test was performed by the method of Campbell and Turner (1953) using antigen prepared at Muguga by their method, but using the T3 strain of *M. mycoides* grown in tryptose broth medium. Veronal buffer was used as the diluent. The complement titre was taken as the highest dilution (lowest concentration) of complement showing extremely slight fixation (\pm). Results were expressed by the classification used by Campbell and Turner in the "four-tube" test, except that + fixation at 1:10 was termed doubtful and anything less was negative. The degree of fixation in the 1:10 dilution of serum was recorded.

Allergic reaction. This was performed as described earlier (Gourlay, 1964a) and the tests performed in January and April 1963 were carried out using the antigen described. For the tests performed in December 1963, however, the double-strength antigen (Gourlay and Palmer, 1965) was used. The serum agglutination slide test (SAST) and the qualitative agar gel test (AGT) were performed as already described (Gourlay, 1964b). For the AGT, the smaller of the home-made cutters was used with hyperimmune sheep x' serum for detecting antigen and lymph 6183 for detecting antibody and as a positive CBPP control.

RESULTS

January 1963

Thirty-nine cattle were tested by the allergic antigen and serum samples obtained from them. Following autopsy 17 cases were termed definitely CBPP, 6 were termed doubtful CBPP and the remaining 16 were negative. Following the results of the various tests 3 of the negative cattle were reclassified as doubtful CBPP. All the 17 definitely CBPP cases (Table I) had acute or early encapsulated lung lesions. The CFT and AGT (the combined test for antigen and antibody) both detected all cases, the SAST failed to detect one case, whilst the allergic reaction failed to detect 3 cases, all of which had large acute lung lesions. Of the 9 doubtful CBPP cases (Table I), 3 had normal lungs and 2 of these were doubtful to the allergic reaction. Two had only scars on the lung and adhesions, and were positive to the allergic reaction only, 2 had abscesses and were positive to more than one test, 1 had only calcified hydatid cysts and reacted strongly to the SAST. The last animal had severe pleurisy, antigen in the serum and reacted to the allergic antigen. The remaining 13 cattle classed as definitely not CBPP were negative to all the tests.

April 1963

Fourteen cattle were tested and autopsied at Iriri Quarantine, and of these only 3 were definite CBPP cases, 3 were doubtful CBPP cases and the remaining 8 negative. Following test results, however, 6 of the negative cases were included in the doubtful CBPP category as they were positive or doubtful to one or more tests. Details of the definite CBPP cases are given in Table 2 and it is obvious that 2 of them (2 and 3) had only small chronic lesions and these were only detected by the allergic reaction. The third case had a very large acute lesion becoming chronic and was detected by all tests. Of the doubtful CBPP cases (Table 2) only 3 (4, 11 and 12) had any signs of past infection, but number 11 was not detected by any

TABLE 2
COMPARISON BETWEEN SOME DIAGNOSTIC TESTS FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA (APRIL 1963, IRIRI).

"Definite" Cases.					
Animal No.	CFT*	SAST*	Allergic reaction† mm. increase if +ve	AGT* for AG	Autopsy specimens AGT for AG
2	—	—	+12	—	+++
3	—	—	+9	—	+++
9	+++	+++	+6	+++	+++
Total tests	3	3	3	3	3
+	1	1	3	1	3
±	0	0	0	0	0
—	2	2	0	2	0
"Doubtful" Cases.					
1	—	—	±	—	Normal.
4	—	—	±	—	Adhesions.
5	—	—	6.5	—	Normal.
6	—	—	±	—	Normal.
7	—	±	±	—	Normal.
8	—	—	±	—	Normal.
11	—	—	—	—	Adhesions. Large calcified lesion inner surface rib opposite adhesions.
12	—	—	±	—	Adhesions.
14	—	±	—	—	Normal.

† Any reaction less than 3 mm. increase was termed negative.

Reactions 3—4 mm. were termed doubtful.

* CFT Complement fixation test.
SAST Slide agglutination serum test.
AGT Agar gel precipitation test for antigen (AG) or antibody (AB)

Three small CBPP sequestra and adhesions.
CBPP sequestrum 2" × 2" × 2" and adhesions.
Acute and chronic CBPP lesion, whole diaphragmatic lobe.

Lung at autopsy

of the tests. The other 6 cases all had normal lungs, 2 of them were doubtful to the SAST and one to the AGT for antibody also. One was positive to the allergic reaction. The remainder were all doubtful to the allergic reaction only. The 2 "definitely not CBPP cases" were negative to all tests.

At Apopoa, 126 cattle were inoculated with allergic antigen; sera were obtained from 35. Unfortunately it was only possible to autopsy 8 of these as the owners were very reluctant to sell any but very sick animals which they thought were going to die. After considerable difficulty we were able to obtain 2 cattle that were in good condition (H44 and H45). At autopsy all 8 cases were classified as definitely CBPP, details of which are given in Table 3. All these cases had large acute lesions except for H44 and H45 which had early encapsulated lesions. The CFT and AGT detected all the cases, the allergic reaction failed to detect 5 of them and the SAST failed to detect 6 of them.

December 1963

At Apopoa, 113 cattle, all of which had at one time or another over the past months been found positive to the field CFT (Huddart, 1960), were tested and autopsied. Of these 83 were classified as definitely CBPP, 21 were termed doubtful CBPP and only 9 were negative. Following test results the number of doubtful CBPP cases was increased by 5 to 26 and the cases classed as definitely not CBPP were reduced to 4. No acute CBPP lesions were seen in any of the cases and all had old sequestra with fibrosis, pleurisy and adhesions. Certain lungs were almost completely replaced by fibrous tissue. The large number of doubtful CBPP cases was due to the chronic nature of the lesions as it was impossible in many instances to be certain of the cause of the lesions found at autopsy. Considering only the 83 definite CBPP cases (Table 4), it was obvious that none of the tests was very reliable, the CFT and allergic reaction detected 61 (73.5 per cent.), the SAST, 29 (35 per cent.) and the AGT only, 17 (20.5 per cent.). Three of the 83 cases were negative to all the tests and another one was doubtful to one test only. Of the 26 doubtful CBPP cases (Table 4), 7 had normal lungs on macroscopic examination but 2 of these had some adhesions to the pleura.

TABLE 3
COMPARISON BETWEEN SOME DIAGNOSTIC TESTS FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA. (APRIL 1963, APOPOA).

"Definite" Cases

Animal No.	CFT*	SAST*	Allergic reaction† mm. increase if + ve	AGT* for AG	Autopsy specimens AGT for AG	Lung at autopsy
H18	++	++	—	++	+	Acute CBPP lesion, whole lung.
H32	++	—	—	++	+	Acute CBPP lesion, whole lung.
H44	++	—	+ 12	++	++	Encapsulated CBPP lesion 12" × 5" × 7".
H45	++	++	+ 6.5	—	++	Encapsulated CBPP lesion 12" × 5" × 5".
H46	++	—	—	++	++	Acute CBPP lesion, half diaphragmatic lobe.
H67	++	±	+ 4.5	++	++	Acute CBPP lesion, whole lung.
H73	++	—	—	++	++	Large acute and encapsulated CBPP lesions both lungs.
I71	++	—	—	++	+	Acute CBPP lesion whole lung.
Total tests	8	8	8	8	8	
+	8	2	3	7	8	
±	0	0	0	0	0	
—	0	6	5	1	0	

† Any reaction less than 3 mm. increase was termed negative.
Reactions 3—4 mm. were termed doubtful.

* CFT Complement fixation test.
SAST Slide agglutination serum test.
AGT Agar gel precipitation test for antigen (AG) or antibody (AB)

TABLE 4
COMPARISON BETWEEN SOME DIAGNOSTIC TESTS FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA (DECEMBER 1963, APOPOA).
"Definite" Cases.

Animal No.	CFT*	SAST*	Allergic reaction* mm. increase if + ve	AGT* for AG	Autopsy specimens AGT for AG	Lung at autopsy
1	+	+	14.5	—		CBPP sequestrum $2'' \times 2'' \times 1''$.
3	+	+	6	—		CBPP sequestrum $1'' \times 1'' \times \frac{1}{2}''$ and adhesions.
4	tr	—	5.5	—		CBPP sequestrum $4'' \times 4'' \times 3''$.
5	—	—	—	—		Few small sequestra, fibrosis, adhesions and pleurisy both lungs.
6	+	+	6.5	—	+	CBPP sequestrum, fibrosis and adhesions.
8	+	+	8.5	—		CBPP sequestra, fibrosis and adhesions.
9	+	+	8	—	+	and pleurisy both lungs.
10	+	—	8	—		CBPP sequestrum, fibrosis and adhesions.
11	+	—	4.5	—	+	CBPP sequestrum, $6'' \times 6'' \times 5''$.
12	—	—	11	—	+	CBPP sequestrum, fibrosis and pleurisy.
13	+	—	5	—		CBPP sequestrum.
14	+	—	7.5	—		CBPP sequestrum, fibrosis and pleurisy.
17	+	+	5	—		CBPP sequestrum $4'' \times 3'' \times 2''$ and pleurisy.
18	+	+	7	—		CBPP sequestrum $3'' \times 2'' \times 2''$ and fibrosis.
19	+	—	10	—		CBPP sequestrum $4'' \times 5'' \times 5''$ (shelled out).
21	+	+	8	—		CBPP sequestrum, $4'' \times 4'' \times 3''$.
22	tr	—	8	—		CBPP sequestrum, $2'' \times 2'' \times 2''$.
24	tr	—	—	—	+	CBPP sequestrum, fibrosis and pleurisy.
25	tr	—	—	—		CBPP sequestrum, fibrosis
26	tr	+	—	—		CBPP sequestrum, $3'' \times 2'' \times 2''$.
27	+	+	9	—	+	CBPP sequestrum, $4'' \times 4'' \times 3''$.
29	+	+	6	—		CBPP sequestrum, (shelled out).
31	+	+	5	—		CBPP sequestrum, fibrosis and pleurisy.
32	—	—	4.5	—		CBPP sequestrum, $2'' \times 2'' \times 3''$.
33	+	+	9	—		CBPP sequestrum, $3'' \times 3'' \times 2''$.
36	+	+	5	—		CBPP sequestrum, $4'' \times 4'' \times 2''$.
37	+	+	4.5	—	+	CBPP sequestrum, $6'' \times 5'' \times 5''$.
38	+	+	7	—		CBPP sequestrum and pleurisy.
39	+	—	—	—		CBPP sequestrum (shelled out) and pleurisy.

Table 4—continued

Animal No.	CFT*	SAST*	Allergic reaction* mm. increase if + ve	AGT* for AG	Autopsy specimens AGT for AG	Lung at autopsy
40	++	—	+ 6	—	—	CBPP sequestrum 6" × 6" × 6".
41	++	—	+ 5	—	—	CBPP sequestrum 2" × 1" × 2" and pleurisy.
43	++	+	+ 6	—	+	Old sequestrum, adhesions and slight pleurisy.
44	++	—	+ 6.5	—	—	CBPP sequestrum 4" × 2" × 2" and pleurisy.
45	++	±	—	—	—	Very old CBPP sequestrum 4" × 2" × 2" and pleurisy.
47	++	—	±	—	+	CBPP sequestrum 3" × 3" × 2".
48	++	—	+ 6.5	—	+	CBPP sequestrum 3" × 3" × 3" and pleurisy.
49	—	—	—	—	—	CBPP sequestrum 2" × 1" × 1" and 1" × 1" × 1".
50	++	—	+ 4.5	—	—	CBPP sequestrum, fibrosis and pleurisy.
52	++	—	+ 6	+	—	CBPP sequestrum 6" × 6" × 5" and pleurisy.
53	++	—	+ 26	+	—	CBPP sequestrum 6" × 5" × 5".
54	++	±	+ 8	+	—	CBPP sequestrum 8" × 6" × 4".
55	++	+	±	—	+	CBPP sequestrum 1" × 1" × 1".
56	++	—	+ 6	—	—	CBPP sequestrum 5" × 3" × 3".
58	++	—	+ 5.5	—	—	CBPP sequestrum 2" × 2" × 1".
59	++	—	—	—	—	CBPP sequestrum 1½" × 1" × 1", pleurisy and adhesions.
60	++	+	+ 7	+	—	CBPP sequestrum 5" × 5" × 5".
61	++	—	±	—	—	CBPP sequestrum, 3" × 2" × 3" pleurisy.
62	++	—	+ 12	—	—	CBPP sequestrum 4" × 4" × 3".
63	++	—	+ 6	—	—	CBPP sequestrum 3" × 2" × 2".
64	++	—	+ 6	—	—	CBPP sequestrum 4" × 4" × 5".
65	++	—	+ 6	++	—	CBPP sequestrum 4" × 3" × 3".
66	++	—	+ 7	++	—	CBPP sequestrum 5" × 3" × 3", pleurisy.
67	++	±	+ 7	—	—	CBPP sequestrum 5" × 5" × 5".
68	++	—	—	—	—	CBPP sequestrum 3" × 3" × 3", adhesions and emphysema.
69	++	—	±	+	++	Small CBPP lesion, fibrosis and pleurisy.
70	++	++	—	++	—	CBPP sequestrum 3" × 3" × 2".
71	++	++	+ 6.5	—	—	CBPP sequestrum 4" × 4" × 4".
72	++	—	+ 9	+	—	CBPP sequestrum 5" × 4" × 4".
73	++	—	+ 5.5	++	—	CBPP sequestrum 6" × 5" × 5".
74	++	++	—	++	—	Two CBPP sequestra 4" × 4" × 4" and pleurisy.
76	++	++	+ 5.5	±	—	CBPP sequestrum 3" × 2" × 2".
79	++	++	+ 12	+	—	CBPP sequestrum 4" × 3" × 3".

DISCUSSION

In order to weigh the merits of the different tests, it is obvious that only the results of the 111 definite CBPP cases can be considered, as the exact status of the doubtful CBPP cases is unknown even though many of them were almost certainly old cases of CBPP. Similarly there were very few cases in the definitely not CBPP category, 24 altogether, and all were of course negative to all the tests, as this was one of the criterion for inclusion in this group.

From the overall results it is obvious that no single test is entirely satisfactory, supporting the conclusions of Johnston and Simmons (1963) in Australia, and that the stage of the outbreak is important. These stages can be divided roughly into two, the acute and the chronic. The acute stage would include all cases in Tables I (Iri, January 1963) and 3 (Apopoa, April 1963), although the latter group is rather atypical as all but 2 of the cases were acutely ill and probably dying, which is a higher proportion of these cases than would normally be expected. In this acute stage it was apparent that the CFT and AGT (the combined test for antigen and antibody) were the most reliable and were very satisfactory, both detecting 100 per cent. of the cases, and both superior to the SAST, 72 per cent. and the allergic reaction, 68 per cent. In the chronic stage, which would include Tables 2 (Iri, April 1963) and 4 (Apopoa, December 1963) none of the tests was entirely satisfactory, but the CFT and allergic reaction were definitely superior to the others, detecting 72.1 and 74.4 per cent. of cases respectively. The SAST detected 34.9 per cent. and the AGT (combined test) 20.9 per cent.

In the acute stage, the majority of cases possessed circulating precipitating antigen and it is probable that this is responsible for the failure of the SAST and allergic reactions due presumably to neutralisation of the respective antibodies. The CFT is apparently not affected by this to such an extent and thus is a more suitable test under these conditions. The AGT for antigen, which of course detects this circulating antigen, is very good under these conditions and if it is combined with the test for antibody the results are as good as those of the CFT. Precipitating antibody, in amounts sufficient to be detected by the AGT, is present shortly after precipitating antigen disappears from the circulation in most instances, although in some cases both antigen and antibody are present simultaneously. This disappearance of precipitating antigen is presumably related to the encapsulation of the lung lesion. The precipitating antibody does not, apparently, persist for long in amounts sufficient to be detected by the AGT. This is borne out by earlier work (Gourlay, 1964b) which showed that precipitating antibodies disappeared very rapidly from the sera of hyperimmune sheep.

In the chronic stages, all tests were unsatisfactory and it is possible that this is due to the gradual disappearance of the respective antibodies in the absence of antigenic stimulus once encapsulation is complete. The rate of decline of the different antibodies to a level below the sensitivity of the respective tests could explain the variation in results obtained with the various tests. As already mentioned the precipitating antibodies do not last long and apparently disappear first, followed by the agglutinating, complement fixing and finally the allergic antibodies. On this basis, therefore, the allergic reaction and the CFT are the most satisfactory tests for the chronic cases. The former is still, however, in the early

stages of its development and considerable work is still required both on its application and on improving the antigen itself. From these results it also follows that the antibodies responsible for each of these tests are different. Repeated testing at suitable intervals would increase the reliability of all the tests, as presumably at some time each individual animal would be at the stage of the disease detectable by the appropriate test. The number of false negatives could also be decreased by using more than one test, particularly if one of the tests for detecting antibody was combined with the AGT for antigen.

The combined AGT which gave excellent results in acute cases had certain advantages over the CFT. It is very simple and very little equipment is required and it can be readily performed in the field with either whole blood, citrated blood or serum and a strong positive result can be detected within as little as 3 hours. A negative result, however, cannot be confirmed before 24 hours. The use of the AGT for antigen performed on autopsy specimens, either lung lesions or associated lymph nodes, also gave good results.

CONCLUSIONS

In acute stages of the disease, the complement fixation test and the agar gel test for antigen and antibody both detected 100 per cent. of Contagious bovine pleuropneumonia cases, the slide agglutination serum test detected 72 per cent. and the allergic reaction 68 per cent. In the chronic stages none of the tests were entirely satisfactory. The allergic reaction and the CFT were the best, detecting 74 and 72 per cent. of cases respectively, while the slide agglutination serum test detected 35 per cent. and the agar gel test 21 per cent.

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EVALUATION OF DIAGNOSTIC TESTS FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA

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INTRODUCTION

Gourlay (1965a) compared the efficiency of several diagnostic tests for detecting contagious bovine pleuropneumonia (C.B.P.P.). The study included two herds of cattle naturally infected with C.B.P.P., and serological test results were correlated with necropsy findings. Both the agar gel test for antigen and antibody, and the complement fixation test (C.F.T.) detected 100% of the acutely infected cattle. In chronic C.B.P.P., no tests were entirely satisfactory. The allergic reaction and the C.F.T. were the most efficient in chronic C.B.P.P. and detected 74% and 72%, respectively, of the cattle.

During C.B.P.P. outbreaks in Uganda in 1964, and in Kenya in 1966, we reevaluated the different tests to find the most efficient one for field use. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Cattle

Cattle involved in this study were East African Zebu from herds naturally infected with C.B.P.P. One herd located in Iriri, Karamoja District, Uganda, was tested in October 1964; the other herd, located at Archer's Post, Isiolo District, Kenya, was tested in May 1966. Both herds included cattle in the acute stages of the disease with many deaths occurring daily.

Experimental Procedures

Skin tests were done for immediate and delayed allergic reactions as described by Shifrine and Gourlay (1965) and Gourlay and Shifrine (1965). After these tests were read, the cattle were killed, and blood was collected from the jugular vein. At necropsy, C.B.P.P. infections were classified on macroscopic examination as being acute, chronic, doubtful, or negative. Specimens of the lungs and mediastinal lymph nodes were collected for attempted isolation of *Mycoplasma mycoides*, the causative agent of C.B.P.P. The only cattle classified as positive were those with unmistakable C.B.P.P. lesions or with *M. mycoides* in the lungs or bronchial and mediastinal lymph nodes. Cattle with no lung lesions but with *M. mycoides* in the lymph nodes were termed peracute. Cattle with evident encapsulation or sequestered lesions were termed chronic, and all others with C.B.P.P. lung lesions were termed acute. Cattle with no lung lesions and no evidence of *M. mycoides* but with positive reactions to any of the serological tests were termed doubtful. These cattle could

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have either false positive reactions (Gourlay and Shifrine, 1966) or undetected microscopic lesions. Only cattle with no lesions and negative test reactions were classified as negative.

The sera and tissues were refrigerated and brought to the laboratory at Muguga within two days where all serological tests were performed.

The agar gel double diffusion precipitin test (A.G.T.) and the slide agglutination serum test (S.A.S.T.) were done following the procedures used by Gourlay (1964). For detection of circulating antigen in infected cattle, bovine hyperimmune anti-*M. mycoides* serum was used. For detection of antibodies, we used 10 mg./ml. water of *M. mycoides* cells, frozen and thawed three times. Although Huddard (1963) published a field modification of the C.F.T. for diagnosing C.B.P.P., no published data are yet available on its value in the field; therefore, we used the method of Campbell and Turner (1953) with the modification of Gourlay (1965b). For isolation of *M. mycoides*, the tissue was cut into small pieces with sterile scissors, and the pieces were then put into Newings tryptose broth (Gourlay, 1964) containing 100 i.u. penicillin and 1:2,000 thallium acetate/ml.

RESULTS

Results of the tests on cattle from Uganda are presented (Table I). The skin tests were not repeated as all had been positive for both immediate and delayed allergic reactions.

Table I.—Results of positive serological tests on 54 cattle with C.B.P.P. (Uganda, 1964)

Number	Cattle C.B.P.P. status	C.F.T.	S.A.S.T.	A.G.T. for	
				Ag	Ab
6	Negative	0	0	0	0
6	Doubtful	0	0	5	1
3	Peracute	0	1	2	1
13	Acute	13	13	10	8
26	Chronic	25	23	19	21

C.B.P.P. = contagious bovine pleuropneumonia.

C.F.T. = complement fixation test.

S.A.S.T. = slide agglutination serum test.

A.G.T. = agar gel diffusion test, for antigen (Ag) or antibody (Ab). For detecting circulating Ag, bovine hyperimmune anti-*M. mycoides* serum was used. For detecting antibody, 10 mg./ml. water of *M. mycoides* cells, frozen and thawed three times, was used.

M. mycoides was isolated from the enlarged lymph nodes of three cattle with no macroscopic lesions in the lungs. Sera from these cattle were positive for antigen or antibody in the A.G.T.; one was also positive in the S.A.S.T. (Table I). These were classified as peracute infections.

Among the acute infections, only one serum was negative in the A.G.T. for both antigen and antibody.

In the chronic infections, only one serum was negative in the A.G.T. for both antigen and antibody, but it was positive in the S.A.S.T. All three sera that were negative in S.A.S.T. were positive in A.G.T.

Of the six doubtful C.B.P.P. cattle, all were positive in A.G.T. but negative

in the C.F.T. and S.A.S.T. (Table I). The mediastinal lymph nodes of these cattle were enlarged.

Results of the tests done on the cattle from Kenya are presented (Table II). All the sera from the chronic and acute infections were positive in either A.G.T. or S.A.S.T.

Table II.—Results of serological tests on 57 cattle with C.B.P.P.
(Kenya, 1966)

Number	Cattle	C.F.T.	S.A.S.T.	A.G.T. for	
	C.B.P.P. status			Ag	Ab
5	Negative	0	0	0	0
28	Acute	27	27	24	4
24	Chronic	23	18	23	7

C.B.P.P. = contagious bovine pleuropneumonia.

C.F.T. = complement fixation test.

S.A.S.T. = slide agglutination serum test.

A.G.T. = agar gel diffusion test, for antigen (Ag) or antibody (Ab). For detecting circulating Ag, bovine hyperimmune anti-*M. mycoides* serum was used. For detecting antibody, 10 mg./ml. water of *M. mycoides* cells, frozen and thawed three times, was used.

DISCUSSION

All cattle from Uganda gave positive skin test although six of these were negative to all other tests and normal on necropsy. As this skin test was later shown to be non-specific (Shifrine and Gourlay, 1965), it was not used in the outbreak in Kenya. We are currently trying to purify extracts of *M. mycoides* in an effort to render this test specific.

The C.F. test was negative when sera from two chronic, one acute and three peracute infections were tested. From the latter three, *M. mycoides* was isolated and the sera were positive in the A.G.T. Thus, out of 94 C.B.P.P. cattle tested (this figure excludes the negative and doubtful cattle), there were six C.F.T. negative sera. Turner and Etheridge (1963), evaluated the S.A.S.T., slide agglutination blood test (S.A.B.T.) and C.F.T. in the diagnosis of C.B.P.P. They found that both S.A.S.T. and S.A.B.T. had the disadvantage of giving negative results with sera from animals with sequestered lesions and non-specific reactions with the S.A.S.T. In our limited studies, we did not find many non-specific reactions using the S.A.S.T. When non-specific reactions were encountered in C.B.P.P.-free cattle, the sera were positive also in the C.F.T. (Gourlay and Shifrine, 1966). The study of Turner and Etheridge (1963), also included cattle involved in a field outbreak of C.B.P.P. Among these there were 57 with pathogenic lesions; 52 of these reacted to C.F.T. and 55 reacted to the S.A.S.T. Out of five recovered animals with only fibrous adhesions, none reacted to C.F.T. and all reacted to the S.A.S.T. Thus, the S.A.S.T. may be more sensitive in both recovered cases and peracute cases.

Turner and Etheridge (1963), reported the S.A.S.T. to be least sensitive with chronic cases of C.B.P.P., probably because of circulating antigen. However, at this stage of the disease, the A.G.T. for antigen is quite sensitive.

In our studies, neither the S.A.S.T. nor the A.G.T., for both antibody and

antigen, detected all positive infections. However, by using the results of both of these tests, all positive cattle were detected.

Our results confirm those of Gourlay (1965a and b) that no single test can detect all C.B.P.P. cattle and the C.F. test is the most sensitive single test for detecting C.B.P.P. However, all C.B.P.P. cattle from the two outbreaks studied, could be detected by using both the S.A.S.T. and the A.G.T.

The results presented here indicate that the use of S.A.S.T. in conjunction with A.G.T. for detecting antigen is the method of choice for detecting C.B.P.P. in the field. It also has the advantage of being easier to perform than the C.F.T.

SUMMARY

The slide agglutination serum test in conjunction with agar gel diffusion test for antigen and antibody detected 100% of cattle naturally infected with contagious bovine pleuropneumonia. The complement fixation test, however, failed to detect 6% of these cattle.

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Résumé

En utilisant les deux tests diagnostiques: sérologique d'agglutination et épreuve de diffusion sur gélose pour l'antigène et les anti-corps, on peut déceler la péripneumonie contagieuse chez 100% des bovins infectés, tandis qu'avec la réaction de fixation de complément seule, 6% d'entre eux échappent à la détection.

A Solid Medium Test for Measuring Growth Inhibition and Neutralization of *Mycoplasma mycoides* by Immune Bovine Serum

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(Accepted for publication 29 December 1966)

SUMMARY

The growth of *Mycoplasma mycoides* var. *mycoides* was inhibited and neutralized by immune serum from cattle which had recovered from infection with *M. mycoides*. Although mycoplasmal neutralization is not necessarily the same as virus neutralization, it is suggested that this terminology be retained since it is accepted and is descriptively appropriate. The effect of immune serum on colony growth was determined by incubating test serum in plastic cylinders on nutrient agar with pre-incubated microscopic *M. mycoides* colonies. Optimal inhibition and neutralization occurred when serum and colonies were incubated at 30° before final incubation at 37°. Inhibition of growth and neutralization also occurred at 37° and 21°; inhibition but no neutralization occurred at 5°.

INTRODUCTION

Blood from cattle affected with contagious bovine pleuropneumonia (CBPP) has been reported to be bactericidal to *Mycoplasma mycoides* var. *mycoides* (Priestley, 1952). Inhibition (Edward & Fitzgerald, 1953; Bailey *et al.* 1961; Cottew, 1963) and 'neutralization' (Edward & Fitzgerald, 1954) of growth of *Mycoplasma* species by hyperimmune sera incorporated in liquid media have also been reported. The possibility that these phenomena may have been caused by agglutination does not appear to have been excluded.

The specific inhibition of *Mycoplasma* species by hyperimmune rabbit serum incorporated in the solid growth medium was reported by Edward & Fitzgerald (1953). This phenomenon has been used to elucidate the serological relationship of several *Mycoplasma* species (Huijsmans-Evers & Ruys, 1956; Clyde, 1964) and to detect specific antibodies in sera of individuals infected with *M. pneumoniae* (Herderschee, 1963).

A review of the literature about 'neutralization' and inhibition of *Mycoplasma* species by antisera indicates that these phenomena, when observed in liquid media, have not been clearly differentiated from agglutination, that neutralization has not been demonstrated on solid media, and that factors which affect the sensitivity of these phenomena have not been fully investigated. A concurrent report presents a study of mycoplasmal inhibition and neutralization in liquid media (Gourlay &

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Domermuth, 1966). The work described here was designed to elucidate further the nature of mycoplasmal neutralization and inhibition by immune sera on solid medium.

METHODS

Organism. A highly virulent strain of *Mycoplasma mycoides*, referred to as Gladysdale (Turner, 1961), was used as the test organism in this work. Seed culture was grown in modified Newing's tryptose broth (Gourlay, 1964) at 37°. The maximum viable titre (colony count) was reached in 72 hr. At this time, the culture was divided into small samples and stored at -70° until used.

Immune sera and control. A pool of immune sera was prepared from grade cattle (crosses between Zebu and European cattle) which had recovered from subcutaneous infection with the Gladysdale strain of *Mycoplasma mycoides* (Gourlay & Domermuth, 1966). This *M. mycoides* antiserum was selected as a test serum because the donor animals had proved to be immune to subsequent subcutaneous challenge with *M. mycoides*. Pooled freeze-dried normal bovine serum (Nutritional Biochemical Corporation, batch number 5920) was obtained from the U.S.A., a country free from contagious bovine pleuropneumonia. Both pools of sera were tested for *M. mycoides* antibodies by the complement-fixation test (c.f.t.; Gourlay, 1965). The immune serum had a c.f.t. titre of 1/1280 and the normal serum had no titre to *M. mycoides*.

Preparation of nutrient agar plates for application of test sera. Solid medium (Gourlay, 1964), modified to contain 1000 i.u. penicillin/ml., thallium acetate 0.5 mg./ml. and agar 1.1% (w/v), was dispensed to a depth of 4.0 mm. in small Petri plates (6.0 cm. internal diameter). The agar surfaces were inoculated by flooding with 1.0 ml. of a 1/10 dilution of seed culture (see above). The inoculum was allowed to remain on the surface of the agar for 15 min., excess of inoculum then poured off and the plates inverted and incubated for 7 or 14 hr at 37°. After this initial period of incubation, the plate tops were flamed and the plates returned to an upright position. Six plastic cylinders (4.5 mm. internal diameter × 6.0-7.0 mm. height) were then inserted 2.0-3.0 mm. in the agar of each plate. The plastic cylinders were sterilized before use by boiling for 20 min. in thallium acetate solution (2 mg./ml.) followed by boiling in sterile distilled water, and then vacuum-dried.

Determination of neutralization and/or inhibition of Mycoplasma mycoides by test sera. The two test sera, anti-*M. mycoides* (immune) and *M. mycoides* negative (normal), described above were heated at 56° for 45 min. and penicillin (1000 i.u./ml.) added to each. Serial doubling dilutions of serum, 1/1 (undiluted) to 1/128 were made in a liquid nutrient medium, prepared by omitting the agar from the solid medium described above. Two drops of each serum dilution were then delivered into each plastic cylinder. Two sets of plates with each of these dilutions were incubated at 5°, 21°, 30° and 37° for 24 hr and two similar sets for 72 hr. After incubation, the test sera were removed from one complete set of plates by flooding each plate with 10 ml. of the nutrient liquid medium which was used as serum diluent. The plastic cylinders were then removed, and the washing medium was removed by decantation and replaced with fresh liquid medium. The plates were then allowed to stand overnight at 21° (ambient), the liquid medium removed, and the plates inverted before and during final incubation. Final incubation of these and identical unwashed plates was at 37° for 24 hr (plates previously incubated with test serum and colonies at 30° and

37°) or 48 hr (plates previously incubated with test serum and colonies at 5° and 21°). After this final incubation, the remaining plastic cylinders were removed and all plates washed for 10 min. with M/15 KH_2PO_4 containing 0.04 % (w/v) sodium azide. The plates were then stained with neutral red (equal parts of 0.5 % neutral red in 95 % ethanol and M/15 KH_2PO_4 plus 0.85 % NaCl in distilled water; fresh stain prepared weekly) for 5 min., washed with the $\text{KH}_2\text{PO}_4 + \text{NaN}_3$ solution, and examined with a dissecting microscope (magnification $\times 12.5$) to determine the effect of the test sera on colony growth.

Absorption of test sera with Mycoplasma mycoides. Test sera were twice absorbed with freeze-dried *M. mycoides* (20 mg./ml.) for 1 hr at 37°, followed by 3 hr at 21° and 18 hr at 5°. After absorption the sera were twice centrifuged at 2000g for 30 min. The supernatant sera were retained and tested for neutralizing and inhibitory activities as described above except that only the most sensitive form of the test was used (30° incubation of test sera and mycoplasma colonies).

Effect of complement on neutralizing and inhibitory activities of test sera. One per cent of 5 % (v/v) of guinea-pig serum (complement-fixing activity of 100 minimal haemolytic doses as determined for the complement-fixation test; Gourlay, 1965) was added to the solid medium and to test serum dilutions. Inactivated (56° for 45 min.) control sera were similarly added to control plates. The effect of these components on neutralization and inhibition was determined by the most sensitive test procedure as above.

Examination for growth-inhibitory antibodies in serum of normal cattle and of cattle which had been infected with M. mycoides. One hundred samples of sera with no c.f.t. titre against *M. mycoides* and 10 sera from cattle endobronchially infected with *M. mycoides* were tested for inhibitory antibodies by the most sensitive test procedure.

RESULTS

The appearance of plates prepared for the application of test sera. Plates inoculated in the manner described produced an average of 25 colonies/mm.². When incubated at 37°, the organisms in the inoculum developed into colonies which grew into the agar and after 6 hr could not be washed away with test serum or nutrient medium. Such colonies were therefore usable for the test. They became visible, therefore unusable for the test, at 16 hr. As judged by continued incubation and observation of 7 hr (37°) pre-incubated colonies, slow growth of *M. mycoides* occurred at 30° and no growth occurred at 21° during the 2-week observation period.

Determination of neutralization and inhibition of Mycoplasma mycoides by test sera. Absence of growth of colonies in the presence of test serum is called inhibition; absence of growth of colonies after washing the test serum from the agar is called neutralization. The term neutralization is used to conform with previously used and accepted terminology (Edward & Fitzgerald, 1954); however, this terminology does not necessarily conform in meaning to that used in virology.

Growth of *Mycoplasma mycoides* colonies was inhibited by *M. mycoides* antiserum in all variations of the test system used (Fig. 1).

Neutralization occurred when colonies and antiserum were incubated together at 21°, 30° and 37°, but not when incubated at 5° (Fig. 1). No neutralization or inhibition was produced by normal serum; partial and complete neutralization and inhibition

of growth were produced by immune serum. Younger (7 hr) colonies were more sensitive to the inhibitory and neutralizing effects of serum than older (14 hr) colonies (Fig. 1).

Partial inhibition and neutralization were produced by immune serum dilution 1/64, complete inhibition by dilution 1/32 and complete neutralization by dilution 1/2 (Fig. 1). The appearance of uninhibited and partially inhibited colonies is shown in Pl. 1. Colonies which are completely inhibited or neutralized do not grow to visible size.

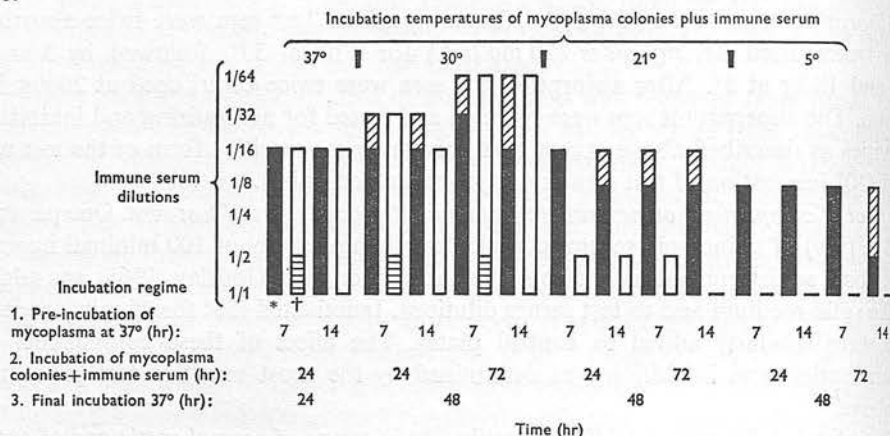


Fig. 1. Effect of physical variables on neutralization and inhibition of *Mycoplasma mycoides* colony growth by homologous antiserum from *M. mycoides* immune cattle. * Serum retained during final incubation: ▨, partial inhibition; ■, complete inhibition. † Serum removed during final incubation: □, partial neutralization; ▤, complete neutralization.

Absorption of immune serum with Mycoplasma mycoides. The neutralizing and inhibitory activities of immune serum were completely removed by absorption with *M. mycoides*.

Effect of complement. The presence or absence of complement produced no detectable change in the neutralizing or inhibitory capacity of immune serum.

Growth-inhibitory antibodies in serum of normal cattle and of cattle infected with M. mycoides. The growth of *Mycoplasma mycoides* was not inhibited by sera from 100 normal cattle but was inhibited by sera from all 10 infected cattle.

DISCUSSION

A sensitive method of detecting *in vitro* neutralization and inhibition of *Mycoplasma mycoides* var. *mycoides* by immune bovine serum has been developed. The method while utilizing the basic method of Edward & Fitzgerald (1953), has been modified by using plastic cylinders as test serum reservoirs to maintain appropriate serum concentrations. This modification restricts the dilution of serum and permits several sera to be tested on each Petri plate, thus causing the test to be of greater potential value in the diagnosis of contagious bovine pleuropneumonia and other mycoplasma infections which produce antisera of low inhibitory titre. The use of a temperature of 30° instead of the customary 37° for incubating organisms and sera also significantly increased the sensitivity and thus the applicability of the test.

'Neutralization' of *Mycoplasma* species by immune serum has been long regarded as a unique characteristic of the Mycoplasmataceae (Edward & Fitzgerald, 1954). The data presented in the present report support and strengthen Edward & Fitzgerald's (1954) findings about 'neutralization', as the present information was obtained by observing the effect of immune serum on microscopic colonies rather than in liquid medium where agglutination may have been partially or wholly responsible for diminution of mycoplasmal titre (see Gourlay & Domermuth, 1966). That all inhibitory antibody was washed from the agar before the final incubation was effectively demonstrated, as no neutralizing effect was observed on colonies incubated with immune serum at 5° (Fig. 1). If an effective antibody concentration had been present after the removal of immune serum by washing, this would have been readily apparent and some inhibition of colonies would have occurred during the final incubation at 37°.

The maximum inhibition and neutralization observed in these tests occurred at 30°, a point very near 27°, which was reported to be the lower growth limit for *Mycoplasma mycoides* (Dujardin-Beaumetz, 1900). This observation suggests that maximum neutralization and inhibition are favoured by slow growth of the test organisms; however, the fact that some neutralization was observed at 21° indicates that the phenomenon was not completely growth-dependent as might be deduced from studies in liquid medium. The reason for this apparent discrepancy is not known.

It would be of interest to determine what antigen(s) are responsible for the production of inhibitory antibody and the nature of the mechanism of inhibition. In addition, the relationship of inhibition to immunity should be elucidated as this knowledge is of great potential value to understanding the pathogenesis of contagious bovine pleuropneumonia. The serious nature of the non-specificity of conventional diagnostic tests for contagious bovine pleuropneumonia has only recently been elucidated (Shifrine & Gourlay, 1966); since conventional mycoplasmal growth inhibition tests indicate that inhibition is species-specific (Clyde, 1964), it is possible that the test described here will eliminate false positive reactions when used in the diagnosis of this disease.

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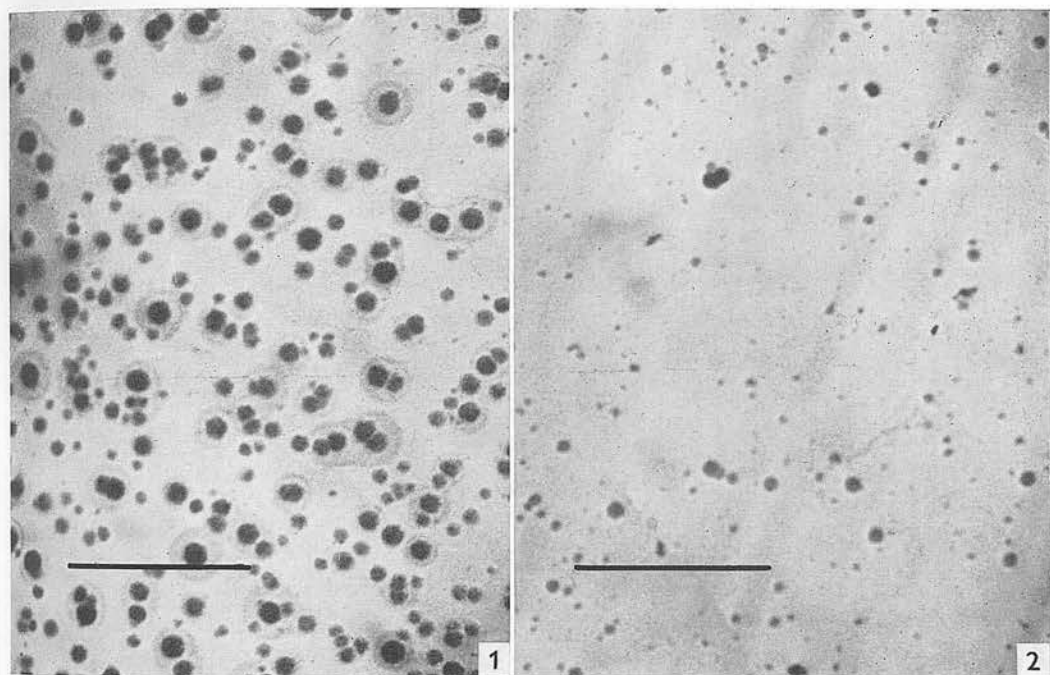
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EXPLANATION OF PLATE

Inhibition and neutralization tests on *Mycoplasma mycoides* colonies.

Fig. 1. Normal serum: colonies are uninhibited and normal in appearance.

Fig. 2. Immune serum: colonies are partially inhibited by immune serum as evidenced by reduction in size. When colonies are completely inhibited or neutralized, no colony growth is visible.



GROWTH INHIBITION AND "NEUTRALIZATION" OF *MYCOPLASMA MYCOIDES* BY IMMUNE BOVINE SERUM: I. DEVELOPMENT OF A LIQUID MEDIUM TEST

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INTRODUCTION

In 1954, Edward and Fitzgerald¹ reported that when antisera to various species of pleuropneumonia-like organisms (*Mycoplasmataceae*) were incorporated in culture medium, growth of the homologous organism was inhibited. Complement was not required for this inhibition. They further suggested that there was a similarity between inhibition of growth of mycoplasma and virus neutralization. Bailey, Clark, Felts and Brown² used an inhibition-of-growth test based on that of Edward and Fitzgerald to study the growth-inhibitory properties of specific antibody on *Mycoplasma hominis*. Huijsmans-Evers and Ruys³ described a method of inhibiting human genital mycoplasma on solid medium by application of antiserum-saturated paper discs, and later Clyde⁴ modified and extended this method to identification of mycoplasma isolated from the human pharynx and from tissue culture.

As antibodies detectable by the serological tests commonly used for contagious bovine pleuropneumonia (CBPP)—namely, the complement fixation test, agglutination test, and precipitin test—appear to give no indication of the immune status of the individual animal, it was thought that the development of a quantitative growth-inhibition or neutralization test might be of value in studies on immunity to the disease. This paper describes the development of a test using a dilution method in broth, and describes studies on the phenomenon of growth inhibition and neutralization of *Mycoplasma mycoides* var. *mycoides*.

METHOD

Organism. For test inocula, the highly virulent Gladysdale strain⁵ of *M. mycoides* was grown at 37°C in modified Newings tryptose broth.⁶ When growth reached the thread phase, the culture was shaken to form a homogeneous suspension and freeze-dried in 0.5 ml amounts. The titer of the freeze-dried culture after reconstitution in distilled water was 10⁸ BCID₅₀ (broth culture infective dose) organisms/ml as determined by triplicate broth titrations. The avirulent KH3J strain of *M. mycoides*⁷ was used as a fresh broth culture in one experiment.

M. mycoides used for absorption of test sera was obtained by growing the Gladysdale strain in modified Newings tryptose broth, harvesting the organisms in a Lister Vaccine Clarifier (APSM 12¶) and freeze-drying the organisms obtained.

Serum Samples. Developmental work on this test was performed with known anti-*M. mycoides* and *M. mycoides* negative sera. Immune anti-*M. mycoides*

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serum was pooled from 7 Grade cattle (cross between Zebu- and European-type cattle) that had been inoculated subcutaneously with 2 ml of Gladysdale 3-day culture, had developed extensive Willems reactions and recovered. They were bled for serum between three and four months after inoculation. This serum was chosen as animals inoculated in this manner have proved to be immune to subsequent subcutaneous and endobronchial challenge. The serum was stored at -25°C . The *M. mycoides* negative serum (normal) was obtained as a freeze-dried powder from the U.S.A. (Nutritional Biochemicals Corporation, Cleveland, Ohio), a country free from CBPP. Later work was done with sera from grade cattle before and after experimental infection with *M. mycoides*.

Absorption of Test Sera with *M. mycoides*. Anti-*M. mycoides* and *M. mycoides* negative sera were absorbed two times with 20 mg/ml dried *M. mycoides* organisms (Gladysdale strain) at 37°C for one hour, room temperature for three hours and 6°C for 18 hours and centrifuged twice at 2000 g for 30 min.

Serological Tests. The complement fixation test (CFT)⁸ titer of the immune serum was 1/1280 and the slide agglutination serum test (SAST)⁶ was + (weakly positive). The normal serum was negative in both tests.

Broth. Modified Newings tryptose broth⁶ was used for the experiments on the effect of using nutrient and nonnutrient diluent for the serum and for the determination of the specificity of the test by absorption of the immune serum. For the serum dilutions only of all other experiments, this medium was further modified by replacing the NaCl with 0.13% KH_2PO_4 and increasing the Na_2HPO_4 to 0.87% to increase the buffering capacity. The earlier modification of the broth⁶ was still used for titrations. For all serum dilutions and titrations, thallium acetate (1/2000) was added to the broth.

The Test

Test Inoculum. One ampule of freeze-dried culture of *M. mycoides* was reconstituted with 0.5 ml of broth and allowed to imbed for 60 minutes at room temperature. It was then shaken well using a Vortex J.R. Mixer (model K500-J*) which was used for all shaking. A 1/10 dilution of the organisms constituted the test inoculum of which two drops (approx. 0.02 ml each) were added to each test serum dilution. The inoculum (two drops) was titrated by triplicate ten-fold dilutions in broth, the end point was taken as the highest dilution showing visible growth within six days and the 50% end point calculated by the method of Reed and Muench.⁹

Mixing System. The test serum was inactivated at 56°C for 45 minutes after which 1,000 I.U. per ml of penicillin and 1/2000 thallium acetate were added. Filtration was unnecessary. A serum dilution of $\frac{1}{4}$ was then prepared, 0.25 ml of serum being added to 0.75 ml of broth. Two drops of the test inoculum were added to the dilution, and this constituted the mixing system, which was shaken and placed at 30°C for three days. The mixing system was shaken briefly each day.

Measuring System. After three days, the mixing system was shaken well and the organisms titrated in triplicate in 9 ml amounts of tryptose broth using 0.25 ml of the mixing system in the first dilution and ten-fold dilutions thereafter. The titrations (measuring system) were placed at 37°C and examined daily for six days for growth of *M. mycoides*. The 50% end point was determined as for the test inoculum. It was necessary to shake the first dilution of the titrations on the Vortex mixer in order to obtain consistent results. A known *M. mycoides* negative serum was included in each test. A diagrammatic representation of the test is given in FIGURE 1.

* Scientific Industries Inc., Springfield, Mass.

THE TEST

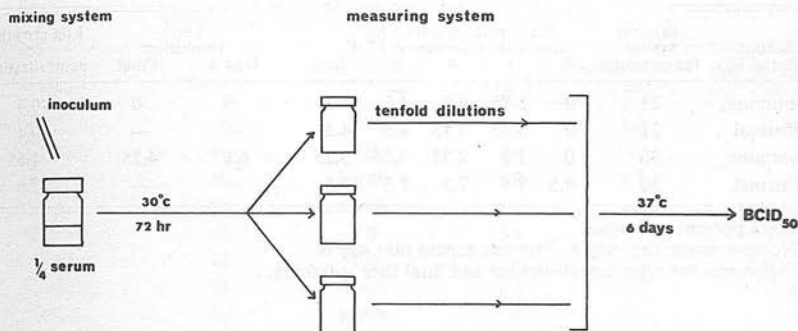


FIGURE 1.

Inhibition was calculated from the difference between the titers of the organisms in the *M. mycoides* negative and immune sera at three and six days. Growth or neutralization was calculated from the difference between the final six-day titer of the organisms from the immune or normal sera and the titer of the initial inoculum. The fact that only 0.25 ml was titrated was compensated for by adding 0.6 log to the titer of the organisms from the sera.

Modified Test. The following modification of the test was used mainly to determine the inhibition of growth in the presence of serum.

Mixing System. Doubling dilutions of inactivated sera in broth were prepared in 6 ml amounts from undiluted (1/1) to 1/256. Each dilution was then divided into three 2 ml amounts. Two drops of the inoculum were added to each triplicate dilution (mixing system), and after shaking, they were then placed at 30°C for three days, being briefly shaken each day.

Measuring System. After three days at 30°C, the mixing system was shaken well and 0.25 ml was subinoculated from each dilution into a bottle containing 9 ml of broth (measuring system). Both the mixing system and the measuring system bottles were then placed at 37°C and were examined daily for visible growth of *M. mycoides*, and the end point taken as the highest concentration of serum showing growth of organisms in at least two of the three tubes within six days of placing them at 37°C. An *M. mycoides* negative serum was included in each test. By means of this test, growth or inhibition of growth in the presence of serum was estimated in the mixing system, and neutralization in the absence of serum was estimated in the measuring system.

RESULTS

The Effect of Different Temperatures of Incubation of the Mixing System

In earlier unpublished work,¹⁰ temperatures of 5°C, 21°C, and 37°C were used for incubation of the mixing system, and only at 37°C did inhibition occur. Unfortunately, results were not consistent. In subsequent work,¹¹ consistent and promising results were obtained on solid medium at 21°C and 30°C. In view of this, 21°C and 30°C were used in these new experiments. Two sets of the immune and *M. mycoides*-negative sera (diluted 1/4) were prepared. After the addition of

TABLE 1
THE EFFECT OF INCUBATING THE MIXING SYSTEM FOR 72 HOURS AT 21°C AND 30°C
(INOCULUM TITER $10^{5.5}$ BCID₅₀ ORGANISMS/0.04 ml)

Serum (diluted 1/4)	Mixing system temperature	Measuring system titer* day after titration at 37°C					Log† inhibition		Log growth‡ or neutralization
		2	3	4	5	6	Day 3	Final	
Immune	21	0	3.75	4.5	4.5	4.5	0	0	-0.4
Normal	21	0	3.25	3.75	4.5	4.5	—	—	-0.4
Immune	30	0	1.5	2.75	3.25	3.25	6.0	4.25	-1.65
Normal	30	6.5	7.5	7.5	7.5	7.5	—	—	+2.6

* Log₁₀ percent end point.

† Normal serum titer day 6—immune serum titer day 6.

‡ Difference between inoculum titer and final titer +0.6 log.

the test inoculum, one set was placed at 21°C and the other set at 30°C for three days. The resulting titrations (TABLE 1) show no inhibition or neutralization by the immune serum at 21°C when compared to the normal serum but definite inhibition and neutralization at 30°C. This was repeated six more times with different numbers of organisms in the inocula, and in all experiments, similar results were obtained. However, on two occasions at 21°C, there was a transient two log inhibition on the third day. It had disappeared by the fourth day.

*The Effect of Varying the Length of Time of Incubation of the
Mixing System at 30°C*

In this experiment, double the normal amount of diluted sera and double the inoculum were used. Aliquots of 0.25 ml of the mixing system were titrated after 24, 48, 72, and 96 hours. The results given in TABLE 2 indicate that both inhibition and neutralization increase in proportion to the length of incubation up to 72 hours, after which time they remain static. When this experiment was repeated, however, the increase in inhibition and neutralization increased up to and including the 96-hour sample. This experiment was repeated twice more at only 24 and 72 hours using different numbers of organisms with essentially similar results.

TABLE 2
THE EFFECT OF VARYING THE LENGTH OF TIME OF INCUBATION OF THE MIXING SYSTEM AT 30°C
(INOCULUM TITER $10^{5.5}$ BCID₅₀ ORGANISMS/0.04 ml)

Serum (diluted 1/4)	Mixing system hours at 30°C	Log inhibition*		Log growth or neutralization†
		Day 3	Final	
Immune	24	1.5	1.0	-0.15
Normal	24	—	—	+0.85
Immune	48	3.75	2.75	-0.4
Normal	48	—	—	+2.35
Immune	72	6.25	5.0	-1.65
Normal	72	—	—	+3.35
Immune	96	5.0	5.0	-1.4
Normal	96	—	—	+3.6

* Normal serum titer day 6—immune serum titer day 6.

† Difference between inoculum titer and final titer +0.6 log.

TABLE 3

THE EFFECT OF USING NUTRIENT (BROTH) AND NON-NUTRIENT (VERONAL BUFFER)
DILUENTS FOR SERUM DILUTIONS
(INOCULUM TITER $10^{5.0}$ BCID₅₀ ORGANISMS/0.04 ml)

Serum	Dilution	Mixing system Diluent	Log inhibition* Day 3	Final	Log growth or neutralization†
Immune	½	VB	1.0	1.0	+0.1
Normal	½	VB	—	—	+1.1
Immune	½	broth	3.0	4.5	-0.9
Normal	½	broth	—	—	+3.6
Immune	¼	VB	3.5	1.75	-0.9
Normal	¼	VB	—	—	+1.1
Immune	¼	broth	4.5	4.25	-0.65
Normal	¼	broth	—	—	+3.6

* Normal serum titer day 6—immune serum titer day 6.

† Difference between inoculum titer and final titer +0.6 log.

*The Effect of Using a Nutrient and Non-nutrient Diluent for the
Serum in the Mixing System*

The mixing system consisted of duplicate ½ and ¼ dilutions of serum, one in veronal buffer pH 7.3¹² and the other in broth.

The results given in TABLE 3 showed that no neutralization and poor inhibition were obtained with veronal buffer at one-half, whereas with the broth at one-half, the inhibition and neutralization were good. With veronal buffer at one-quarter, however, good neutralization but relatively poor inhibition were obtained compared with the broth at one-quarter. This was repeated with similar results.

The Effect of Varying the Number of Organisms in the Test Inoculum

Five sets of one-quarter dilutions of immune and normal sera were each inoculated with ten-fold dilutions of the inoculum in broth from undiluted to 10^{-4}

TABLE 4

THE EFFECT OF VARYING THE NUMBER OF ORGANISMS IN THE TEST INOCULUM

Serum (diluted ¼)	No. organisms Log BCID ₅₀ /0.04 ml	Log inhibition* Day 3	Final	Log growth or† neutralization
Immune	6.0	5.5	5	-2.4
Immune	5.0	6.0	5.75	-2.65
Immune	4.0	6.0	4.5	-1.9
Immune	3.0	5.0	5	-2.4
Immune	2.0	3.5	3.5	-1.4
Normal	6.0	—	—	+2.6
Normal	5.0	—	—	+3.1
Normal	4.0	—	—	+2.6
Normal	3.0	—	—	+2.6
Normal	2.0	—	—	+2.1

* Normal serum titer day 6—immune serum titer day 6.

† Difference between inoculum titer and final titer +0.6 log.

The results of inhibition and neutralization given in TABLE 4 were consistent within 1 log, no matter how many organisms were used, except perhaps in the case of 10^2 BCID₅₀ organisms; and it appears that this number of organisms may be below the minimum for the test.

The Effect of Using Young or Old Organisms in the Test Inoculum

M. mycoides was grown in buffered broth at 37°C, and while in the thread form was vigorously shaken using the Vortex mixer. An inoculum was prepared from a portion of the culture by diluting it in broth to an estimated 10^5 BCID₅₀ organisms/0.04 ml. The remaining cultures were then incubated at 37°C for a further 24 and 48 hours, when aliquots were taken and inocula prepared as before. Each inoculum was titrated after preparation.

Immediately after the inocula had been prepared, they were inoculated into immune and normal one-quarter serum dilutions (mixing system). The results, given in TABLE 5, show that the age of the organisms had little effect on the results of the test. This experiment was repeated using a different number of organisms in the inoculum with similar results.

TABLE 5
THE EFFECT OF USING YOUNG AND OLD ORGANISMS AS TEST INOCULA

Serum (diluted 1/4)	Test organisms	No. organisms Log BCID ₅₀ /0.04 ml	Log inhibition* Day 3	Final	Log growth or† neutralization
Immune	Young	5.5	5.0	3.75	-2.15
Normal	Young	—	—	—	+1.6
Immune	24 hours	7.5	4.75	4.5	-2.65
Normal	24 hours	—	—	—	+1.85
Immune	48 hours	4.25	5.0	4.75	-2.1
Normal	48 hours	—	—	—	+2.85

* Normal serum titer day 6—immune serum titer day 6.

† Difference between inoculum titer and final titer +0.6 log.

A Comparison Between Test Inocula Grown in Glucose and Glucose-free Broth

The Gladysdale organisms were subinoculated into ordinary and glucose-free broths. Three days later, the organisms from both broths were lyophilized at the same time, and these were used for inocula.

Duplicate dilutions of immune and normal test sera were made, one series in ordinary broth and the other in glucose-free broth. Organisms grown in ordinary broth were inoculated into the ordinary broth dilutions, and glucose-free-grown organisms were inoculated into the glucose-free dilutions. This experiment was performed three times, and each time the glucose-free organisms grown in broth were slightly more susceptible than the glucose ones, but the difference each time was within the error of test (see below).

*A Comparison between the Gladysdale and KH3J Strains of
M. Mycoides as Test Inocula*

The two strains acted identically in the test.

TABLE 6
COMPARISON BETWEEN SERA UNABSORBED AND ABSORBED WITH *M. mycoides**
(INOCULUM TITER $10^{5.0}$ BCID₅₀ ORGANISMS/0.04 ml)

Serum (diluted 1/4)	Log inhibition† Day 3	Final	Log growth or‡ neutralization
Immune	4.5	3.0	-1.4
Immune absorbed	2.0	1.5	+0.1
Normal	—	—	+1.6
Normal absorbed	2.0	2.0	-0.4

* All sera compared to normal unabsorbed sera.

† Normal serum titer day 6—immune serum titer day 6.

‡ Difference between inoculum titer and final titer +0.6 log.

Determination of the Specificity of the Test by Absorption of Immune and Normal Serum with M. mycoides

The results (TABLE 6) showed that absorption of the immune serum removed much of the inhibitory and all the neutralizing ability of the serum. Also absorption of the normal serum increased its inhibitory and neutralizing ability. This experiment was repeated using an inoculum containing few organisms, with similar results.

The Effect of Complement

Four series of serum dilutions, two immune and two normal, were prepared. To one immune and one normal series, 5% guinea pig serum was added, and to the other 5%, heated (56° for 45 minutes) guinea pig serum was added. The undiluted guinea pig serum contained 100 minimal hemolytic doses per ml, as determined for the complement fixation test.⁸ Each dilution was then inoculated with two drops of $10^{4.75}$ BCID₅₀ organisms/0.04 ml.

As can be seen from TABLE 7, no significant difference was produced by the addition of complement to the mixing system.

Examination of Sera from Cattle before and after Infection with M. mycoides

Sera from 10 cattle before and after infection with *M. mycoides* were examined. Five of the cattle were inoculated subcutaneously with broth culture of the

TABLE 7
THE EFFECT OF COMPLEMENT (INOCULUM $10^{4.75}$ BCID₅₀ ORGANISMS/0.04 ml)

Serum (diluted 1/4)	Complement	Log inhibition* Day 3	Final	Log growth or‡ neutralization
Immune	+	4.5	3.75	-0.9
Normal	+	—	—	+2.85
Immune	—	4.0	3.75	-1.15
Normal	—	—	—	+2.6

* Normal serum titer day 6—immune serum titer day 6.

† Difference between inoculum titer and final titer +0.6 log.

TABLE 8

COMPARISON OF SERA FROM CATTLE BEFORE AND AFTER INFECTION WITH *M. mycoides*
BY THE SUBCUTANEOUS AND ENDOBRONCHIAL ROUTES
(INOCULUM TITER $10^{4.75}$ BCID₅₀ ORGANISMS/0.04 ml)

Animal number	Route of inoculation	Serum sample	Inhibition		Log growth or neutralization	SAST*	CFT† titer
			Day 3	Day 6			
327	Endobronchial	before	—	—	+1.1	—	0
		after	2.0	2.5	-1.4	+++	1/640
343	"	before	—	—	+2.1	—	0
		after	6.5	4.25	-2.15	++++	1/320
344	"	before	—	—	+2.1	—	0
		after	4.5	2.75	-0.65	—	1/1280
347	"	before	—	—	+1.85	—	0
		after	3.75	3.0	-1.5	+	1/2560
374	"	before	—	—	+3.1	—	0
		after	4.25	3.5	-0.4	+++	1/2560
402	Subcutaneous	before	—	—	+2.35	—	0
		after	6.0	3.25	-0.9	++	1/160
416	"	before	—	—	+2.1	—	0
		after	1.5	1.75	+0.35	+++	1/320
419	"	before	—	—	+2.6	—	0
		after	3.0	3.0	-0.4	+	1/320
531	"	before	—	—	+1.35	—	0
		after	3.25	2.0	-0.65	—	1/40
533	"	before	—	—	+2.85	—	0
		after	5.25	2.75	+0.1	+	1/640
Normal	Immune	—	—	—	+4.1	—	0
		—	4.75	4.75	-0.65	+	1/1280

* SAST — slide agglutination serum test.

† CFT — complement-fixation test.

T3 strain of *M. mycoides*,¹³ and five were inoculated endobronchially with Gladysdale lung material.¹⁴ The post-infection sera were collected between two and four weeks after inoculation.

Each post-infection serum sample was compared with the pre-infection serum sample from the same animal. The results (TABLE 8) show that in all cases, inhibition was demonstrated in the post-infection serum, although in some cases, this was only slight. Neutralization, however, was absent in two sera from the subcutaneously inoculated cases, but was demonstrated in all the sera from the endobronchially inoculated cattle.

There did not appear to be a quantitative correlation between the antibodies responsible for inhibition or neutralization and those responsible for either agglutination or complement fixation.

Reproducibility of the Test

The test was performed six times with the immune and normal sera. In the first series of tests, different numbers of organisms were used in the test inocula and different batches of broth were used for the reconstitution of the freeze-dried ampule and for the titrations. In the second series of tests, the same number of organisms and the same batch of broth were used. The results showed that in the first

series, the range of inhibition and neutralization was 1.25 log, and in the second series, the range was 0.75 log.

Microscopic Examination of the Mixing System

Dark-ground microscopic examination was made of the organisms in the $\frac{1}{4}$ dilutions of the normal and immune sera at 1, 24, 48, and 72 hours after the addition of the organisms. The mixing system was shaken on the Vortex for five seconds before each sample was removed. No difference was noted between the appearance of the organisms in the two sera at one hour, and no aggregation of the organisms was visible. At 24 hours, many small bodies were seen in both sera; but whereas in the normal serum, numerous growing forms were present, they were not seen in the immune serum. No significant aggregation was seen in either serum. After 48 and 72 hours, organisms in both sera were mainly of the small coccoid type, but fewer appeared to be present in the immune than the normal serum.

The addition of SAST antigen⁶ to the one-quarter dilutions of the two sera showed that considerable agglutination occurred and could be easily seen by dark-ground microscopy. However, there was no visible difference between samples placed at 21°C or 30°C for one hour and then shaken in the Vortex for five seconds.

Modified Test

The immune and normal sera were used and the results are given in TABLE 9. Inhibition was observed in the presence of immune serum to a titer of one-eighth, as the highest concentration of serum in which *M. mycoides* would grow in six days was one-sixteenth. In the normal serum, the organisms grew in all the dilutions, including the one-quarter dilution within one day. No neutralization was detected, as growth was obtained in the subinoculations (measuring system) from both the undiluted (one to one) immune and normal sera.

DISCUSSION

The term neutralization has been used throughout this report in order to conform to previously used and accepted terminology.¹ It is realized, however, that there may be a difference between virus neutralization and the "neutralization" of *M. mycoides*. The possibility that inactivation of the organisms may occur in the absence of multiplication in the immune serum is not excluded; nevertheless,

TABLE 9
EXAMINATION OF IMMUNE AND NORMAL SERA BY THE MODIFIED TEST
(INOCULUM TITER $10^{5.5}$ BCID₅₀ ORGANISMS/0.04 ml)

Serum	Highest concentration of serum showing growth					
	Mixing system* Days at 37°			Measuring system† Days at 37°		
	1	2	3	4	5	6
Immune	1/128	1/64	1/32	1/32	1/32	1/16
Normal	1/4	1/4	1/4	1/4	1/4	1/4

* It was impossible to observe growth of *M. mycoides* in serum concentrations greater than 1/4 with reliability, so they were discarded.

† Growth in at least two tubes.

this phenomenon is the direct result of specific antibodies although the mechanism is as yet unknown. Furthermore, it is possible that growth inhibition and neutralization are only steps in the same process, neutralization resulting from inhibition.

Cottew,¹⁵ using a growth-inhibition test, reported that inactivation of the sera at 56°C for 30 minutes destroyed the inhibitory activity; and he concluded that two factors—one a heat-labile one, presumably complement, and the other heat-stable—together with bovine conglutinin caused an aggregation, and that it was this aggregation of the organisms that may greatly reduce colony counts made to measure viability. It seems that in many of the so-called growth-inhibition tests described by various workers, the phenomenon of agglutination or aggregation has not been sufficiently considered as a possible explanation for the inhibition obtained. The reported observations herein indicate that inhibition and neutralization can occur with inactivated serum, but the question of the effect of agglutination is rather more complex. The fact that no inhibition or neutralization occurs at 21°C (except for the transient inhibition occasionally seen at day 3) when agglutination can occur, the inhibition noted in the mixing system of the modified test when agglutination or aggregation would not affect the results, and the results obtained using the sera from experimentally infected cattle where no quantitative correlation was observed between the test results and the SAST and CFT results, indicate that agglutination probably does not play a significant part in the test. It was interesting to note that no agglutination was observed in the mixing system although marked agglutination was seen when the SAST antigen was used instead of viable organisms. The SAST antigen is a heated suspension of organisms.

The lack of inhibition or neutralization at 21°C (except for the slight transient inhibition) and the activity at 30°C indicates either that the antibodies responsible are relatively inactive at 21°C, or more likely that multiplication of the organisms is necessary for the phenomenon to occur, as the minimum temperature at which *M. mycoides* will grow is 27°C.¹⁶ The evidence that inhibition and neutralization were greater in nutrient as compared to nonnutrient diluents and that neutralization increases with the length of time at 30°C also supports the idea that multiplication or growth is necessary. If growth is necessary, it does not appear that the stage of growth is important, but rather the actual growth or multiplication of the organisms, as young and old organisms were equally susceptible.

It is of interest that the number of organisms used in the test inocula made no significant difference to the result, indicating possibly that a constant proportion of the population of organisms is susceptible to inhibition and neutralization. The results of the modified test, where there was a surviving fraction even using undiluted serum, would support this. If this is so, it is evident that the non-susceptible organisms do not multiply sufficiently at 30°C in three days to effect the test. This may be the reason why earlier work using 37°C for initial incubation was unsatisfactory.

From the results using the modified test, it is apparent that inhibition after three days at 30°C and six days at 37°C in the presence of serum occurs at a minimum concentration of this serum of one-eighth. Therefore, in the test proper, it would not be expected that the presence of the small amount of serum in the first tube of the measuring system, i.e., 1/144, would inhibit growth.

It proved unnecessary to filter the test sera unless they were heavily contaminated, as heating the serum at 56°C for 45 minutes plus the addition of penicillin and thallium acetate to the broth was usually sufficient to prevent growth of contaminating organisms.

It was noted that certain normal sera from local cattle possessed inhibitory

and neutralizing ability as compared to the normal serum obtained from the U.S.A. The reason for this is not known but may be associated with a number of factors—for example, the nutritional value of the serum or the presence of non-specific or natural antibodies. This latter factor appears unlikely, as the animal possessing the greatest inhibitory or neutralizing ability in its preinfection serum (No. 327, TABLE 8) succumbed the quickest to the experimental infection with *M. mycoides*.

The use of buffered broth as diluent for the serum in the mixing system appeared to increase the level of inhibition and neutralization. The reproducibility of the test was most affected by variation in the batches of broth. This was presumably due to different batches of pig serum, as this is the only variable ingredient of the medium. This suggests that a bank of pooled pig serum would be of value for preparation of broth for this test.

It has been shown that the Gladysdale strain of *M. mycoides* grown in broth containing 0.5% added glucose produces considerably thicker threads than *M. mycoides* grown in broth containing no added glucose.¹⁷ Furthermore, the KH3J strain produces no threads even in 0.5% glucose broth.¹⁷ However, it appears that this ability to form threads or the thickness of threads does not effect the inhibition or neutralization of the organisms in vitro, as the KH3J strain and Gladysdale strain grown in glucose or glucose-free broth were equally susceptible to inhibition and neutralization with the immune serum.

Absorption of the immune serum with *M. mycoides* removed much of the inhibitory and all the neutralizing ability of the serum. However, it was also seen that absorption of the normal serum increased its inhibitory and neutralizing ability. The reason for this is unknown, but it does not affect the results obtained with the immune serum but rather accentuates them.

SUMMARY

Inhibition of growth and "neutralization" of *Mycoplasma mycoides* by inactivated immune bovine serum occurred at 30°C but not at 21°C, and no significant difference was noted between young and old cultures of the organisms. Organisms grown in broth with and without added glucose were equally susceptible. The virulent Gladysdale and avirulent KH3J strains of *M. mycoides* acted identically in the test. The degree of inhibition and "neutralization" was consistent no matter how many organisms were used in the test. The addition of complement had no effect on the degree of inhibition or "neutralization." Agglutination did not appear to contribute to the decrease in viable count obtained with immune serum. There was no quantitative correlation between the test results and results of the complement-fixation of agglutination tests on the same sera. Reproducibility of the test within a range of 0.75 log could be obtained.

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Serological Relationship between Galactans from Normal Bovine Lung and from *Mycoplasma mycoides*

A GALACTAN (pneumogalactan) isolated from bovine lung^{1,2} precipitated 28 per cent of pneumococcal polysaccharide type XIV antiserum³. This suggested that both polysaccharides have galactose residues of similar linkages, possibly β -1:6, β -1:3 or β -1:3:6⁴, and agreed with the postulated structures of type XIV pneumococcal polysaccharide⁴ and of lung galactan².

Recently it was reported^{5,6} that a galactan was isolated from *Mycoplasma mycoides*, the causative organism of contagious bovine pleuropneumonia (CBPP). It was decided, therefore, to determine whether a serological relationship existed between the galactans from normal bovine lung and from this organism causing lung lesions in cattle.

Purified pneumogalactan was kindly supplied by Hoffman-La Roche, Inc., Nutley 10, New Jersey. Since the isolation method of pneumogalactan is not described, we extracted, with hot phenol⁷, an acetone powder of bovine lung obtained from the U.S.A., a country free of CBPP (California Corporation for Biochemical Research, 3625 Medford St., Los Angeles); this material is referred to as normal bovine lung extract. *M. mycoides* cells were used for preparation of antiserum (x') in sheep⁸. Sera from animals naturally infected with CBPP were also used (sera Nos. 21 and 42). As our attempts to produce antisera against pneumogalactan have so far failed, the results presented here are of experiments with pneumogalactan, *M. mycoides* antigens and antisera against *M. mycoides*.

Table 1 shows the results of complement-fixation, indirect haemagglutination⁹ and tube agglutination tests, using *M. mycoides* antigen with immune sera and sera absorbed with excess pneumogalactan. These results show that the pneumogalactan absorbs a considerable proportion of the relevant antibodies against *M. mycoides*.

In the agar-gel precipitin test, serum against *M. mycoides* (x') gave homologous lines with hot phenol-extracted carbohydrate from *M. mycoides*¹⁰, pneumogalactan, and normal bovine lung extract. Sera 21, 42,

Table 1. RECIPROCAL TITRES OF *M. mycoides* ANTISERA, BEFORE AND AFTER ABSORPTION WITH PNEUMOGALACTAN, AGAINST *M. mycoides* ANTIGEN

<i>M. mycoides</i> antiserum	Complement fixation	Indirect haemagglutination	Agglutination
x' before absorption	80	640	640
after absorption	10	80	80
'21' before absorption	80	320	80
after absorption	20	80	40
'42' before absorption	160	320	320
after absorption	20	80	40

and x' formed precipitin lines with hot-phenol-extracted carbohydrate: after absorption with 30 mg of pneumogalactan no lines were formed with either serum 21 or 42 and only very faint lines were produced with serum x' .

Animals naturally infected with CBPP (Nos. 21 and 42) gave positive skin test reactions¹¹ with both hot-phenol-extracted carbohydrate from *M. mycoides* and pneumogalactan.

The cross-reactivity of pneumogalactan and the galactan from *M. mycoides* indicates that both these polymers have some common linkages. We are now investigating the possibility that the pneumogalactan plays a part in the pathogenesis of CBPP, either in an allergic type reaction, or by localizing the organisms in the lung through an antibody-antigen reaction. It would also be of interest to determine whether human lung contains a galactan which might cross-react with the pneumococcal polysaccharides.

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ANTIGENIC CROSS-REACTIONS BETWEEN THE GALACTAN FROM *MYCOPLASMA MYCOIDES* AND POLYSACCHARIDES FROM OTHER SOURCES

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INTRODUCTION

In an earlier paper (Shifrine and Gourlay, 1965a) we mentioned the isolation of a gram positive coccobacillus from apparently normal cattle that had produced strong immediate-type skin reactions on inoculation of lipopolysaccharide from *Mycoplasma mycoides*. Sera from these cattle were also positive to the complement fixation test (CFT) and serum agglutination slide test (SAST) for contagious bovine pleuropneumonia (CBPP). Also in a preliminary publication (Shifrine and Gourlay, 1956b) we reported on the serological relationship between polysaccharides from normal bovine lung and *M. mycoides*. In this paper we give more details of this and further details of the gram positive bacterium. We also report on antigenic cross-reactions between the galactan of *M. mycoides* and polysaccharides from other sources. The significance of these various findings in the diagnosis and pathogenesis of CBPP is discussed.

MATERIALS AND METHODS

Strain of M. mycoides. The Gladysdale strain was used throughout this work.

Serological methods. The Campbell and Turner complement fixation test (CFT) was carried out with the modification as previously described (Gourlay, 1965). The tube agglutination test (AT), the slide agglutination serum test (SAST) and the agar gel precipitin test (AGT) were performed as described by Gourlay (1964a) and the skin test by the method of Gourlay (1964b). The indirect haemagglutination test (IHA) used was that described by Perreau, Provost, Regnault and Orue (1964). For use in the AGT organisms A and B were passaged 10 times on nutrient gelatin slopes, then harvested and treated in an ultrasonic disintegrator for 15 minutes before use. The most purified extracts of the various polysaccharides were used for absorptions, AGT and skin tests. Absorptions were carried out with 10 mg. of antigen per 1 ml. of serum at 37°C. for 2 hours, followed by 4°C. for 18 hours. This was repeated 3 times.

Isolation of the Gram positive bacterium. Fourteen apparently healthy cattle at Muguga that had had no contact with CBPP were tested by various diagnostic methods and the results are given in Table 1. Five gave strong positive skin tests on inoculation of *M. mycoides* lipopolysaccharide (Shifrine and Gourlay, 1965a), 7 were positive and the rest doubtful to the CFT, 2 were weakly positive to the SAST and 2 had precipitating antigen in their sera. Two of the cattle were killed; one, A67, within a few days of testing and the other, A59, one month later. On autopsy A67 had numerous slight pneumonic lesions on the tips of both diaphragmatic lobes and on the tip of the right cardiac lobe. All other organs appeared normal. A59

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appeared completely normal on autopsy. From the lungs of A67 two colony types were isolated which gave precipitin lines in the AGT against *M. mycoides* antiserum and were named A and B.

Polysaccharide extracts. The galactan from *M. mycoides* and the polysaccharide from organisms A and B were extracted with hot phenol (Gourlay and Shifrine, 1965). Bovine lung extract (BLE) was prepared from normal bovine lung acetone powder.* The powder was extracted with hot phenol and the extract was dialysed against water and lyophilized. The polysaccharide was further purified by precipitating it with 2 volumes of ice-cold 95 per cent. ethanol. Agar extract was prepared by stirring a 2 per cent. (w/v) agar (Difco) suspension in water at 4°C. for 2 days, followed by centrifugation and lyophilization of the supernatant fluid. The extract was further purified by hot phenol extraction and ethanol precipitation. Rabbit and human lungs were homogenized with minimal amounts of distilled water in a Waring blender and insoluble material removed by centrifugation. The supernatant fluid was lyophilized and then extracted with hot phenol followed by ethanol precipitation. Gum guar† was used without further purification. Galactan from bovine lung, i.e. pneumogalactan (PG), was kindly donated.‡

Preparation of antisera. Antisera against organisms A and B were prepared in the following manner. Two rabbits and 2 cattle which possessed no antibodies against *M. mycoides* in the CFT nor against organisms A and B in the AGT were inoculated with organisms which had been passaged 4 times on nutrient gelatin slopes instead of agar slopes. They were inoculated on 3 occasions intramuscularly with antigen in Freund's adjuvant and, in addition, the rabbits were also inoculated intravenously with antigen in normal saline.

Attempts to prepare antisera against the other polysaccharides were made as follows. Two rabbits were each inoculated intravenously with their own erythrocyte stroma coated with polysaccharide extracts of *M. mycoides*, BLE, PG and the aqueous extract of agar by the method of Staub (1964). Since no demonstrable antibodies were produced by this method, the rabbits were further inoculated 3 times intramuscularly with the coated stroma in Freund's adjuvant. For BLE, however, the hot phenol extract (10 mg.) in Freund's adjuvant was used, the dose being 1 ml. each time. The rabbits were bled 7 days after the last injection.

Cattle were inoculated with *M. mycoides* galactan in a manner identical to those used for rabbits, but tenfold amounts were used. Cattle were also inoculated intramuscularly with agar and agar extract (40 mg./ml.) in Freund's adjuvant, and gum guar 20 mg./ml. in saline. The dose was 5 ml. each time.

For hyperimmune sera, cattle and sheep were immunized with *M. mycoides* as described earlier (Gourlay, 1964a). Before use in the agar gel precipitin test (AGT) the sera were absorbed 3 times with constituents of the media (pig serum, yeast extract and tryptose—10 mg./ml.) in which the organisms were grown for immunization.

RESULTS

Cross-reaction between M. mycoides and the Gram-positive Coccobacillus

The type A colony, on nutrient agar, after 2 days growth at 37°C., was 3 mm. in diameter, circular, umbonate, granular, slightly radially striated with lobate, slightly crenated edges and cream-coloured. The colony became primrose yellow after standing in the light. The colony grew at room temperature, though more slowly, and after 4 days was about the same size as after 2 days at 37°C. The type B colony, on nutrient agar, after 2 days growth at 37°C. was 1.5 mm. in diameter, circular, smooth, convex with entire edges, creamy-grey in colour and

* Purchased from California Biochemical Corporation, Los Angeles, California, U.S.A.

† Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

‡ Hoffman-la Roche Inc. Nutley 10, N.J., U.S.A.

slightly opalescent. The colony also became primrose yellow in the light. As with type A, it also grew at room temperature and was about 1.5 mm. in diameter after 4 days growth.

Development of these organisms appeared better on solid than in liquid medium. Following growth in nutrient broth the A type colony was transformed into smooth colony type when plated on to solid medium: it looked identical to the B type colony and was possibly the rough form of the same organism. Types A and B organisms were both gram-positive coccobacilli, but type A showed beaded filaments.

The organisms were grown in nutrient broth for 2 days and each culture inoculated intraperitoneally into 6 mice (0.5 ml. each) and 2 rabbits (1 ml. each), and subcutaneously into 2 rabbits (1 ml. each) and 1 ox (10 ml.). The

TABLE 1
RESULTS OF SKIN TESTS

CFT and SAST, with *M. mycoides* antigen, and AGT (for antigen) using hyperimmune sera against *M. mycoides*, on 14 normal cattle

Animal No.	LPS skin test. Increase in skin thickness after 2 hours (mm.)	CFT*	AGT*	SAST*
A58	6	+	—	—
A59	7	++	—	—
A61	1	+	—	—
A62	3	+	—	—
A63	2	++	—	—
A64	6	++	—	—
A66	2	++	+	+
A67	10	++	—	—
A69	6	+	—	—
A72	3	++	—	—
A73	2	+	+	—
A74	3	+++	—	+
A77	2	+	—	—
A78	2	+	—	—

* CFT Complement fixation test; fixation at 1:10 dilution of serum.

AGT Agar gel precipitin test for antigen.

SAST Slide agglutination serum test.

number of viable organisms in each case were, type A 1.5×10^{11} /ml., type B 1.9×10^{12} /ml. None of the animals died or showed signs of sickness.

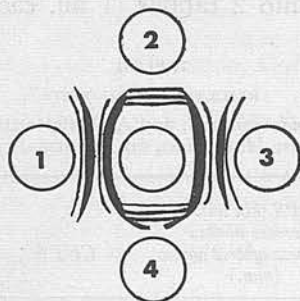
Cattle immunized with organisms A and B gave strong immediate allergic reactions of 6.5 and 8 mm. thickness increase in the skin test using lipopolysaccharide (LPS) from *M. mycoides* (Shifrine and Gourlay, 1965a). They were challenged 2 weeks after the last immunizing injection by the subcutaneous inoculation of 2 ml. of a broth culture of *M. mycoides* (Gladysdale strain). They both developed extensive Willems reactions at the inoculation site and died.

Using hyperimmune sheep and ox sera prepared against *M. mycoides* in the AGT, organisms A and B produced 3 common precipitin lines, which also formed lines of identity with lines produced by *M. mycoides* organisms (Fig. 1) and 3 of the lines of LPS from *M. mycoides*. Against sera from cattle naturally infected

with CBPP, precipitin lines were also produced by organisms A and B, but no lines were produced against normal sheep and cattle sera.

In the AGT, using hyperimmune sheep and ox sera, hot phenol extracts of organisms A and B gave lines of identity with some of the lines produced by hot phenol extracts of *M. mycoides*. However, following incubation of A and B (1 mg. in each /ml.) with cellulase (Nutritional Biochemical Co., 1 mg./ml. of 0.2 M acetate buffer pH 4.5 at 37° for one day) no precipitin bands were produced in AGT against *M. mycoides* antisera, or against the homologous antisera. To further ensure that agar was not responsible for the cross-reactivity in the AGT

Fig. 1.



Photosketch of precipitin lines produced by organisms A and B and *M. mycoides*.

Centre well *M. mycoides* ox hyperimmune serum.

Well 1 *M. mycoides* cells 10 mg./ml.

Well 2 A cells 50 mg./ml.

Well 3 *M. mycoides* cells 10 mg./ml.

Well 4 B cells 50 mg./ml.

TABLE 2

RECIPROCAL TITRES OF ANTI-A, B AND *M. mycoides* SERA

Before and after absorption with homologous and heterologous antigens using the indirect haemagglutination test with erythrocytes coated with *M. mycoides* lipopolysaccharide

Antiserum against	Unabsorbed sera	Reciprocal titres absorbed with		
		A	B	<i>M. mycoides</i>
A	80	40	0	0
B	80	80	10	0
<i>M. mycoides</i>	2560	640	1280	0

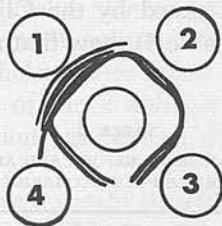
we used serum from an animal naturally sensitized with A and B organisms (A66), and as antigen source we used sera from CBPP naturally infected animals. Serum A66 gave precipitin lines with the *M. mycoides* antigen in these sera.

The rabbit sera prepared against types A and B gave titres of 1/40 and 1/20 respectively against *M. mycoides* antigen in the CFT and titres of 1/80 in the IHA test. The results of the IHA on cattle serum from a field case of CBPP and the rabbit sera, after absorption with 10 mg. of cells A, B and *M. mycoides*, are shown in Table 2. Sera from cattle A66, A67 and A74 reacted in the IHA to give titres of 1/160, 1/160 and 1/40 respectively. Sera from normal cattle gave negative results at the 1/10 dilution.

Cross-reaction between M. Mycoides Galactan and Agar, Gum Guar and Polysaccharides from Bovine, Human and Rabbit Lungs

Rabbits and cattle which were inoculated with the polysaccharide from *M. mycoides*, agar, gum guar and PG did not produce any demonstrable antibodies against *M. mycoides*, as determined by CFT, AT, AGT and skin test, or against their homologous or other heterologous antigens in the AGT or skin test. In cattle, only PG was used in the skin test. BLE, however, did elicit antibodies in rabbits against *M. mycoides* in the CFT, giving titres of 1/160 and, in the AT,

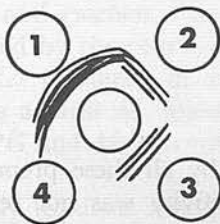
Fig. 2.



Photostetch of precipitin lines produced by polysaccharides from various materials.

Centre well	<i>M. mycoides</i> ox hyperimmune serum.
Well 1	<i>M. mycoides</i> galactan 0.05 mg./ml.
Well 2	RLE 10 mg./ml.
Well 3	HLE 10 mg./ml.
Well 4	Gum guar 20 mg./ml.

Fig. 3.



Centre well	<i>M. mycoides</i> ox hyperimmune serum.
Well 1	<i>M. mycoides</i> galactan 0.05 mg./ml.
Well 2	PG 40 mg./ml.
Well 3	PG 40 mg./ml.
Well 4	Nil.

titres of 1/10 and 1/128. In the AGT both anti-BLE rabbit sera gave at least 2 precipitin bands against the homologous antigen and also against PG, agar, gum guar, *M. mycoides* galactan, human lung extract (HLE) and rabbit lung extract (RLE). When the various polysaccharides were tested in the AGT against sera of sheep and cattle hyperimmunized against *M. mycoides*, they all gave precipitin bands of identity with certain bands produced by *M. mycoides* galactan. The latter gave bands identical to those produced against polysaccharide antigen

P.1/2/3 isolated from urine of cattle infected with *M. mycoides* (Gourlay and Palmer, 1965). Agar and gum guar gave at least 2, PG at least 3 and BLE at least 4 lines: HLE and RLE produced lines identical to those of BLE.

Figs. 2 and 3 show photosketches (these are drawings over photographs followed by bleaching) of the lines produced by various polysaccharides against *M. mycoides* hyperimmune ox serum. Only 2 thick lines are visible against HLE and RLE. In certain instances, however, these two could be seen to be split into 2 more, but were difficult to photograph. Attempts to use BLE as antigen against *M. mycoides* immune serum in the CFT and AT failed.

Serum of an ox hyperimmunized against *M. mycoides* and pooled rabbit sera prepared against BLE were absorbed with BLE, HLE, PG and *M. mycoides* galactan and the absorbed sera tested by the CFT, AT and SAST against *M. mycoides* antigen. The results (Table 3) show that antibodies against *M. mycoides*

TABLE 3
RECIPROCAL TITRES OF ANTISERA BEFORE AND AFTER ABSORPTION WITH
VARIOUS POLYSACCHARIDES

Serum against	Test*	Unabsorbed sera	Sera absorbed with			
			<i>Mycoides</i> galactan	BLE	PG	HLE
<i>M. mycoides</i>	BLE	80	0	0	10	10
	SAST	+++	—	ND	—	—
	AT	128	64	ND	16	16
	CFT	640	10	20	40	40
	SAST	++++	—	ND	+	ND
	AT	1024	128	ND	128	ND

* CFT Complement fixation test.
SAST Slide agglutination serum test.
AT Agglutination test.
ND Not done.

were absorbed from both sera by all these preparations. Agar made the sera anti-complementary and this activity was not removed by kaolin. Gum guar was too viscous for absorption.

DISCUSSION

Heslop (1922) reported a slight serological relationship between *Mycoplasma mycoides* and vaccinia virus, which was confirmed by Provost (1958). Turner (1956) reported a similar relationship between *M. mycoides* and some strains of *Actinobacillus lignieresii*. Heidelberger (1960) found that bovine antiserum against *M. mycoides* cross-reacted with polysaccharides from *Azotobacter chroococcum*, *Cryptococcus neoformans* A, *Rhizobium radicicolum*, *Acrosiphonia*, *Albizzia*, *Brachychiton*, *Fagara*, flax straw hemicellulose, and barley and oat glucans. He remarked: "Only a few substances were tested in this serum, but a remarkably high proportion of positive reactions was obtained."

To the above list of micro-organisms and polysaccharides with which *M. mycoides* antibodies will react we can now add the coccobacillus or more specifically the polysaccharide from the coccobacillus, described in this report, and agar, gum guar and polysaccharides from bovine, human and rabbit lungs.

Whereas the coccobacillus obtained from the ox does not appear to be a virulent organism, in that it did not kill any of the experimental animals it may, on circumstantial evidence, have sensitized the group of cattle from which it was obtained. The cattle reacted to the serological tests in a rather similar manner and, except for an isolated case in 1963, in which a control animal reacted to the CFT and SAST skin tests (Gourlay, 1964b), and 3 cattle from a separate herd at Muguga in 1961, which gave strong transient positive CFT reactions (Brown, 1961), all previous control animals, and many hundreds have been tested, have been negative to these tests. Whether this organism did sensitize these animals or not it, nevertheless, cross-reacts serologically with *M. mycoides*. It was isolated from only one of the 2 animals killed, but the second one was killed a month after the infection was noticed and the organisms may well have disappeared by that time.

Despite the use of coated erythrocyte stroma and adjuvant we were unable to demonstrate any antibodies in rabbits or cattle to the different polysaccharides, other than that from organisms A and B, with the exception of BLE, probably indicating that they were haptens. BLE elicited antibody response, probably because it was a less pure product.

In the AGT, using the polysaccharides as antigens and sera prepared against *M. mycoides*, we showed that agar, gum guar, PG, BLE, *M. mycoides* galactan and water extracts from human and rabbit lungs all possessed some common precipitin lines. Again, antibodies against BLE, essentially a less purified PG preparation, also produced some common lines between agar, gum guar, PG, BLE, *M. mycoides* galactan and extracts from human and rabbit lungs. The above results were substantiated by the results of absorption experiments. Using antiserum against *M. mycoides* produced in an ox and antisera against BLE produced in rabbits, the titres against *M. mycoides* antigen were reduced after absorption with BLE, HLE, PG and *M. mycoides* galactan in the CFT, AT and SAST.

We have reported previously (Shifrine and Gourlay, 1965b) that the titres of sera produced in sheep against *M. mycoides* and sera from cattle naturally infected with *M. mycoides* were reduced in the CFT, AT and IHA, against *M. mycoides* antigens, by absorption with PG. The results given in this paper, together with the above results, indicate that *M. mycoides* galactan, BLE, HLE, PG and, probably agar, gum guar and RLE, have some reactive sites (antigenic determinants) in common. The only evidence, so far as the latter three materials are concerned, is the result of the AGT, since absorption experiments with gum guar and agar were not possible due to the extreme viscosity of the solutions, anti-complementary activity of agar and insufficient amounts of RLE.

The polysaccharides used in this study are heterogeneous, although some of the linkages have been elucidated. The postulated linkages for PG (Wolf from, Sutherland and Schlamowitz, 1952) are β 1-3 and β 1-6 galactopyranose; those of agar are β 1-3 linked D-galactopyranose connected by 1-4 linkage to

L-galactose (Jones and Peat, 1942) and those of gum guar are β 1-4 linked D-mannopyranose units linked with D-galactopyranose units by 1-6 linkages (Ahmed and Whistler, 1950). The common linkages in these heterogeneous polysaccharides are 1-3 and 1-6 and it is therefore possible that *M. mycoides* galactan also contains these linkages. Buttery and Plackett (1960) indicated that the sugars in *M. mycoides* galactan might be mainly in furanose form. Our studies of the cross-reactivity of this galactan with known poly-galactopyranoses would indicate that *M. mycoides* galactan may contain some galactopyranose rings also. It is possible that this galactan is present in both ring types.

Of more interest to us is the possible role that PG might play in the pathogenesis of contagious bovine pleuropneumonia (CBPP) in cattle and its possible role in immunity to the disease. If it does play a part it may do so in a number of ways, for example, in an auto-immune allergic type reaction, or as a method of localization of the organisms in the lungs through the bivalent sites in the antibody. Alternatively the similarity between the antigens of *M. mycoides* and PG may produce a partial immune tolerance of the host to the parasite. These ideas are being currently investigated by us.

Sera from some of the cattle possibly sensitized with the coccobacillus and certainly not infected with CBPP were positive to all the common tests used for diagnosis of CBPP. It is evident that the galactan of *M. mycoides* is antigenically related to a number of polysaccharides found in nature, including polysaccharides from certain pathogenic and non-pathogenic micro-organisms, as well as food-stuffs. The presence of these antigenically similar polysaccharides may account for the false positive results obtained at times in the diagnosis of CBPP. There have been 3 instances of non-specific sensitization of cattle at Muguga alone in the last four years, indicating that this phenomenon may be fairly common. However, as the reactions are apparently only transient they may not be noticed or their significance appreciated unless tests are performed on sera from cattle from CBPP free areas.

CONCLUSIONS

Antigenic cross-reactions are reported between polysaccharides from *Mycoplasma mycoides*, agar, gum guar, normal bovine, human and rabbit lungs and from a gram-positive coccobacillus isolated from apparently normal cattle.

Polysaccharides antigenically related to the galactan of *M. mycoides* occurring in micro-organisms and foodstuffs may account for false positive results obtained at times in the diagnosis of CBPP. It is considered that the pneumogalactan from normal bovine lung may play a role in the pathogenesis of CBPP.

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PRELIMINARY EXPERIMENTS WITH ANTIGENS OF *THEILERIA PARVA* (THEILER, 1904)

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SUMMARY

The agar gel precipitin test was used to study the antigens of *Theileria parva*. Fractions were prepared from pre- and post-infection blood and lymph glands from the same ox. Antisera were prepared on cattle by inoculating post-infection fractions prepared from blood and spleen in Freund's adjuvant. At least four specific precipitin bands were observed; the antigens associated with at least two of the bands were heat labile and the remainder heat stable. A number of the antigens in the blood and spleen were common.

The agar gel precipitin test and the intradermal skin test indicated that *T. parva* possessed antigens in common with pneumogalactan from normal bovine lung and with *Mycoplasma mycoides*, the causal organism of contagious bovine pleuropneumonia.

Serological investigations concerning East Coast Fever (E.C.F.) have been very limited. Most of these investigations have been designed to develop a diagnostic test; for instance, Lichtenheld (1911) obtained fixation of complement with the serum of animals suffering from E.C.F. using an antigen extracted from the lymph nodes of animals dying from the infection. This was confirmed by Neitz (1962) but has not been developed as a diagnostic tool. No further publications have appeared on the subject, although it is known that some work has been done in different laboratories.

The work described in this paper was not designed to develop a diagnostic tool but was rather an attempt to begin the study of the antigenic structure of *Theileria parva*. The work of Sherman (1964) on *Plasmodium lophurae* and the extensive work of Zuckerman, reviewed by her in 1964, encouraged us in the belief that such an investigation might be profitable. This paper records preliminary observations made with the agar gel precipitin test (AGT) and the intradermal skin test.

MATERIALS AND METHODS

Parasites. The strain of *T. parva* used was that which has become known as *T. parva* (Muguga). This strain has been maintained in these laboratories for many years by alternate bovine/tick passages, according to the methods described by Bailey (1960). Its behaviour is well known and it has been

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characterized in a number of different publications (see Brocklesby & Bailey, 1962). The parasite is highly pathogenic for exotic breeds of cattle, causing a mortality rate of 96 per cent when ten *Rhipicephalus appendiculatus* adult ticks are used to transmit the infection.

Mycoplasma mycoides concentrated organisms. The Gladysdale strain was used and was grown and harvested as described previously for the T₃ strain antigen (Gourlay, 1964).

Pneumogalactan from ox lung. Pneumogalactan (PG) was kindly supplied by Hoffman-La Roche Inc., Nutley 10, N.J., U.S.A.

Cattle. Experimental cattle were Grade, which are crosses between East African Shorthorn Zebu and breeds of European origin, in which the latter dominate.

Preparation of piroplasm fractions

Whole blood was collected into tubes containing dry ethylene diamine tetra-acetic acid (EDTA) (1 mg. for 5 ml. blood) from normal cattle and from cattle infected with *T. parva*. The erythrocytes were deposited by centrifugation at 5°C in a refrigerated centrifuge at 1250 g for 30 minutes, the buffy coat and plasma were removed and the erythrocytes were washed three times in normal saline. The packed erythrocytes were lysed by the addition of two volumes of distilled water and piroplasm deposit 1 (piro 1) was produced by centrifugation at 1250 g for 30 minutes. The supernatant remaining after the removal of piro 1 was further centrifuged at 37,000 g for 60 minutes, giving piroplasm deposit 2 (piro 2). This consisted of two layers, a small solid button at the bottom of the tube and a layer of syrupy material above it. The deposits were freeze-dried.

Microscopic examination of Giemsa-stained smears of these two fractions showed that piro 1 consisted mainly of comparatively large piroplasms, that were in association with erythrocytic stroma and were possibly in the process of phagotropy, whereas piro 2 consisted of individual piroplasms that were smaller and more densely stained. Separation was not complete and many piroplasms of both types, as well as intermediate forms, were present in both fractions.

Preparation of spleen and lymph node fractions

A spleen was collected from an ox infected with *T. parva*. Smears of this spleen contained many macroschizonts and microschizonts; free micromerozoites were frequent. The spleen was decapsulated and chopped up into small pieces with scissors; 0.25 molar sucrose was added and the chopping continued. This material was then squeezed gently through gauze into a measuring cylinder. This crude spleen extract was then centrifuged at 9.4 g for 3 minutes, giving spleen deposit 1 (spleen 1). The supernatant was then further centrifuged at 150 g for 5 minutes, giving spleen deposit 2 (spleen 2); the supernatant thus produced (spleen supernatant 2) was centrifuged again at 1475 g for 10 minutes, giving spleen deposit 3 (spleen 3). The last supernatant was finally centrifuged at 37,000 g for 15 minutes, giving spleen deposit 4 (spleen 4). All deposits were freeze-dried.

Smears of these four fractions were examined: spleen 1 consisted of densely staining nuclear debris, red cell ghosts and a few areas like spleen 2 which contained hypertrophic lymphocytes harbouring micro- and macro-schizonts (the latter always with large coarse pieces of chromatin); spleen 3 consisted of intact and heavily parasitized erythrocytes, many coarse macroschizonts and frequent microschizonts in all stages of development; spleen 4 revealed a background of collagenous material, many free micromerozoites with their residual bodies and rare small macroschizonts.

Lymph nodes were finely chopped with scissors, frozen and thawed four times and chopped again, centrifuged at 150 *g* for 5 minutes and the supernatant, after treatment in an ultrasonic disintegrator* for 10 minutes at 4°C, was used as antigen in the AGT.

Preparation of antisera

Antisera were first prepared in rabbits but were unsatisfactory as it was not possible to absorb out all the non-specific antibodies produced; ultimately cattle were used for the preparation of antisera.

Sera from cattle that had recovered from a previous infection with *T. parva* were first examined in the AGT to determine whether any precipitin bands would be produced against the various piroplasm or spleen deposits; in no case were precipitin bands seen.

(a) *Piroplasm antisera*. For the preparation of piroplasm antisera 230 mg. of piro 1 and piro 2 "syrup" freeze-dried materials were each reconstituted in 10 ml. distilled water and treated for 15 minutes in the ultrasonic disintegrator at 4°C. Following ultrasonic disintegration the materials were each mixed with 10 ml. of complete Freund's adjuvant. The two antigens thus produced were each inoculated intramuscularly into an ox, in two doses 2 weeks apart, the animal being bled for serum 2 weeks after the last inoculation. The immunization process was repeated in the same animals after an interval of 3 weeks. No precipitin bands were produced by serum from the ox immunized with piro 2, and therefore two more cattle were immunized, this time with 300 mg. each of piro 2. One of these animals received piro 2 which had been treated in the ultrasonic disintegrator for 15 minutes, while the other received untreated material. Both materials were mixed with Freund's adjuvant as before.

(b) *Spleen antisera*. For preparation of spleen antiserum 200 g. each of spleen 2, 3 and 4 were mixed together and half of this pooled material was dialysed against distilled water at 4°C for 48 hours. The dialysed material was then treated by ultrasonic disintegration for 15 minutes at 4°C. Each of the two aliquots of spleen material were mixed with equal quantities of complete Freund's adjuvant and inoculated intramuscularly into two cattle as already described.

Agar gel precipitin test

The medium used was 1 per cent (w./v.) Special Agar Nobel (Difco) dissolved in veronal buffer (Meyer, Croft & Gray, 1948) of pH 7.3-7.4 and

* Measuring and Scientific Equipment Ltd, Spencer Street, London S.W.1.

containing 0.04 per cent merthiolate. The medium was poured into flat-bottomed Petri dishes, which had previously been treated with silicone*. Wells were cut in the agar by means of Feinberg agar-gel cutters†. The plates were placed at room temperature 21–22°C and lines of precipitation which formed were recorded by means of accurate drawings. The plates were examined daily for up to 14 days after preparation. The AGT results were preserved by the method earlier described by Gourlay (1964), the finished preparation being used as a negative in preparing the photographic print. Antigens were used at 50 mg./ml. strength in all cases, unless otherwise stated.

Skin test

The fraction in 0.1 ml. of normal saline was inoculated intradermally in the side of the neck of immune or susceptible cattle. Calipers were used to measure the increase in skin thickness 2, 24 and 48 hours after inoculation.

RESULTS

Piroplasm antigens

Control antigens were prepared from the same animal that was later used to produce the piroplasm antigens, as follows. A normal ox was bled and the parasite-free blood treated in the manner described for the preparation of piroplasm fractions. The same animal was then infected with *Theileria parva* and when sufficient piroplasms were present, about 200 per 1000 red cells, blood was again collected and piroplasm fractions prepared. The hyper-immune sera prepared against piroplasm fractions were absorbed with the fractions (control antigens) prepared from the parasite-free blood. The AGT was then set up using the absorbed sera and the fractions prepared from the piroplasm-infected and parasite-free blood.

Piro 1 antiserum gave three or four precipitin bands when diffused against piro 1 and only two lines against piro 2; these two lines gave a reaction of identity with two of the lines produced by piro 1 (Fig. 1). No bands were produced against the pre-infection fractions (control antigens). Piro 2 antisera produced no precipitin bands against piro 1 and 2. Antisera prepared against the spleen fractions produced five precipitin bands when diffused against piro 1 and no band against piro 2. Three of the bands produced by the spleen antisera gave reactions of identity with the bands that were produced by the piro 1 antiserum (Fig. 2). No precipitin bands were produced when piro 1 antiserum was diffused against spleen antisera.

When piro 1 was placed in a boiling water bath for 15 minutes and then compared with an unheated fraction in the AGT using piro 1 antiserum, it was evident that antigens responsible for at least two precipitin bands had been destroyed. Boiling had no effect on the precipitin bands produced by piro 2 and they were identical with the bands produced by piro 1 after heating.

* "Silicone Repelcote". Hopkins & Williams Ltd, Chadwell Heath, Essex, England.

† Shandon Scientific Company Ltd, 6 Cromwell Place, London S.W.7.

PLATE I

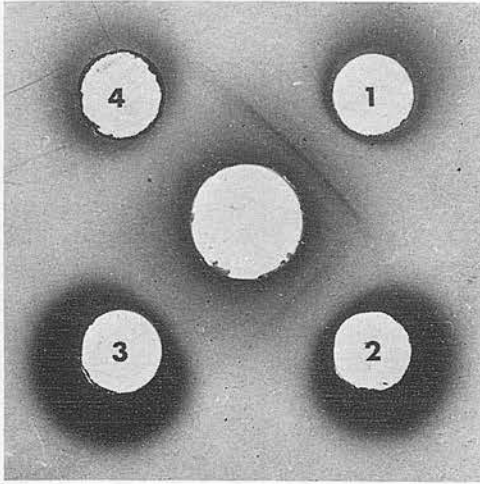


Fig. 1. Precipitin bands produced by piroplasm antigens. Centre well: piro 1 antiserum. Well 1: post-infection piro 1 antigen 50 mg./ml. Well 2: post-infection piro 2 antigen 50 mg./ml. Well 3: pre-infection piro 2 antigen 50 mg./ml. Well 4: pre-infection piro 1 antigen 50 mg./ml.

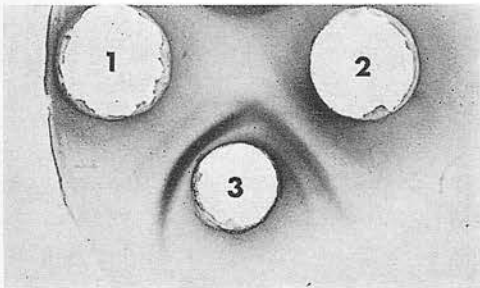


Fig. 2. Comparison between precipitin bands produced by piroplasm and spleen antisera. Well 1: piro 1 antiserum. Well 2: spleen antiserum. Well 3: post-infection piro 1 antigen 50 mg./ml.

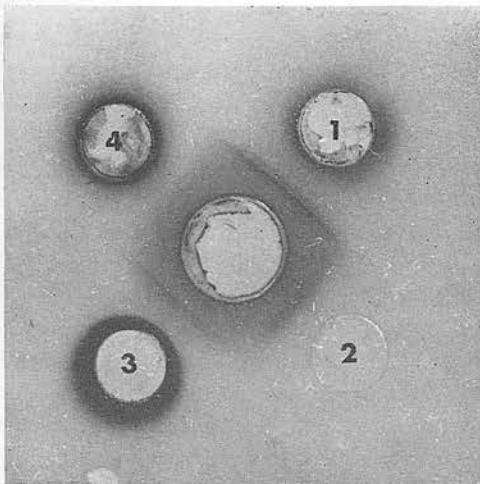


Fig. 3. Comparison between precipitin bands produced by piroplasm antigens and pneumogalactan. Centre well: piro 1 antiserum. Well 1: post-infection piro 1 antigen 50 mg./ml. Well 2: pneumogalactan 40 mg./ml. Well 3: post-infection piro 2. Well 4: *M. mycoides* antigen.

Spleen and lymph gland antigens

Antisera prepared against the spleen fractions gave two precipitin bands against spleen 1 and four bands against spleens 2, 3 and 4 but this specificity could not be confirmed since pre-infection spleen material was not available.

A precrucial lymph node was therefore surgically removed from a normal ox and stored at -25°C . The same animal was then infected with *T. parva* and shot when parasites were plentiful. The other precrucial lymph node was then collected. The two lymph nodes were then treated as described under Materials and Methods, and set up in the AGT with sera prepared against spleen and piro fractions. The spleen antisera produced a weak band against the infected lymph node antigen but not against the pre-infection lymph node fraction. Piro 1 antisera, that had been absorbed with control antigen, produced one precipitin band when diffused against spleen 1, spleen 2 and spleen 3 but no band against spleen 4. The bands produced against these different spleen antigens gave a reaction of identity with each other and with the bands produced by piro 2 antigen. Piro 1 antiserum also gave one weak band when diffused against infected lymph node antigen, but no band when diffused against pre-infection lymph node antigen.

Relationship between Theileria parva, Mycoplasma mycoides and pneumogalactan from normal bovine lung

Piro 1 antiserum absorbed with control antigens was tested in the AGT against piro 1, piro 2, pneumogalactan from normal lung (40 mg./ml.) and concentrated organisms of *Mycoplasma mycoides*. Two precipitin bands were produced with the pneumogalactan and these gave a reaction of identity with the bands produced by piros 1 and 2 (Fig. 3). Three very weak lines were produced by *Mycoplasma mycoides* and these lines joined up with those produced by piro 1. Piro 1 antiserum absorbed with control antigens, was tested in the AGT against constituents of the medium in which the *Mycoplasma* were grown, pig serum (20 per cent), tryptose (10 per cent) and yeast extract (10 per cent), but no precipitin bands were seen. *M. mycoides* antisera however, did not produce any bands against piro 1.

Skin tests

A total of nineteen ECF immune and three susceptible cattle were inoculated intradermally with piro 1, piro 2 and spleen supernatant 2, at 5–10 mg./0.1 ml. Piro 1 produced slight immediate and delayed reactions in about half of the immune cattle. This appeared to be related to the length of time after recovery from infection that they were tested, the longer the time the less the reaction. The maximum reaction was an increase of 6 mm. at 24 hours. Piro 2 did not produce any reaction, either immediate or delayed, while the spleen fraction was only used in two immune and one susceptible animal and produced moderate delayed reactions in the immune cattle but also gave a slight reaction in the susceptible animal.

ECF immune animals gave large immediate skin reactions when inoculated with *M. mycoides* carbohydrates (Shifrine & Gourlay, 1965) at 1 mg./0.1 ml.

The reactions occurred in all of ten cattle inoculated; the largest was an increase in skin thickness of 18 mm. and the smallest 4.5 mm. Four of the cattle which gave the largest skin reactions to *M. mycoides* antigen were also inoculated with pneumogalactan (PG) at 1 mg./0.1 ml. and they also reacted, showing increases in skin thickness of 9-10 mm. 2 hours after inoculation.

Seven cattle that had previously been infected with *M. mycoides* which still reacted to the skin test using *M. mycoides* carbohydrate and PG were subsequently infected with *T. parva*. When in the middle of the fever reaction to *T. parva* they were re-tested by skin test with *M. mycoides* carbohydrate and PG, and it was noticed that the skin reactions were greatly reduced or non-existent, depending on the stage of the fever reaction. For example ox No. A490 before infection with *T. parva* gave increases in skin thickness of 11.5 and 9 mm. with PG and *M. mycoides* respectively. On the ninth day of fever following infection with *T. parva* the reactions were only 4 mm. and 3 mm. respectively and on the twelfth day of fever they were reduced to 2 mm. and 1 mm.

DISCUSSION

By absorbing piro 1 antiserum with control antigens and including control antigens in the AGT it would appear that the precipitin bands produced by this serum and piro 1 were specifically associated with *T. parva* infection. In addition, presumably the three bands produced by spleen antisera diffused against piro 1 antigen, which showed reactions of identity with the bands produced by piro 1 antiserum, were also specific. All other precipitin bands must be presumed non-specific as there was no control spleen material available for absorption. Unfortunately antiserum was not prepared against lymph gland.

There appeared to be a difference between the antigenicity of piro 1 and piro 2 fractions. Piro 1 produced antibodies against itself and against piro 2, whereas piro 2 did not. The morphological difference between these two fractions was that piro 1 consisted mainly of large piroplasms in association with erythrocyte stroma whereas piro 2 consisted of individually small densely stained piroplasms. The piro 2 antigens were not destroyed by boiling, whereas piro 1 appears to possess at least two heat labile antigens in addition to the heat stable ones present in piro 2. Antiserum against piro 1 gave a single thick band against spleens 1, 2 and 3 and this band joined up with the two bands produced by piro 2.

Spleen antisera gave precipitin bands against piro 1 but not against piro 2. Three of the bands were identical to those produced by piro 1 antiserum against piro 1. Therefore it would appear that the antibodies common to the two sera were those to the heat-labile antigens. Spleen antisera gave a weak band against infected lymph node fractions, but not against normal uninfected lymph node material. We were, however, unable to determine whether this band was identical to the weak band produced by the piro 1 antiserum when diffused against the same lymph node, as the antigen was apparently too weak.

It appears probable that the heat stable (carbohydrate?) element in the

piroplasm fractions is responsible for the cross-reaction with pneumogalactan and also with the galactan from *M. mycoides*. We were, however, unable to demonstrate any precipitin band when antisera prepared against *M. mycoides* organisms was diffused against piroplasm fractions. Although only very weak bands were produced by piro 1 antiserum against *M. mycoides* organisms, the carbohydrates from *M. mycoides* and the pneumogalactan gave large immediate skin reactions in cattle previously infected with *T. parva* and, furthermore, during infection with *T. parva* the skin reactivity previously produced against *M. mycoides* was reduced. We do not, however, know whether this reduction was due to loss of ability to react from sickness or due to an antigen-antibody reaction.

The cross-reactivity between the carbohydrate of *T. parva* and pneumogalactan raises interesting questions as to the part this might possibly play in the pathogenesis of East Coast Fever, possibly in an auto-immune allergic reaction in the lung or, perhaps, the similarity of these antigens may produce a partial immune tolerance of the host to the parasite.

Interpretation of the various bands in the AGT is difficult but the work does indicate that the technique would be worth following. The experiments described are no more than preliminary ones and are reported now since the work has been concluded owing to our departure from Kenya. The technique employed gives a minimum number of antigens; more resolution could probably be obtained by immuno-electrophoretic methods.

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Expériences préliminaires avec des antigènes de *Theileria parva* (Gourlay et Brocklesby)

Résumé. La technique de précipitation en milieu gélifié a été employée dans l'étude des antigènes de *Theileria parva*. Des fractions ont été préparées à partir du sang et des ganglions lymphatiques du même bovin avant et après infection. Les antisérums ont été produits chez des bovins par inoculation de fractions préparées après infection à partir de sang et de rate dans l'adjuvant de Freund. Au moins quatre lignes spécifiques de précipitation ont été observées; les antigènes associés à au moins deux des lignes étaient thermo-labiles et les autres thermo-résistants. Une communauté a été observée entre plusieurs antigènes sanguins et spléniques. La technique de précipitation en milieu gélifié et l'épreuve intradermique suggèrent que *T. parva* ait des antigènes en commun avec la galactane du poumon bovin normal et avec *Mycoplasma mycoides*, l'agent causal de la péripneumonie infectieuse bovine.

Einleitende Experimente mit *Theileria parva* Antigene (Theiler, 1904)
(Gourlay und Brocklesby)

Zusammenfassung. Die Antigene von *Theileria parva* wurden mittels Agargelpräzipitation untersucht. Fraktionen wurden aus verschiedenem, vor und nach Infektion gesammeltem, Blut- und Lymphknotenmaterial eines Rindes hergestellt. Antisera wurden im Rind durch Einimpfung von nach der Infektion gesammelten Blut- und Milzfraktionen im Freundschens Adjuvant hergestellt. Mindestens vier spezifische Präzipitinbände wurden beobachtet. Die mit mindestens zwei der Bände verbundenen Antigene waren hitzeunbeständig, während die anderen hitzebeständig waren. Es gab mehrere gemeinsame Antigene im Blut und in der Milz.

Agargelpräzipitation und intrakutane Hautprobe wiesen antigenische Gemeinschaft mit *T. parva*, mit Galaktan der normalen Rinderlunge und mit dem Erreger von Lungenseuche (*Mycoplasma mycoides*) auf.

Experiencias preliminares con antigenes de *Theileria parva*
(Gourlay y Brocklesby)

Resumen. Se ha empleado la prueba de difusión en gel por estudiar los antigenes. Se ha preparado fracciones a partir de sangre y de linfonodos de un mismo bovino antes y después de la infección. Se han producido antisueros en bovinos por inoculación de fracciones obtenidas post infección de sangre y bazo en adjuvante de Freund. Al menos cuatro líneas específicas de precipitación fueron observadas; los antigenes asociados con al menos dos líneas eran termolabiles y las restantes termoresistentes. Se observó una comunidad entre un numero de antigenes de la sangre y del bazo. La prueba de difusión en gel y la prueba intradérmica han mostrado que *Theileria parva* posee antigenes en comun con la galactona de pulmón bovino normal y con *Mycoplasma mycoides*, el agente causal de la perineumonía contagiosa de los bovinos.

PASSIVE TRANSFER OF IMMUNITY AND FORMATION OF LUNG LESIONS IN CATTLE FOLLOWING INTRAVENOUS INOCULATION OF ANTIBODY AND *MYCOPLASMA MYCOIDES*

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INTRODUCTION

Evidence of the passive transfer of immunity in contagious bovine pleuropneumonia (C.B.P.P.) is very scanty and there appears to be no information on this since the experiments carried out at the end of the last century. Nocard, Roux and Dujardin-Beaumetz (1899) (quoted by Nocard and Leclainche, 1903) reported that the serum of a cow that had been inoculated with increasing doses of *Mycoplasma mycoides* var. *mycoides* culture, giving a total of 5 litres, acquired immunising properties. A dose of 40 ml. inoculated into an ox produced an immunity to subcutaneous challenge lasting up to 10 days, and a dose of 100-200 ml., repeated at intervals of 24 hours, if necessary, cured or inhibited the further development of an artificially produced inoculation swelling.

Earlier we had reported that antibodies against *M. mycoides* cross-reacted with pneumogalactan isolated from normal bovine lung (Shifrine and Gourlay, 1965; Gourlay and Shifrine, 1966) and suggested that this phenomenon might play a part in the pathogenesis of C.B.P.P.

The following paper reports studies on the above phenomena by the intravenous injection of *M. mycoides* antiserum into cattle, followed by the intravenous injection of *M. mycoides* culture. Turner (1959) states: "All authors agree that the pulmonary disease never follows simple subcutaneous, intradermal, intramuscular, intracerebral or intravenous inoculation. The intravenous route is without any effect if care is taken to avoid local infection."

MATERIALS AND METHODS

Strain of *M. mycoides*. The Gladysdale strain (Turner, 1961) was used and was grown in modified Newing's tryptose broth (Gourlay, 1964). Organisms in the thread phase were used and cultures were shaken well before inoculation. The B.C.I.D.₅₀ (broth culture infective dose 50) titre of the cultures were between $10^{9.5}$ and $10^{10.5}$ organisms/ml.

Cattle

Grade cattle (cross between Zebu and European type cattle) were used throughout.

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Ox Serum

M. mycoides immune antiserum was pooled from seven cattle that had been inoculated subcutaneously with 2 ml. of *M. mycoides* three-day old culture (Gladysdale strain), developed extensive Willems reactions and later recovered. They were bled for serum between three and four months after inoculation. *M. mycoides* hyperimmune antiserum was prepared in cattle, as described earlier (Gourlay, 1963). Cattle that had 12 months previously been inoculated with T2 avianised C.B.P.P. vaccine (Piercy and Knight, 1956) then challenged subcutaneously with the T3 strain of *M. mycoides* (Piercy and Knight, 1957) from which they survived after developing large Willems reactions, were inoculated intravenously with T3 antigen (Gourlay, 1964) three times each week for three weeks and bled for serum four days after the last injection.

All sera were sterilised by filtration.

Cattle Inoculation

Serum was inoculated intravenously into the jugular vein on one side of the body and *M. mycoides* culture was inoculated intravenously into the jugular vein on the opposite side of the body 24 hours later. Great care was taken to prevent leakage of any culture into the tissues outside the vein by inoculation through a length of polythene tubing which was passed through a needle penetrating into the vein, followed by washing the tubing with normal saline before withdrawal. Penicillin was given for three days following the serum inoculations.

RESULTS

In the first experiment two cattle were inoculated with 250 ml. of normal ox serum and two cattle with 250 ml. of a 3 : 2 mixture of immune and hyperimmune sera. Twenty-four hours later two of the cattle, one from each group, were inoculated with 20 ml. of viable *M. mycoides* culture and the other two cattle with 20 ml. of *M. mycoides* culture which had been inactivated at 56° C. for 60 minutes. The two cattle that received the inactivated culture showed no signs of ill health and when shot four weeks later showed no signs of pleuritis or visible or palpable lung lesions. The ox that received normal serum followed by viable culture developed an extensive Willems reaction over the inoculation site and had to be destroyed *in extremis* 11 days after the inoculation. On autopsy there were no signs of pleuritis or lung lesions. The remaining ox which received hyperimmune/immune serum followed by viable culture became ill, developed severe dyspnoea and eventually collapsed and had to be destroyed four weeks after the *M. mycoides* injection. On autopsy pleuritis with adhesions to the cardiac and diaphragmatic lobes of the right lung were seen and the cardiac lobe had a lesion of C.B.P.P. $2 \times 2\frac{1}{2} \times 2\frac{1}{2}$ in. in size and the diaphragmatic lobe had a lesion of C.B.P.P. $6 \times 4 \times 4$ in. in size. The associated lymph glands were enlarged. *M. mycoides* was isolated in pure culture from both lung lesions. Two control cattle that received only 20 ml. of viable culture developed extensive Willems reactions over the inoculation site and had to be destroyed *in extremis* 14 and 17 days later. Neither showed any signs of pleuritis or lung lesions on autopsy.

One further ox was given 250 ml. of hyperimmune/immune serum and 24 hours later was given 300 ml. of viable culture. A small reaction 4×6 in. developed over the injection site, with fever, and the ox collapsed after eight days and was destroyed

in *extremis* nine days after the *M. mycoides* injection. On autopsy the only abnormalities seen were slightly enlarged and haemorrhagic mediastinal lymph glands.

In the second experiment immune serum alone was used instead of the hyperimmune/immune mixture. Two cattle were inoculated with 250 ml. and one with 100 ml. of immune serum and one each with 250 ml. and 100 ml. of normal serum. They were then inoculated 24 hours later, together with three control cattle, which received no serum, with 20 ml. of viable *M. mycoides* culture. All the cattle, which did not receive immune serum, developed extensive Willems reactions at the site of injection and either died or were destroyed in *extremis* 17–28 days after the last injection. On autopsy only one of these cattle had any abnormality in the thoracic cavity and this was a control ox that had received only viable *M. mycoides* and was destroyed in *extremis* 20 days after the last injection with an extensive Willems reaction. It had a lesion of C.B.P.P. in the diaphragmatic lobe of the left lung $2 \times 2 \times 1$ in. in size and from which *M. mycoides* was isolated in pure culture. Of the three cattle that had received immune serum, the two that had had 250 ml. showed no signs of illness and when destroyed 38 days after the last injection showed no abnormalities in the thoracic cavity. The remaining ox which received 100 ml. of immune serum was destroyed in *extremis* 13 days after injection of *M. mycoides*, following an acute illness. On autopsy multiple acute areas of pneumonitis were seen throughout the lungs, three of which were cultured and from which *M. mycoides* was isolated.

In the third experiment two cattle were inoculated three times at four-day intervals with a total of 400 ml. of immune serum. Five days later 20 ml. of viable *M. mycoides* culture was inoculated. They did not develop Willems reactions and showed no signs of illness and, when destroyed 42 days after the *M. mycoides* injection, no signs of pleuritis or lung lesions were seen on autopsy.

DISCUSSION

A total of 17 cattle were inoculated, of which 15 received viable *M. mycoides*. Of these 15, two received hyperimmune/immune serum, five received immune serum, three received normal serum and five were control cattle that received no serum prior to injection with viable *M. mycoides* culture.

All eight cattle that received either normal serum or no serum developed extensive Willems reactions at the inoculation site following injection of *M. mycoides* and died or were destroyed in *extremis*, whereas six out of seven cattle that received hyperimmune or immune serum did not develop Willems reactions. The seventh ox, which received the very large inoculation (200 ml.) of *M. mycoides* developed only a small Willems reaction. It would appear from these results that the inoculation of hyperimmune or immune serum had transferred passive immunity to the subsequent inoculation of *M. mycoides* and would therefore confirm the early report of Nocard *et al.* (1899), (quoted by Nocard and Leclainche, 1903). Despite the care taken to prevent subcutaneous leakage of viable *M. mycoides* during injection into the jugular vein, Willems reactions formed. It appears probable, therefore, that lesions did not develop from leakage of organisms into the subcutaneous tissues but leakage through the puncture wound after the needle had been withdrawn.

Of the three cases in which lung lesions developed two had previously been given hyperimmune or immune serum and of these the one that had received hyperimmune serum developed the largest lesions. Neither of these two developed Willems reactions. The third animal was one that received only viable *M. mycoides*

without prior serum injection and also developed an extensive Willems reaction. The lung lesion was small but definite.

The ox that had received the large inoculation of *M. mycoides* following hyper-immune/immune serum was destroyed *in extremis*, without developing an extensive Willems reactions. Enlarged and haemorrhagic mediastinal lymph glands were the only abnormality seen on autopsy. The reason for this death is unknown.

From these results it is obvious that lung lesions can form following intravenous inoculation of the Gladysdale strain of *M. mycoides*, but the part played by the preinoculation of antiserum is not clear, as a lung lesion also developed in one animal that had not received the antiserum. It is possibly significant that the inoculation of antiserum in two cases inhibited the formation of Willems reactions but did not, however, prevent the formation of a lung lesion, indicating that possibly the antiserum had "sensitised" the lungs to subsequent infection with *M. mycoides*.

SUMMARY

Intravenous inoculation of hyperimmune or immune ox serum into cattle 24 hours prior to intravenous inoculation of the virulent Gladysdale strain of *M. mycoides*, transferred passive immunity as judged by the absence of Willems reactions over the inoculation site.

Lung lesions of C.B.P.P. developed in two cattle that had received hyper-immune or immune serum prior to the intravenous inoculation of *M. mycoides*, but also a small lesion developed in one ox that had not received prior inoculation of serum.

ACKNOWLEDGMENT

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Résumé

L'inoculation intraveineuse du sérum bovin immun ou hyperimmun dans les bovins 24 heures avant l'inoculation intraveineuse de la souche virulente "Gladysdale" de *M. mycoides* a transféré une immunité passive, à en juger par l'absence d'une réaction de Willems sur le point d'inoculation.

Des lésions pulmonaires se sont développées chez deux bovins qui avaient reçu du sérum immun ou hyperimmun avant l'inoculation intraveineuse de *M. mycoides*, mais une petite lésion s'est aussi développée chez un bœuf qui n'avait reçu aucune inoculation préalable du sérum.

Morphology of *Mycoplasma mycoides* Thread-phase Growth

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SUMMARY

Examination of threads of *Mycoplasma mycoides* var. *mycoides* by optical and electron microscopy revealed organisms within a homogeneous mucinous matrix. This matrix stained with the periodic acid-Schiff (PAS) reagent method was almost electron-transparent and was probably lipo-polysaccharide (galactan) material. Treatment of threads with specific antibody produced a clearly visible microscopic precipitate strongly outlining the threads. By electron microscopy this precipitate was electron-dense, homogeneous, and finely granular; it could frequently be seen surrounding individual mycoplasma cells as a well-defined capsule.

INTRODUCTION

Tang, Wei, McWhirter & Edgar (1935) described the formation of 'islands' and 'threads' visible to the naked eye during the growth of *Mycoplasma mycoides* var. *mycoides* in liquid medium. Later, Turner (1959) called them 'masses of intricately branched mycelial filaments' and described them as 'whitish, translucent, elongated objects which may hang from the surface of the medium or from the walls of the tube, or may originate from a mucinous deposit at the bottom. On removal from the incubator, convection currents cause them to circulate slowly through the crystal-clear medium as easily deformable, elongated objects recalling wisps of smoke. As incubation proceeds, these "wispy growths" become dispersed and growth becomes diffuse throughout the medium.' The morphology of the thread has been largely ignored, although the mycelial filaments which form the threads have been studied intensively. This present paper describes studies on threads of *M. mycoides* before and after the addition of specific antibody.

METHODS

Mycoplasma mycoides. The T2 vaccine strain (Piercy & Knight, 1956) was used. Threads were grown in modified Newing's tryptose broth (Gourlay, 1964) which contained 0.5% glucose.

Microscopical examination. Threads were removed from the broth with a Pasteur pipette and were either placed intact or gently spread on a glass slide. The preparations were air-dried and fixed in Schaudin's fluid and then stained overnight with 10% Giemsa or by the periodic acid-Schiff (PAS) reagent method, followed by Giemsa. Slides were examined by light microscopy. Wet preparations under a coverslip were examined by dark-ground and phase-contrast microscopy.

Electron-microscopical examination. Threads were placed in 3% (v/v) glutaraldehyde for about 18 hr, washed in 0.1 M-phosphate buffer (pH 7.3) for about 7 hr, and finally put into 1% (w/v) osmium tetroxide in 0.1 M-phosphate buffer (pH 7.3) for about

18 hr. After fixation the threads were washed briefly in distilled water and embedded in 2% agar at 45°. Pellets of agar containing the threads were then dehydrated and embedded in Araldite. Sections were cut with a Reichert OMU2 ultra-microtome, stained with uranyl acetate and examined with a Philips EM 75 electron microscope.

The effect of specific antibody and complement. Immediately after removal from the liquid medium threads were placed into undiluted anti-*M. mycoides* hyperimmune sheep serum (Gourlay, 1964) previously heated at 56° for 30 min. or into 2.2% globulin in phosphate-buffered saline (pH 7.2) precipitated by ammonium sulphate from the sheep serum. Complement, as 20% fresh guinea-pig serum, was occasionally added to the serum before the addition of the threads. Threads were left in the serum at room temperature for 90 min. and then removed and fixed as described above. Occasionally, incubation of the thread and antiserum was done on a glass slide under a coverslip and the reaction observed by dark-ground and phase-contrast microscopy. Controls were set up with normal sheep serum and inactivated guinea-pig serum.

RESULTS

Growth characteristics of threads in broth

When about 10^9 organisms were inoculated into broth in serial tenfold dilutions, growth usually occurred in the low dilutions within 1 to 2 days, as a diffuse turbidity, and in the higher dilutions by day 4 or 5. The characteristic growth in these higher dilutions was the appearance of one or more white, translucent, pear-shaped 'islands' from which threads developed. The threads lengthened and thickened, sometimes attaining a length of about 3 cm. and a thickness of about 1 mm. The islands and threads could be easily seen suspended in the clear medium (Pl. 1, fig. 1). After further incubation the threads slowly dispersed and the medium became opaque with a mucinous deposit at the bottom of the tube.

Examination of threads

Optical microscopy. The slightly mucinous consistency of the thread could be seen when it was lightly spread on a glass slide. Under the low power of the microscope smears stained with PAS-Giemsa showed a bright red-streaked mucinous mass in which the more deeply stained purple mycelial filaments and groups of organisms could be discerned (Pl. 1, fig. 2). Under high power of the microscope, Giemsa-stained smears showed a mass of deeply staining mycoplasma cells apparently held in a faintly staining homogeneous matrix (Pl. 1, fig. 3).

Electron microscopy. Sections of threads contained mycoplasma cells as finely granular bodies without any marked clear inner zones (Pl. 2, fig. 6). They were relatively closely packed in the centre of the thread, but became progressively further apart towards the periphery.

Examination of threads after immersion in antiserum

Optical microscopy. After immersion in antiserum and with dark-ground illumination the rather diffuse hazy outline and milky white colour of the thread slowly began to sharpen and within a few minutes became refractile and brighter, reminiscent of a crystallization process (Pl. 1, fig. 4). Normal serum did not have this effect (Pl. 1, fig. 5). The addition of complement to the specific antiserum did not appear to affect the 'crystallization process'.

Electron microscopy. After exposure to antiserum an electron-dense homogeneous precipitate was clearly seen at the edge of the thread, where the mycoplasma cells were arranged close together within the precipitate (Pl. 2, fig. 7). Some precipitate free from mycoplasma cells could also be seen outside the thread margin. In addition to the electron-dense precipitate, a fine granulation was also seen, both outside and within the thread (Pl. 2, fig. 7). Mycoplasma cells which had become separated from the thread were observed within the electron-dense precipitate beyond the edge of the thread. This precipitate took the form of an electron-dense homogeneous capsule round each organism but frequently embraced more than one organism when they were in close proximity (Pl. 3, fig. 9).

Examination of threads that had been immersed in normal sheep serum showed no precipitate and no evidence of a capsule round any mycoplasma cell. Fine granular material was, however, evident within and without the thread substance. Towards the centre of the thread a clear halo, outlined by this granular material, was frequently seen around individual mycoplasma cells (Pl. 3, fig. 8). This halo was sometimes observed also around mycoplasma cells in the centre of a thread that had been submerged in specific antiserum. The addition of complement to either the normal serum or specific antiserum appeared to have no effect on the mycoplasma cells nor on the precipitate.

Threads that had been immersed in globulin had an electron-dense precipitate at the margin, but there was very little granulation within or outside the thread.

DISCUSSION

Mycoplasma mycoides threads appear to be composed of a mass of filamentous-phase mycoplasma cells within a slightly mucinous homogeneous matrix. The individual cells are granular without any marked clear zones inside them, unlike the cells observed at a later stage of growth when the threads have dispersed. The matrix is almost electron-transparent, only becoming electron-dense when treated with specific antiserum. It stains with PAS, and it has also been shown that the addition of glucose to the medium causes thicker threads to be formed (Gourlay & MacLeod, 1966) indicating that the threads may be formed by products of glucose metabolism. A specific carbohydrate has been isolated from *M. mycoides* (Kurotchkin, 1937; Dafaalla, 1957), and Buttery & Plackett (1960) showed that it was a galactan and amounted to 10% of the dry weight of the organism. A similar galactan-containing lipo-polysaccharide was also shown to be present in blood and exudates of cattle infected with *M. mycoides* (Gourlay, 1964). Following evidence that the addition of the lipo-polysaccharide to viable organisms had an aggressive and virulence-enhancing effect *in vivo*, it was suggested that this material might be present in the form of a capsule round the organisms (Gourlay, 1963, 1965*b*). Plackett, Buttery & Cottew (1963) made the same suggestion following their studies on carbohydrates of some Mycoplasma strains, although they produced no evidence to support this view. They did, however, state that no clear evidence of any capsule had been seen in their electron micrographs. Domermuth, Nielsen, Freundt & Birch-Andersen (1964), on the other hand, suggested that the small amount of amorphous or floccular extracellular substance that they observed during their ultrastructural studies with different Mycoplasma strains (including *M. mycoides* var. *mycoides*) might be a capsule or slime layer.

From the evidence presented here, it would seem that the matrix is probably the galactan-containing lipo-polysaccharide material. There does, however, appear to be some difference between the matrix near the mycoplasma cells and that further away, as is shown by the halo and capsule formation. This difference may be qualitative or only quantitative. The stage of growth of the organisms may be important, since the capsules observed here were associated with actively-growing organisms in the filamentous phase. The addition of specific antiserum produces a precipitation of the capsule, perhaps analogous to the Quellung or specific capsular reaction of the pneumococcus.

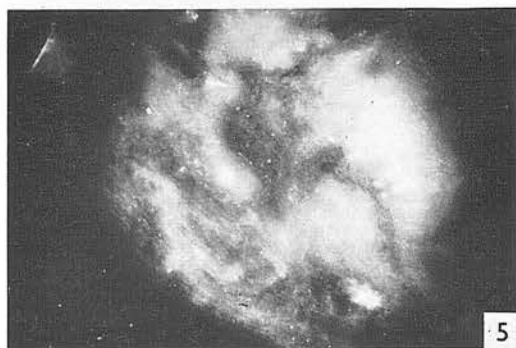
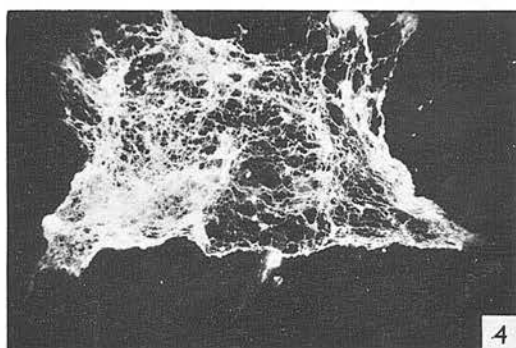
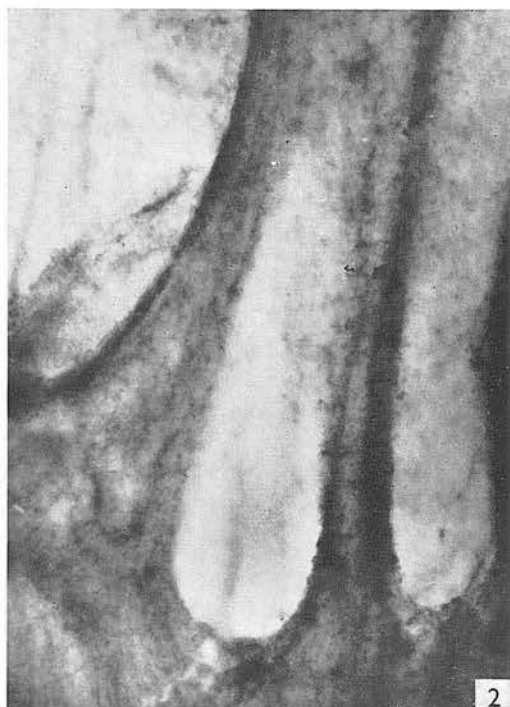
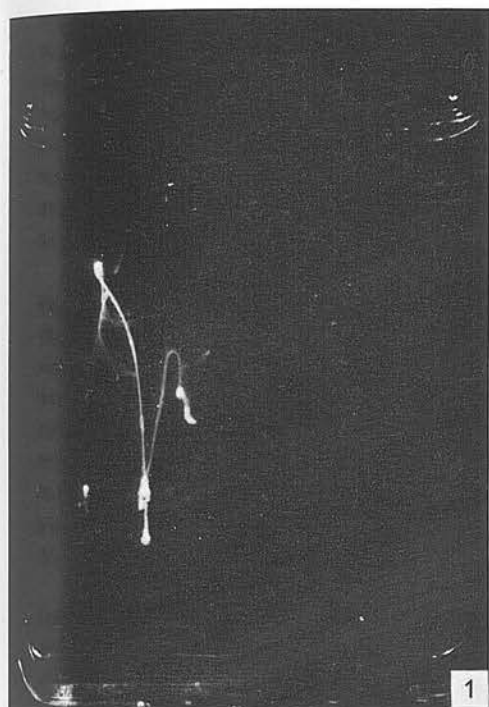
It is probable that all strains of *Mycoplasma mycoides* do not produce an equally large capsule, since it was shown (Gourlay & MacLeod, 1966) that, whereas the highly virulent Gladysdale and the T2 vaccine strains of *M. mycoides* produced threads, the avirulent KH3J and the T1 vaccine strains did not, even in liquid medium with added glucose. It has been observed that the Gladysdale strain produces about 5 times the volume and about 13 times the dry weight of centrifuge deposit of the KH3J strain for a similar concentration of particles (Gourlay, unpublished). Both virulent and avirulent strains, however, have the same serologically common galactan-containing lipo-polysaccharides (Gourlay, 1965*a*), so perhaps the difference is only quantitative.

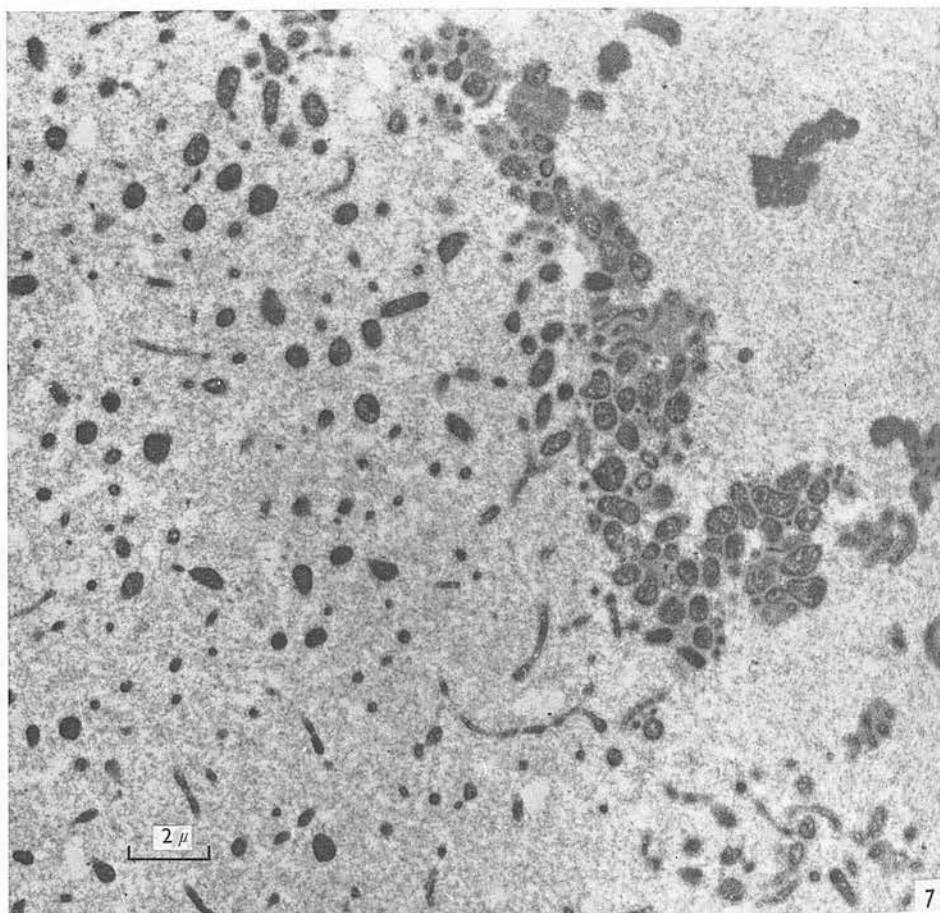
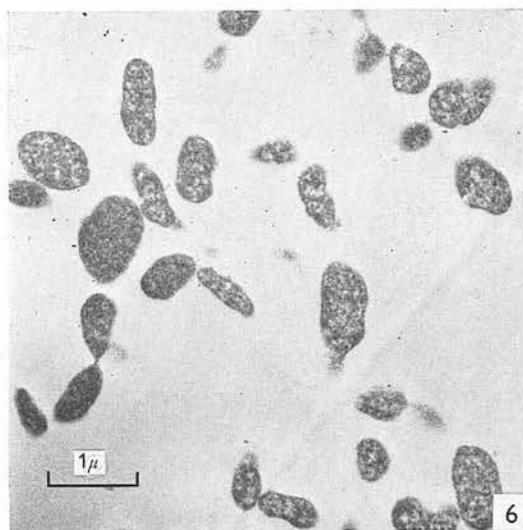
The granulation observed in electron micrographs of threads that had been immersed in either normal or specific antiserum was almost absent from threads that had been immersed in globulin, indicating that the granulation was probably due to the precipitation, by the fixation fluids, of serum components other than specific antibody which had penetrated the thread.

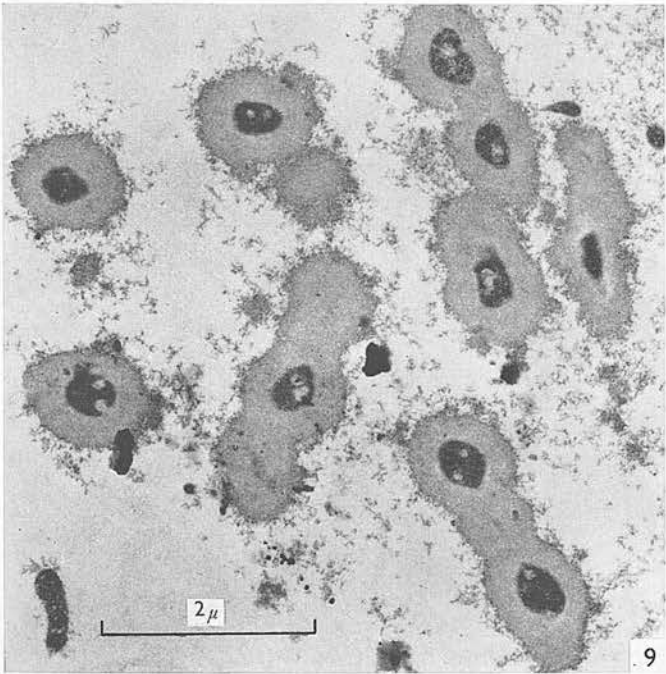
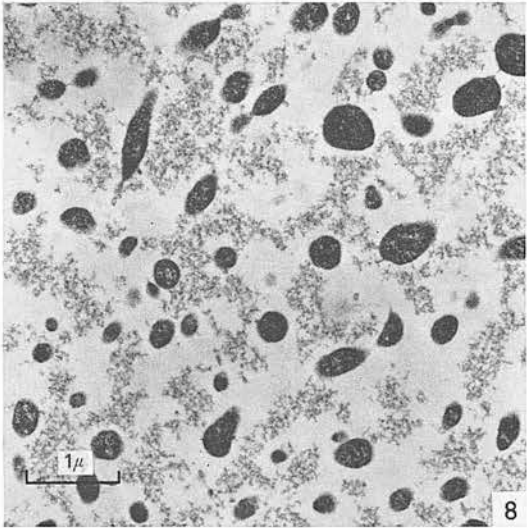
We thank members of the Electron Microscopy Section for advice and assistance, Mr I. Jebbett for some of the photography, and members of the Histology Section and Miss Sara Wyld for technical assistance.

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EXPLANATION OF PLATES

Mycoplasma mycoides var. *mycoides*

PLATE I

- Fig. 1. Islands and threads in broth culture. Direct illumination against dark background. $\times 2$.
- Fig. 2. Thread spread on a glass slide showing mycelial filaments within a mucinous streaked mass of matrix. PAS-Giemsa, $\times 116$.
- Fig. 3. Thread spread on a glass slide showing mycoplasma cells within a faint matrix. Giemsa, $\times 660$.
- Fig. 4. Wet preparation of a piece of thread immersed in antiserum. Dark ground illumination, $\times 23$.
- Fig. 5. Wet preparation of a piece of thread immersed in normal serum. Dark ground illumination, $\times 23$.

PLATE 2

- Fig. 6. Electron micrograph. Section through the edge of a thread following immersion in antiserum, showing dense precipitate at the margin and granular material within and outside the thread. $\times 5400$.
- Fig. 7. Electron micrograph. Section through the centre of an untreated thread, showing mycoplasma cells without marked clear inner zones within an electron-transparent matrix. $\times 15,000$

PLATE 3

- Fig. 8. Electron micrograph. Section through the centre of thread following immersion in normal serum, showing halo formation outlined by granular material. $\times 11,200$.
- Fig. 9. Electron micrograph. Section through free mycoplasma cells at the edge of a thread, following immersion in antiserum, showing dense homogeneous capsules round mycoplasma cells. $\times 14,700$.

THE PRODUCTION OF T₁ BROTH CULTURE CONTAGIOUS BOVINE PLEUROPNEUMONIA VACCINE

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Since Walker (1921) found that attenuation of virulent *Mycoplasma mycoides* occurred by repeated subculture in broth, such attenuated strains have been used in both Africa and Australia as vaccines for the control of contagious bovine pleuropneumonia (C.B.P.P.).

A serious disadvantage of broth culture vaccine is that the shelf-life is limited to 1-2 months and transportation on ice to remote areas may be difficult. Difficulties over transportation and keeping qualities were resolved when Sheriff and Piercy (1952) attenuated *M. mycoides* by repeated sub-culture in embryonated hens' eggs, and the infected embryos harvested and freeze-dried. However, avianised C.B.P.P. vaccine has been found to set up active C.B.P.P. in cattle inoculated with egg embryo vaccines prepared with the T₁ (Piercy and Knight, 1958), T₂ (Sanders, 1961), T₃ (Roe, 1958), and V₅ (Hudson, 1964) strains of *M. mycoides*. This ability of avianised C.B.P.P. vaccine to set up active C.B.P.P. has been shown to be associated with the presence of egg fluids in the vaccine (Hudson, 1964). Because of this danger, in East Africa, C.B.P.P. vaccines are now once more prepared in broth culture form using the avianised vaccine strains. The method of production of T₁ vaccine, which is widely used, is described herein.

MATERIALS

Equipment

Items required in addition to normal laboratory apparatus include a Horm * filter, a Brewer † automatic pipetting machine and a 20 litre Pyrex ‡ reagent bottle.

T₁ Strain of *M. mycoides*

Freeze-dried ground up fowl embryos infected with the T₁ strain (Piercy and Knight, 1956) of *M. mycoides* in the forty-fifth egg passage was reconstituted with distilled water, and 10-fold serial dilutions were prepared in broth and incubated at 37° C. for five days when the highest dilution showing growth was sub-cultured and incubated a further three days. The culture was then freeze-dried in 0.5 ml. amounts and stored at -25° C. until required. These ampoules constitute the seed-bank.

Broth for the Growth of *M. mycoides* (Gourlay, 1964)

	Per cent
Bacto-Tryptose (Difco)	2.00
Dextrose	0.50
Sodium chloride	0.50
Disodium phosphate anhydrous	0.25
Glycerol	0.50
Difco yeast extract	0.10
Water to	100.00

* F. R. Hormann and Co., Inc., Milldale, Connecticut, U.S.A.

† Baltimore Biological Laboratory Inc., Baltimore, U.S.A.

‡ James A. Jobling, Sunderland, England.

The above ingredients are dissolved in water by steaming and 10 volumes of pig serum (inactivated at 56° C. for 30 minutes) added to 100 volumes of the solution together with 100 I.U. penicillin per ml. of the final volume of broth. The broth is then filtered through a Seitz sterilising filter pad under negative pressure in 2 litre amounts into 4 litre flasks. The pH of the final product is adjusted to 7.6 and flasks of broth are incubated for 48 hours at 37° C. to check for contaminants and then stored at 4° C. When required for vaccine production flasks of broth are placed in a warm room at 37° C. for 18–24 hours before use; this acts as a further check on sterility, increases the rate of flow through the filter and the temperature of the broth is near to 37° C. when inoculated with the *M. mycoides* culture.

Boxes for Transportation of Vaccine

Details of boxes used for vaccine are given in Fig. 1. Each box consists of a plywood outer case with a metal inner container, the intervening space being filled with Styropor,* a synthetic insulation material. The metal inner container has a space in the middle for an ice tank which is partially filled with water and frozen at – 25° C. before fitting into the box when vaccine is despatched. On either side of the ice tank are spaces for seven bottles of vaccine. When vaccine is packed, each bottle is surrounded by corrugated paper. The lid of the box is also lined with Styropor. When vaccine is despatched the lid is sealed with lead seals.

METHODS

The production and testing of a batch of C.B.P.P. vaccine takes 16 days (Table 1).

Table I.—Time-table for the Production and Testing of Contagious Bovine Pleuropneumonia Culture Vaccine

Day	Procedure
1	Open ampoule of freeze-dried attenuated <i>M. mycoides</i> culture (seedbank), reconstitute and prepare 10-fold dilutions. Incubate three days at 37° C.
4	Sub-inoculate above culture into 6 × 100 ml. bottles of broth. Incubate for two days.
6	Filter broth, seed with culture and dispense in 200 ml. amounts. Incubate for three days.
9	Cap down vaccine and seal. Set up test for contaminants and titrate.
9–14	Examine mice daily.
12	Examine culture for contaminants.
14	Despatch vaccine.
16	Read titration.

Growth of Seed

An ampoule of the seed-bank (freeze-dried second broth passage of Tr—forty-fifth egg passage *M. mycoides*) is opened and reconstituted by the addition of

* Badischen Anilin and Soda Fabrik AG, Ludwigshafen am Rhein, Germany.

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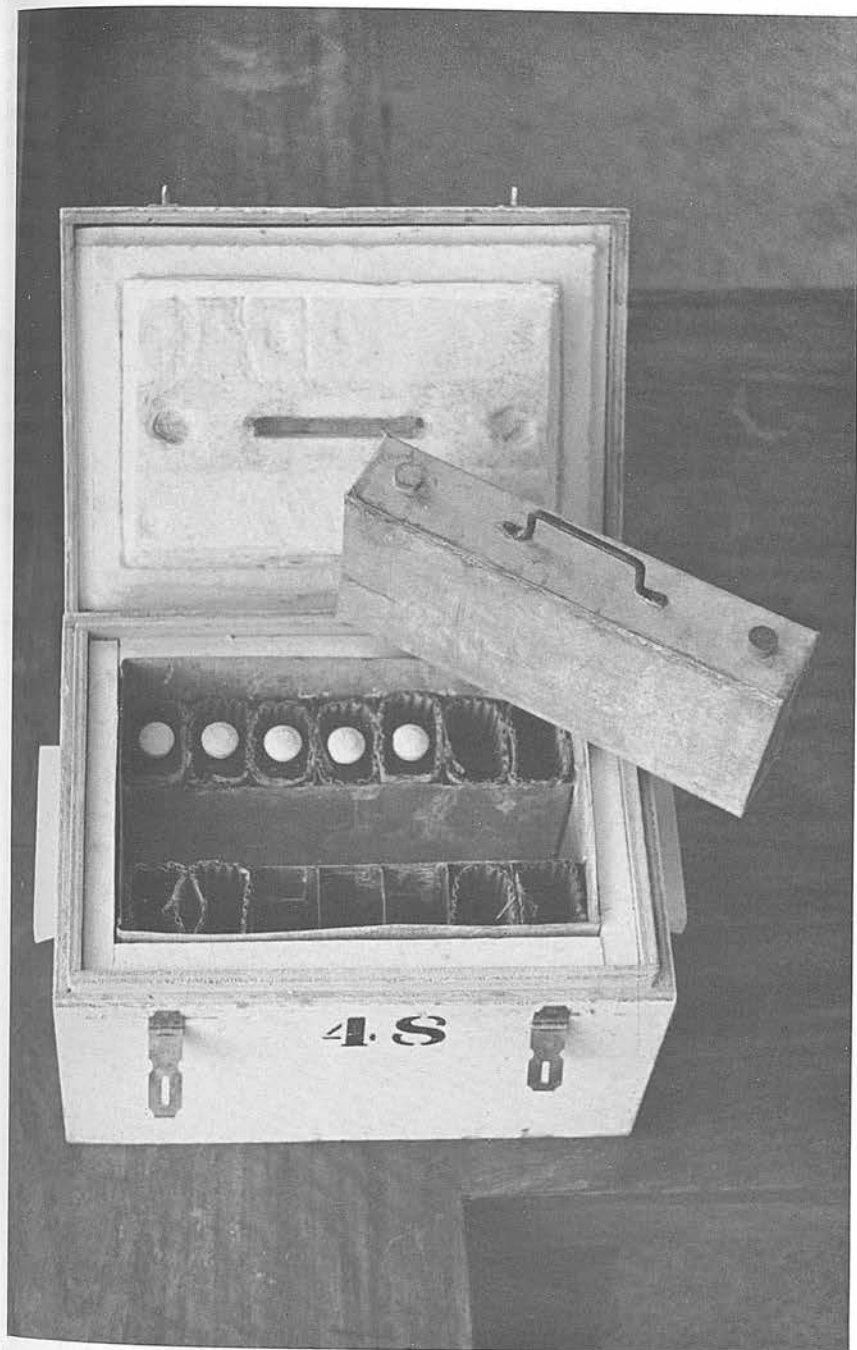


FIG. 1.—Insulated box for transportation of vaccine.

0.5 ml. of sterile distilled water and added to 9 ml. of broth and three series of 10-fold dilutions made up to 10^{-8} . These are incubated for three days at 37°C . (the temperature at which all incubations are carried out). From one of these series the highest dilution showing growth of *M. mycoides* is used for the sub-inoculation of 1 ml. of culture into each of six 4-oz. screw cap bottles each containing 100 ml. of broth. After incubation for two days these are used for seeding the batch of vaccine.

Seeding, Dispensing and Incubation of Vaccine

Broth, previously incubated at 37°C . for 18–24 hours is filtered under a positive pressure of 15 lb./sq. in. through two thicknesses of sterilising pads in a Horm filter into a 20 litre Pyrex reagent bottle (Fig. 2). Crystalline penicillin is added during filtration to produce a concentration of 200 I.U./ml. of broth. After filtration seed culture is added aseptically directly to the filtered broth through an inlet tube, using a 50 ml. hypodermic syringe, at a ratio of 10 ml. of seed culture per litre of broth. The reagent bottle is then shaken thoroughly to mix the broth and culture and then connected up aseptically to the automatic pipetting machine (Fig. 3). The broth is then dispensed in 200 ml. amounts into 8-oz. sterile medical flat bottles which are stoppered with non-absorbent cotton wool plugs and incubated for 72 hours. The bottles are examined daily for growth of *M. mycoides* and for the presence of contaminants.

Sealing Down Vaccine

After incubation the cotton wool plugs are removed from the bottles and replaced aseptically by "Subaseal" * (size 29) rubber caps and then sealed with "Viskap" † plastic covers (size 3A cut 1 inch). The bottles are allowed to stand in a warm room (temperature about 30°C .) until the "Viskaps" have dried and contracted to form a tight seal. The bottles are then labelled and stored at 4°C . The expiry date of the vaccine is 30 days after sealing down. The dose of the vaccine is 0.5 ml. inoculated into the tail tip.

Checks for Contaminants

On the day the vaccine is sealed down three mice are each inoculated intraperitoneally with 0.25 ml. of vaccine, and are examined daily for five days for signs of sickness or death. Bacteriological examination for contaminants includes Gram staining and the inoculation of two bottles of nutrient broth, two bottles of thio-glycolate medium and one blood agar plate. In addition the vaccine is examined microscopically under dark ground illumination.

Potency Estimations

On the day the vaccine is sealed down it is titrated by making three 10-fold series of dilutions in broth up to 10^{-11} , which are incubated for one week before being read, and the end points calculated by the method of Reed and Muench (1938). Although the vaccine is sent out after five days, that is two days before the final titration result is known, the titre calculated at five days is only slightly less than the final figure and is a good indication of the potency of the vaccine. The vaccine is also titrated weekly until the expiry date.

* William Freeman and Co., Ltd., Barnsley, England.

† Viscose Development Co., Ltd., London, England.

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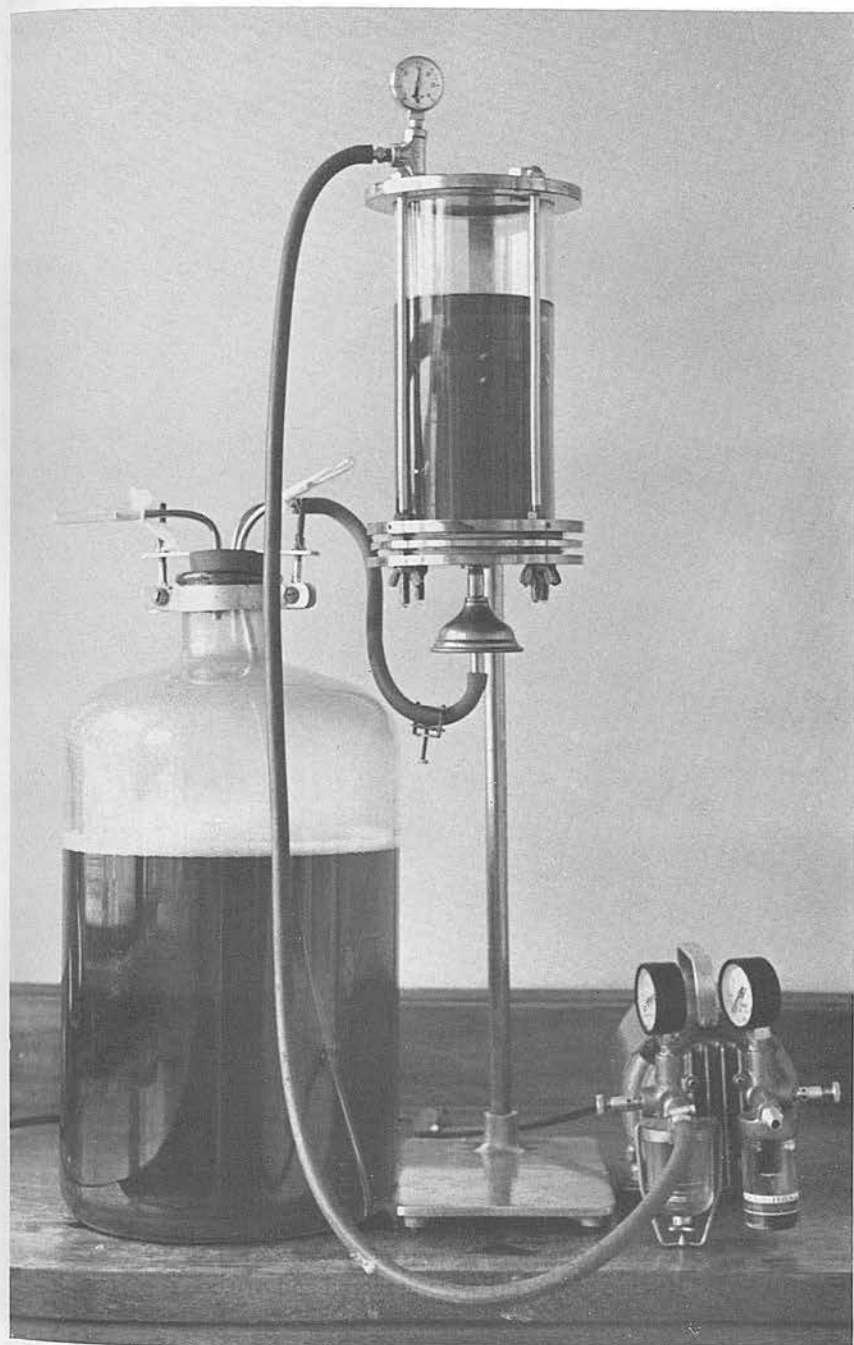


FIG. 2.—Filtration of broth prior to seeding.

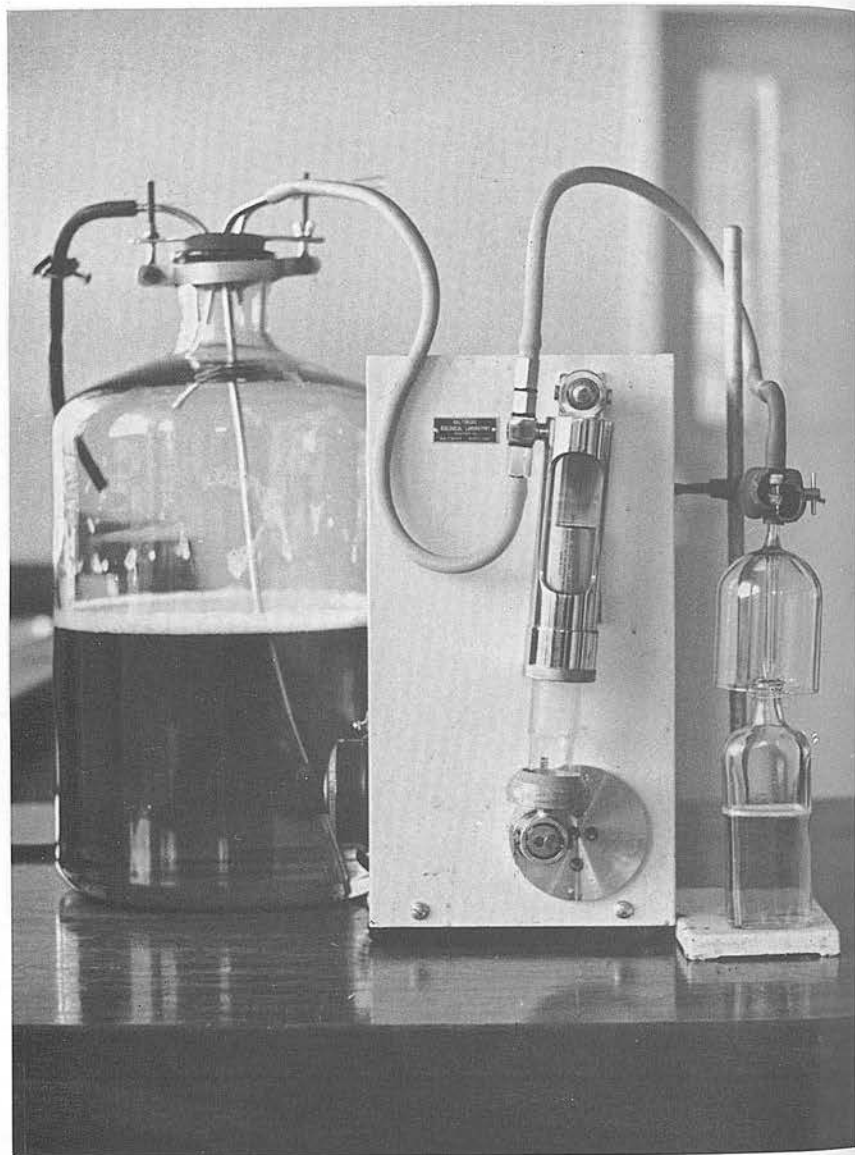


FIG. 3.—Dispensing of seeded broth into bottles.

RESULTS

Vaccine Batches

Between July 1964 when production of T₁ culture vaccine was started and March 1965, 38 batches of vaccine were prepared and 916,000 doses issued.

Keeping Quality of the Vaccine

From results of titrating 10 batches of vaccine from the day of sealing down and at weekly intervals up to five weeks later, the regression curve was calculated. The

mean regression curve based on these figures is $\hat{Y} = 10.06 - .24X$. In other words, 28 days after the vaccine is sealed down the titre has dropped by about 1 log. base 10.

DISCUSSION

Pleuropneumonia culture vaccine can be easily prepared but its production is time consuming and more than two weeks notice is required to supply. In addition the shelf-life is only 30 days and problems of transport in the field may be difficult to resolve. As culture vaccine can be lyophilised easily and with little fall of titre (Palmer and Gourlay, 1964) such a product would be the one of choice. Stocks of such vaccine could be held and orders filled immediately instead of two weeks later.

SUMMARY

Details are given of the production and testing of T1 C.B.P.P. culture vaccine at Muguga.

ACKNOWLEDGEMENTS

We should like to thank Mr. John Njumba and Mr. Harun Kahui for their help in the preparation of this vaccine. This paper is published by permission of the Director of the East African Veterinary Research Organisation.

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Résumé

Le vaccin de culture contre la péripneumonie peut être facilement préparé, mais sa production demande du temps et il faut plus de deux semaines pour répondre à une commande. En outre, sa durée d'emploi est limitée à 1 ou 2 mois, et son transport est difficile.

D'autre part le vaccin avianisé, préparé à partir des souches T1, T2, T3 et V5 de *M. mycoides* s'est révélé dans certains cas capable de provoquer des péripneumonies évolutives, lorsqu'il était inoculé aux bovins.

A cause de ce danger en Afrique de l'Est, les vaccins contre la péripneumonie sont de nouveau préparés en bouillon de culture en utilisant des souches vaccinales avianisées.

La méthode de production du vaccin T1 et les contrôles effectués sont décrits dans cet article.

FERMENTATION OF GLUCOSE BY *MYCOPLASMA MYCOIDES* AND ITS EFFECT ON VIABILITY

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Since broth culture vaccine prepared from the T₁ avianised strain of *Mycoplasma mycoides* var. *mycoides* (Piercy and Knight, 1956) at the forty-fifth egg passage, was first prepared at Muguga in July 1962 and subsequent large-scale production in July 1964, it has been grown in modified Newing's tryptose broth (Gourlay, 1964). During 1964 a few batches of broth culture vaccine were prepared from the T₂ avianised strain of *M. mycoides* (Piercy and Knight, 1956) at the thirty-third egg passage. These batches were produced in a manner identical to that used for the large-scale production of T₁ broth culture vaccine (Brown, Gourlay and MacLeod, 1965). During routine titrations of the vaccine it was noticed, however, that the T₂ vaccine did not maintain its titre of viability as well as the T₁ vaccine and it was decided to investigate the reasons for this. Investigation showed that the loss of viability was associated with excessive lowering of the pH, which was also associated with the glucose content of the medium; furthermore, different strains of *M. mycoides* varied in their ability to ferment glucose.

The following paper gives details of these studies.

MATERIALS AND METHODS

Strains of *M. mycoides*

The T₁ and T₂ strains of *M. mycoides* (Piercy and Knight, 1956) at the forty-fifth and thirty-second egg passages respectively, stored as freeze-dried, ground-up infected chicken embryos, were used and seed was prepared in the manner described earlier (Brown *et al.*, 1965) for the T₁ strain. The Gladysdale strain (Turner, 1961) is the most virulent in our hands and was used as second broth passage from lymph obtained by inoculating infected lung material (Brown, 1964) subcutaneously into an ox. The KH₃J strain of *M. mycoides* (Gambles, 1956) is an avirulent strain obtained as a freeze-dried culture from Nigeria, through the courtesy of Mr. E. P. Lindley.

Broth Culture Medium

Tryptose broth was prepared as described earlier (Brown *et al.*, 1965). Parallel culture studies were performed with this broth (which contained 0.5% added glucose), with similar basic broth containing 0.25% added glucose, and with basic broth alone containing no added glucose.

Later studies were carried out using two modifications of the 0.5% added glucose tryptose broth in order to improve the buffering capacity of the broth. In one the NaCl was replaced by KH₂PO₄, 0.13%, and the Na₂HPO₄ was increased to 0.87%, giving approximately M/15 buffer (Gourlay and Domermuth, 1966) and in

the second three times the quantity of KH_2PO_4 and Na_2HPO_4 were used giving approximately $\text{M}/_5$ buffer.

Titration of Viable *M. mycoides*

Serial ten-fold dilutions of the culture to be titrated were performed in triplicate in tryptose broth medium containing 0.5% added glucose. The end-point was taken as the highest dilution showing growth of *M. mycoides* in six days and the B.C.I.D.₅₀ (broth culture infective dose ₅₀) estimated by the method of Reed and Muench (1938).

pH Estimations

These were performed with a Beckman Zeromatic II pH meter.*

RESULTS

Viability Titres and pH of *M. mycoides* Cultures Grown in Broth with Different Concentrations of Glucose

M. mycoides, KH_3J , T₁, T₂ and Gladysdale strains were grown in broth containing 0.5% and 0.25% added glucose and no added glucose, for two, three, four or five days at 37° C. After growth for these various times the cultures were stored at 4° C. for up to five weeks. Titrations and pH estimations were determined after growth at 37° C. and also at intervals during storage at 4° C.

The results of the titrations after two, three and four days' growth and storage are given in Tables I-III. From these it can be seen that during growth of the organisms at 37° C. the pH of the medium containing 0.5% glucose decreased only very slightly with the KH_3J strain, more with the T₁, more still with the T₂ and very considerably and rapidly with the Gladysdale strain. The broth containing 0.25% glucose was only used for the T₁ and T₂ strains but it also showed a moderate decrease in pH with the T₁ strain and a more marked and rapid drop in pH with the T₂ strain. The broth containing no glucose showed only a slight decrease in pH with all strains. After five days' growth the pH was the same as at four days in all cases, except that with the KH_3J strain in 0.5% glucose broth the pH had dropped from 7.4 to 7.3, with the T₂ strain in broth without added glucose the pH had dropped from 7.3 to 7.2 and with the Gladysdale strain in the same broth the pH had dropped from 7.4 to 7.3.

The viability titres of the various strains in 0.5% glucose broth roughly paralleled the pH, in that once the pH dropped to about 6.3 the viability decreased rapidly. This occurred with the Gladysdale strain after two days' growth at 37° C., by which time its titre had obviously fallen from its peak, with the T₂ strain after three days, while with the T₁ and KH_3J strains this stage was not reached even after five days' growth at 37° C. With 0.25% glucose broth a moderate drop in viability occurred with the T₂ strain, but not with the T₁ strain. In the broth without added glucose growth was slower and, with all but the Gladysdale strain, the maximum titre was not reached until after four days' growth.

The viability on storage at 4° C. again roughly paralleled the pH, in that if it remained above about 6.3 the viability decreased only slightly and slowly but once it dropped much below this figure the loss of viability was marked and rapid. This was particularly well illustrated with the Gladysdale and T₂ strains.

* Beckman Instruments Inc., Fullerton, California.

Table I.—Viability titres and pH of *M. mycoides* cultures grown in media with different concentrations of glucose
Incubation : 2 days at 37° C. Initial pH 7·8.

Storage period at 4° C.	Glucose content of medium	<i>M. mycoides</i> strain							
		KH ₃ J		T ₁		T ₂		Gladysdale	
		Titres *	pH	Titres *	pH	Titres *	pH	Titres *	pH
Nil	0·5	9·5	7·65	10·75	7·1	9·75	6·9	8·5	5·45
	0·25	—	—	10·25	7·45	9·5	7·0	—	—
	Nil	8·75	7·65	9·5	7·8	9·25	7·7	10·0	7·5
1 week	0·5	—	—	10·5	6·75	9·75	6·7	4·5	5·4
	0·25	—	—	10·25	6·8	10·25	6·9	—	—
	Nil	—	—	9·75	7·2	8·5	7·6	9·5	7·5
2 weeks	0·5	—	—	9·25	6·7	9·25	6·5	0·0	5·4
	0·25	—	—	9·75	6·8	9·5	6·8	—	—
	Nil	—	—	9·25	7·1	7·25	7·5	9·5	7·5
3 weeks	0·5	—	—	8·5	6·7	8·5	6·5	0·0	—
	0·25	—	—	9·5	6·8	8·75	6·7	—	—
	Nil	—	—	9·25	7·1	7·5	7·5	8·75	7·4
4 weeks	0·5	—	—	7·25	6·6	8·5	6·5	0·0	—
	0·25	—	—	8·75	6·8	8·5	6·6	—	—
	Nil	—	—	8·5	7·0	6·75	7·5	8·25	7·3
5 weeks	0·5	7·5	7·6	6·75	6·6	7·75	6·3	0·0	—
	0·25	—	—	8·75	6·5	7·75	6·3	—	—
	Nil	7·25	7·6	8·75	7·0	6·75	7·3	7·75	7·25

* Log₁₀ B.C.I.D.₅₀/ml.

Table II.—Viability titres and pH of *M. mycoides* cultures grown in media with different concentrations of glucose
Incubation : 3 days at 37° C. Initial pH 7.8.

Storage period at 4° C.	Glucose content of medium	<i>M. mycoides</i> strain							
		KH ₃ J		T ₁		T ₂		Gladysdale	
		Titres *	pH	Titres *	pH	Titres *	pH	Titres *	pH
Nil	% 0.5 0.25 Nil	9.5 — 8.75	7.5 — 7.7	9.75 10.5 9.75	6.6 6.7 7.3	9.25 9.25 10.5	5.85 6.3 7.3	4.5 — 9.5	5.3 — 7.3
1 week	0.5 0.25 Nil	— — —	— — —	10.25 9.5 9.5	6.75 6.75 7.2	8.25 8.75 9.75	5.6 6.05 7.3	2.75 — 8.5	5.25 — 7.3
2 weeks	0.5 0.25 Nil	— — —	— — —	9.75 9.5 9.25	6.6 6.7 7.1	6.25 7.0 9.25	5.5 6.1 7.3	0.5 — 8.25	5.3 — 7.35
3 weeks	0.5 0.25 Nil	— — —	— — —	9.75 9.5 9.25	6.5 6.65 7.1	5.75 6.25 8.75	5.5 6.1 7.3	0.0 — 8.5	— — 7.3
4 weeks	0.5 0.25 Nil	— — —	— — —	9.75 9.5 8.75	6.4 6.5 7.1	2.75 5.25 7.75	5.5 6.0 7.1	0.0 — 7.75	— — 7.25
5 weeks	0.5 0.25 Nil	8.75 — 6.5	7.4 — 7.6	9.5 8.75 8.75	6.4 6.55 7.1	0.25 2.75 6.5	5.5 5.95 7.1	0.0 — 7.5	— — 7.25

* Log₁₀ B.C.I.D.₅₀/ml.

Table III.—Viability titres and pH of *M. mycoides* cultures grown in media with different concentrations of glucose
Incubation : 4 days at 37° C. Initial pH 7·8.

Storage period at 4° C.	Glucose content of medium	<i>M. mycoides</i> strain							
		KH ₃ J		T ₁		T ₂		Gladysdale	
		Titres *	pH	Titres *	pH	Titres *	pH	Titres *	pH
Nil	0·5 0·25 Nil	9·5 — 8·75	7·4 — 7·7	10·25 10·25 10·25	6·5 6·6 7·15	6·75 8·50 11·50	5·6 6·2 7·3	0·0 — 9·0	5·3 — 7·4
1 week	0·5 0·25 Nil	— — —	— — —	10·25 9·5 9·75	6·5 6·5 7·05	4·75 5·5 10·75	5·5 6·1 7·2	— — —	— — —
2 weeks	0·5 0·25 Nil	— — —	— — —	10·25 9·5 9·75	6·5 6·5 6·95	3·75 5·25 9·75	5·5 6·05 7·2	— — —	— — —
3 weeks	0·5 0·25 Nil	— — —	— — —	10·25 9·5 9·5	6·5 6·4 6·95	3·5 5·25 9·25	5·5 5·95 7·2	— — —	— — —
4 weeks	0·5 0·25 Nil	— — —	— — —	10·25 9·25 10·25	6·4 6·4 6·95	Nil 3·75 8·5	5·5 5·9 7·1	— — —	— — —
5 weeks	0·5 0·25 Nil	— — —	— — —	9·75 9·25 9·75	6·4 6·4 6·95	Nil 3·75 7·75	5·4 5·9 7·1	— — —	— — —

* Log₁₀ B.C.I.D.₅₀/ml.

Cultural Characteristics of the Various Strains in Broth with Different Concentrations of Glucose

After 2-3 days' growth at 37° C. in broth without added glucose the opacity was very slight with all strains and remained so even after five days' growth. The T2 and Gladysdale strains, however, produced fine visible threads after 2-3 days' growth, which later broke up and dispersed but the opacity still remained slight. Only the T1 and T2 strains were used in the broth containing 0.25% glucose and in both cases there was moderate opacity after 2-3 days' growth but whereas the T2 strain produced fine threads the T1 strain did not. Later, when the T2 threads broke up the opacity became marked while the T1 opacity remained moderate after five days' growth.

In the broth containing 0.5% glucose the opacity of the KH₃J strain after 2-3 days' growth was slight and remained so even after five days. No threads were formed. The T1 strain showed marked opacity but also no threads after 2-3 days and it remained the same after five days' growth. The T2 and Gladysdale strains showed only slight opacity at first but thick threads were formed which eventually broke up, giving very marked opacity after five days' growth.

The opacity produced in broth by growth of the organisms does not appear to be due to the effect of acid on the medium constituents, as the gradual addition of 0.2 M HCl to 0.5% glucose broth, to give a final pH of 4.4 did not cause any opacity of the broth.

When a 28-hour 0.5% glucose broth culture (pH 7.1) of the Gladysdale strain of *M. mycoides*, showing marked opacity (B.C.I.D.₅₀ titre 10^{11.5}/ml.) was centrifuged at 8,700 G for 60 minutes in an angle-head centrifuge, the supernatant was completely clear, the substance responsible for the opacity having been deposited. The B.C.I.D.₅₀ titre of the supernatant was decreased by 3.75 Logs.

A 53-hour culture (pH 7.7) of the same strain of *M. mycoides* grown in broth with no added glucose (B.C.I.D.₅₀ titre 10^{10.5}/ml.) but with only slight opacity, was centrifuged in an identical manner, the supernatant was again completely clear but the titre was only reduced by 2.0 Logs.

The Effect on Viability of Adjusting the pH After Growth

The T1 and T2 strains were grown in 0.5% glucose broth and after three days' growth an aliquot of each culture was adjusted by means of N.NaOH to pH 7.1. The effect of this on the viability on storage at 4° C., as compared with the non-adjusted broth, can be seen in Fig. 1. Little effect was seen with the T1 strain but with the T2 strain the viability was greatly improved and it was only slightly less viable than T1 after five weeks' storage.

The Effect of Using Buffered Tryptose Broth on Growth and Viability

The T2 strain was grown in buffered 0.5% glucose broth containing two different concentrations of buffer, one containing approximately M/15 buffer and the other approximately M/5 buffer. The pH before inoculation of the broths was 7.55. Table IV gives the results, after three and five days' growth at 37° C. and again following five weeks' storage at 4° C.

The addition of buffer maintained the pH at near neutrality and improved the viability after three and five days' growth. On storage, however, the M/15 buffer was superior to the M/5 buffer.

Table IV.—Viability titres and pH of *M. mycoides* (T2 strain) grown in buffered broth

Days at 37° C.	Buffer molarity	After growth at 37° C.		After 5 weeks at 4° C.	
		Titre *	pH	Titre *	pH
3	M/15	10·0	7·2	7·75	6·7
	M/5	10·0	7·4	4·75	6·8
5	M/15	10·5	6·8	6·5	6·6
	M/5	10·0	7·0	3·75	6·7

* Log₁₀ B.C.I.D.₅₀/ml.

DISCUSSION

In broth without added glucose the variation in growth and viability of the different strains was very slight, except that the Gladysdale and T2 strains produced fine threads.

When grown in broth containing added glucose the highly virulent Gladysdale and moderately virulent T2 strains formed thick threads, produced a more rapid growth, marked opacity and considerable lowering of the pH. The addition of glucose had no effect on the avirulent KH₃J strain and only slight effect on the almost avirulent T1 strain. The degree of glucose fermentation and the formation of threads may possibly give an indication of the virulence of the strain of *M. mycoides* for cattle.

Loss of viability was associated with the lowering of pH and confirms the finding of Freund (1958). The fact that the loss of viability on storage of the T2 strain was greatly reduced by adjusting the pH to 7·1 immediately after growth is further evidence to support this finding.

The opacity produced in broth during growth of the organisms was not due to the effect of the acid produced on the medium constituents, but apparently to a proportion of organisms which could be deposited by centrifugation at 8,700 G for 60 minutes. It appears that, with the Gladysdale strain at least, more of these easily deposited forms were present in cultures grown in 0·5% glucose broth than in broth with no added glucose and these gave the marked opacity seen in the former. As break-up of threads appears to increase the opacity greatly it is likely that these easily deposited forms responsible for the opacity are frequently derived from the threads. The formation of threads appears to be related to inoculum size; where the inoculum is sufficiently large threads are not formed, but the opacity, nevertheless, becomes as marked in the glucose broth as when threads form.

The use of buffer in the 0·5% glucose broth maintained the pH near neutrality and improved the viability during growth of the T2 organisms. On storage at 4° C., however, the M/15 buffer was superior to the M/5 buffer in which the salt concentration was probably too high. It is possible that less than M/15 buffer would be still more satisfactory.

SUMMARY

Different strains of *M. mycoides* var. *mycoides* vary in their ability to ferment glucose. In broth without added glucose all four strains produced slight opacity and very little acid. The highly virulent Gladysdale and the moderately virulent T2 strains, however, produced fine threads, whereas the avirulent KH₃J and almost

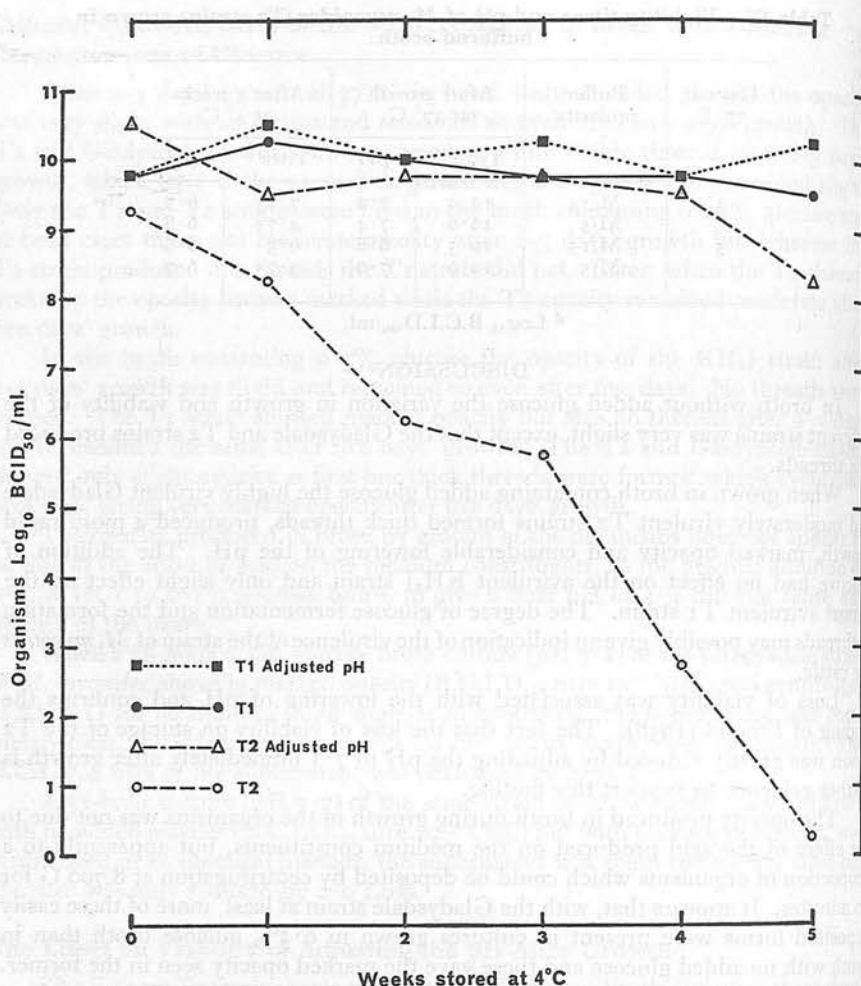


FIG. 1.—The effect of viability of adjusting the pH to 7.1 after growth for three days at 37°C.

avirulent T1 strains did not. In broth with added glucose the Gladysdale and T2 strains produced very marked opacity, considerable acid and thick threads. The T1 strain produced marked opacity, slight acid and no threads and the KH₃J strain produced only slight opacity, relatively little acid and no threads. The production of acid was directly responsible for the loss of viability and this was improved by the addition of buffer to the broth or by adjusting the pH to neutrality immediately after growth. Opacity was not due to the effect of acid on the medium constituents but to that proportion of organisms that could easily be deposited by centrifugation.

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Résumé

Les diverses souches de *M. mycoides* var. *mycoides* varient dans leur pouvoir de faire fermenter la glucose. En bouillon sans addition de glucose, toutes les quatres souches ont produit une faible opacité et très peu d'acide.

La souche "Gladysdale" très virulente et la souche T₂ de moyenne virulence ont produit cependant des filaments fins, tandis que la souche avirulente KH₃J et la souche presque avirulente T₁ n'en ont pas produit. En bouillon avec addition de glucose, les souches Gladysdale et T₂ ont produit une opacité très marquée, beaucoup d'acide et des fils épais. La souche T₁ a produit une opacité marquée, peu d'acide et pas de fils; la souche KH₃J n'a produit qu'une faible opacité, relativement peu d'acide, et pas de fils. La production de l'acide a été directement responsable de la perte de viabilité, et celle-ci a été améliorée soit par l'addition du tampon phosphate au bouillon, soit en ajustant le pH à la neutralité aussitôt après la croissance.

L'opacité n'est pas due à l'effet de l'acide sur les éléments du bouillon, mais à la proportion des organismes qui peuvent être facilement déposés par centrifugation.

The Use of Embryonated Chicken Eggs for the Study of Immunity to *Mycoplasma mycoides**

Sir,—The only host available at present, other than cattle, for experimental studies on contagious bovine pleuropneumonia (CBPP), is the chicken embryo; CBPP has not been transmitted as yet, in the true sense, in laboratory animals.

It has previously been demonstrated that antiviral and antibovine serum antibodies can be passed from the serum of chickens to the egg yolk (Fraser, Jukes, Branion & Malpern, 1934; Brandley, Moses & Jungherr, 1946; Patterson, Younger, Weigle & Dixon, 1962). Taylor and Schelling (1960) and Macleod and Hemsley (1965), used the embryo susceptibility test to determine the incidence of avian encephalomyelitis in chickens. We carried out experiments to determine whether antibodies against *Mycoplasma mycoides* would pass from the serum of hens to the yolk and confer immunity on the embryos to challenge with *M. mycoides*. We thus hoped to circumvent the lack of an experimental animal for the study of immunity to *M. mycoides*.

Twelve white Leghorn hens were each inoculated intramuscularly on three occasions at two-week intervals, with 20 mg. of lyophilised *M. mycoides* (Gladysdale strain) in complete Freund's adjuvant. Embryonated eggs from these hens were used one month after the last injection.

Nine-day-old embryos from normal and immunised hens were inoculated with various dilutions of *M. mycoides* culture, via the allantoic route. The virulent Gladysdale strain and the T₂ vaccine strain (Piercy & Knight, 1956), which had been passaged 33 times in egg embryos, were used. Embryos that died after the second day post inoculation were cultured in PPLO tryptose broth and nutrient broth, and on

nutrient agar and blood agar plates, in order to determine the cause of death. Challenge titrations were done every second week to permit accumulation of sufficient eggs.

Table I shows the result of a typical experiment. Specific deaths among the control embryos occurred between the fourth and eighth days post inoculation. On rare occasions when embryos from immunised hens died, death was usually on the seventh day after inoculation or later.

Similar results were obtained when the T₂ strain was used.

In a second experiment, the LD₅₀ was determined in embryonated eggs from a flock of 12 hens before immunisation; this was 10^{5.5} per ml., calculated by the method of Reed and Muench (1938). These hens were then immunised, using the schedule and strain of *M. mycoides*, as previously described. Embryonated eggs immune to challenge of 10⁸ organisms per ml. first appeared 16 days after the last injection of the hens.

From the above results we consider that this method may be of use in the study of immunity and pathogenesis in contagious bovine pleuropneumonia and possibly in other non-avian diseases where an experimental laboratory animal is not available, but where the chicken embryo can be used.

Further work is in progress to determine the duration of immunity in relation to various schedules of immunisation, with different antigens, fate of inoculum in the immune embryos, and concentration of antibodies in hens and their eggs in relation to immunity.

November 18th, 1965.

Yours faithfully,

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TABLE I
SPECIFIC MORTALITY OF EMBRYONATED HENS FROM IMMUNE AND NORMAL HENS AFTER CHALLENGE WITH *Mycoplasma mycoides* (GLADYSDALE STRAIN)

Log. No. of organisms per dose	No. of eggs	No. of dead eggs† Days after inoculation						% dead
		4	5	6	7	8	9	
Normal eggs								
10 ^{0.5}	10		5	4				100
10 ^{1.5}	10			6	1			100
10 ^{2.5}	10			6	4			100
10 ^{3.5}	10		3	1	3	2		100
Immune eggs								
10 ^{0.5}	10				1			12.5
10 ^{1.5}	10							0

† This refers to specific deaths only.

THE VIRULENCE AND VIABILITY OF *Mycoplasma Mycoides*
STRAINS IN CHICKEN EMBRYOS FROM NORMAL AND
IMMUNIZED HENS

BY

R. N. GOURLAY and M. SHIFRINE

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The Virulence and Viability of *Mycoplasma mycoides* Strains in Chicken Embryos from Normal and Immunized Hens

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SUMMARY. The KH₃J strain of *M. mycoides* var. *mycoides* was avirulent for chicken embryos, whereas the LD₅₀ titre of 4 other strains varied from 10^{5.6} to 10^{6.1}.

The Gladysdale strain was inoculated into immune and normal chicken embryos and there was a decrease in the number of organisms in the immune embryos, from the 4th day after inoculation, when compared with the normal embryos. Sera from immune hens possessed agglutinating and growth-inhibiting antibodies, but yolk extracts from these hens rarely contained agglutinating antibodies. The immunity possessed by embryos persisted at least 17 weeks after immunization of the hens.

GOURLAY & SHIFRINE (1966) showed that chicken embryos from hens immunized with *Mycoplasma mycoides* var. *mycoides* could survive a challenge with *M. mycoides* which killed embryos from normal hens. We report here further work using embryos from normal and immunized hens.

MATERIALS AND METHODS

Immunized hens (Gourlay & Shifrine, 1966) were maintained in individual battery cages, but periodically placed with a cockerel. Hens were bled for serum 6 and 16 weeks after the last immunizing injection. Sera from hens and yolk extracts from

infertile eggs prepared by the method of Patterson *et al.* (1962) were inactivated and tested by the slide agglutination serum test (SAST) (Gourlay, 1964) and the solid medium growth inhibition (GI) test (Domermuth & Gourlay, 1967).

The LD₅₀ titres of the T₃, S₁, KH₃J (Gourlay, 1964, 1965) Gladysdale and Mara strains (Brown, 1964) were determined by inoculating 0.1 ml. of 10-fold dilutions of the organisms by the allantoic route into groups of 10 nine-day old chicken embryos. The LD₅₀ was calculated by the method of Reed & Muench (1938).

The Gladysdale strain was used for viability titrations. In the first experiment, embryos from normal and immune hens were inoculated with 10^{7.5} organisms and a number from each group were removed for titration daily for 5 days after inoculation. In the second experiment, embryos were inoculated with 10^{9.25} organisms and were titrated on the 4th to 6th day after inoculation. In

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the first experiment, the immune embryos were from eggs laid 5 to 7 weeks after the last immunizing injection, and in the 2nd experiment, the eggs were laid 15 to 17 weeks after the last injection.

For titration, the embryo and fluids (less the albumin) were homogenized and serial 10-fold dilutions of mixture were titrated in duplicate in tryptose broth (Gourlay, 1964). The end-point was the highest dilution showing growth of *M. mycoides* within 6 days from which the $BCID_{50}$ (broth culture infective dose 50) titre was calculated.

RESULTS

LD₅₀ of Various Strains of M. mycoides

The average LD_{50} titres of 3 titrations were Gladysdale $10^{5.6}$, S1 $10^{6.1}$, T3 $10^{5.7}$, Mara $10^{5.6}$ and KH3J $>10^{10}$. We were unable to determine the LD_{50} of the KH3J strain as even undiluted broth culture containing 10^{10} organisms/0.1 ml. did not kill 50% of the embryos.

Titre of M. mycoides in Embryos from Normal and Immunized Hens

The results of the 2 experiments (Table I) show a decrease in the average titre of *M. mycoides* in the immune embryos, compared with the normal embryos, on the 4th to 6th days after inoculation. In both experiments, one embryo from each of the immune hens was not used for titration and all survived challenge.

TABLE I
TITRE OF *M. mycoides* IN EMBRYOS FROM NORMAL AND IMMUNE HENS FOLLOWING INOCULATION WITH $10^{7.5}$ ORGANISMS (EXPERIMENT 1) AND $10^{9.25}$ ORGANISMS (EXPERIMENT 2)

Day post Inoc.	Experiment 1				Experiment 2			
	Immune embryos		Normal embryos		Immune embryos		Normal embryos	
	No.	Titre*	No.	Titre*	No.	Titre*	No.	Titre*
1	2 A	6.75	2 A	7.5				
2	4 A	6.125	4 A	6.5				
3	4 A	6.9	4 A	6.5				
4	4 A	3.4	4 A	7.125	9 A	6.6	8 A	7.4
							3 D	8.7
5	2 A	3.5	2 A	6.0	9 A	5.4	10 A	7.7
			3 D	9.5			4 D	9.7
6					2 A	5.75	5 A	8.9

* Average \log_{10} $BCID_{50}$ organisms/ml. of homogenized embryo. A=alive at time of titration. D=dead at time of titration.

Examination of Serum and Yolk Extracts for Antibodies Against M. mycoides

All immunized hens possessed agglutinating antibodies in their sera 6 weeks after the last immunizing injection. However, 16 weeks after the

last injection only half the hens showed any antibodies. GI antibodies were demonstrated in the sera of all immunized hens on both occasions. Only 1 egg out of 30 possessed any demonstrable agglutinating antibodies in the yolk extract. Yolk extracts did not prove suitable for the GI test. Sera from normal hens had no demonstrable agglutinating or GI antibodies.

DISCUSSION

The avirulence of the KH3J strain for egg embryos is of interest as this strain, in contrast to the others tested, is also avirulent for cattle. There is, however, no direct correlation between chicken embryo and cattle virulence. Whereas chicken embryo LD_{50} titres of the latter strains are very similar, their virulence for cattle varies considerably, as judged by subcutaneous challenge, when deaths were Gladysdale, 19 out of 26 (73%) T3 14/64 (22%), Mara, 1/20 (5%) (Gourlay—unpublished data), and S1 9/19 (47%) (Brown, 1966).

Agglutinating antibodies were demonstrated in the sera of all the immunized hens at 6 weeks, but in only half of them at 16 weeks and in only one yolk extract. In some instances embryos from immunized hens withstood challenge at a time when no agglutinating antibodies were detectable in either the hens' sera or yolk extracts. The insensitivity of the SAST, however, does not eliminate the possibility that some agglutinating antibodies were still present. GI antibodies were detected in all immunized hens' sera throughout the period of the experiment. It would appear, therefore, that GI antibodies are more likely to be associated with immunity than agglutinating antibodies. However, the exact relationship between GI antibodies and immunity is difficult to assess due to the technical difficulties experienced with yolk extracts in the GI test.

ACKNOWLEDGMENTS

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Isolation of a *Mycoplasma*-like Organism from Pneumonic Calf Lungs

Sir,—During the examination of pneumonic lesions from calf lungs a number of strains of a hitherto undescribed mycoplasma-like organism were isolated.

Pieces of pneumonic lung were triturated in phosphate buffered saline and inoculated in 10-fold dilutions into glucose serum broth. The first of these organisms to be isolated was from the pneumonic lung of a six-months-old calf (No. 462). Growth was observed in the broth up to the 10^{-6} dilution, which was the highest dilution used. Typical *Mycoplasma* colonies were not observed on subculture to solid medium (consisting of the broth with 0.65 per cent. agarose) and no bacteria were isolated, but a few large colony-like structures up to 300μ in diameter were observed after one to three days incubation (Fig. 1). These were granular, did not possess any obvious centres and were very friable and disintegrated when touched with a wire loop. They did not grow when subcultured with a loop either into liquid or on to solid medium. Sub-inoculation from broth to broth was possible, how-

ever, and colony-like structures were formed when broth cultures were dropped on to solid medium or when an agar block containing colonies was pushed over the surface of a fresh solid medium plate.

A further 14 strains producing morphologically similar colonies have since been isolated from pneumonic calf lungs. On primary isolation growth is generally detectable after seven to 14 days incubation, although occasionally as early as four and as late as 21 days after commencing incubation. Three of these strains appear serologically similar by the Metabolic Inhibition Test (Taylor-Robinson, Purcell, Wong & Chanock, 1966) with serum prepared in rabbits to one of the strains (462) and when compared by the polyacrylamide gel electrophoresis method of Razin and Rottem (1967), and they do not appear to belong to any of the recognised groups of *Mycoplasma* of bovine origin. These three strains ferment glucose and will not grow in broth without added serum or at 22°C . Centrifuge deposits from broth cultures of these strains showed organisms indistinguishable from mycoplasmas both by light and electron microscopy.

These organisms appear to be mycoplasmas but, despite the fact that two of these strains were isolated from the lungs in medium free of penicillin and thallium acetate, and were subsequently subcultured three times on this medium without reverting to bacteria, we cannot exclude the possibility that the calves had been treated at some time with antibiotics, perhaps producing L-forms of some bacteria. Further work is in progress with these strains and a detailed report will be published in the near future (Gourlay & Leach, in preparation).

December 9th, 1968.

Yours faithfully,

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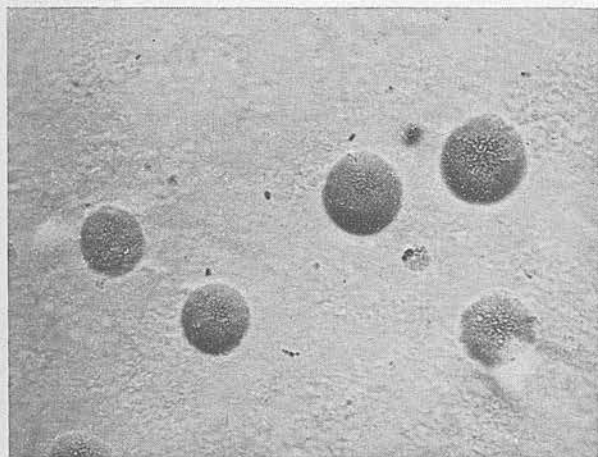


Fig. 1.—Colonies of the 462 strain on solid medium $\times 35$.

A New Mycoplasma Species Isolated from Pneumonic Lungs of Calves (*Mycoplasma dispar* sp. Nov.)

BY

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A NEW MYCOPLASMA SPECIES ISOLATED FROM PNEUMONIC LUNGS OF CALVES (*MYCOPLASMA DISPAR* SP. NOV.)

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PLATES VIII-X

TYPICAL large-colony mycoplasmas are prevalent in the upper respiratory tract of normal cattle and in the lungs of calves with pneumonia (Davies, 1967; Günther *et al.*, 1967; Leach, 1967) and mycoplasma T-strains have also been isolated from the latter source (Gourlay, 1968).

These organisms can be cultivated by the use of the appropriate conventional mycoplasma medium, but such media are not suitable for cultivation of all types of mycoplasma, and several mycoplasmas found in animals require special media for primary isolation. A relevant example is the mycoplasma causing enzootic pneumonia in pigs, named *Mycoplasma hyopneumoniae* (Mare and Switzer, 1965) or *Mycoplasma suis-pneumoniae* (Goodwin, Pomeroy and Whittlestone, 1965). This organism can be cultivated in a special acellular medium modified from tissue-culture fluids, but cannot be grown in conventional mycoplasma medium of the type described by Chanock, Hayflick and Barile (1962).

This suggested that there might be unrecognised mycoplasmas in other animals, and that they might be detected by the use of similar media. With this in mind we used a modification of the medium of Goodwin *et al.* (1965) in attempts to isolate mycoplasmas from the lungs of calves, as part of a current microbiological and pathological investigation of calf pneumonia at Compton. Strains of an unusual type of organism were isolated in this medium. They have the general characteristics of mycoplasmas, but with some atypical features, and do not appear to have been described before. A preliminary report on the isolation of these organisms has been published (Gourlay, 1969).

MATERIALS AND METHODS

Culture media. For the attempted isolation and growth of mycoplasmas from calf lungs, the following glucose calf-serum (GS) broth was normally used: Hanks' buffered salt solution (Burroughs Wellcome and Co.) 40 ml; lactalbumin hydrolysate (Nutritional Biochemicals Corp.), 5 per cent. (w/v) 10 ml; Hartley's digest broth 20 ml; foetal calf serum (heated at 56°C for 30 min.) 20 ml; glucose, 50 per cent. (w/v) 2 ml; calf thymus DNA (B.D.H.—highly polymerised), 0.2 per cent. (w/v) 1 ml; penicillin, 200,000 IU per ml, 0.5 ml; thallium acetate, 5 per cent. (w/v) 0.5 ml; phenol red, 1 per cent. (w/v) 0.2 ml.

These solutions were made in glass-distilled double-deionised water. The pH was adjusted to 7.8 with N-NaOH. Solid medium (GS agar) was prepared by the addition of

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0.65 per cent. agarose to the appropriate broth medium. Modifications of this medium were used for particular purposes as indicated in the text.

Tests were carried out in the acellular fluid or solid media for *M. suis* pneumoniae described by Goodwin *et al.* (1967), which will be referred to as the Cambridge media. Cambridge broth differs from GS broth in containing pig serum and yeast extract but not foetal calf serum or calf thymus DNA, and in the absence of added glucose. The corresponding solid medium is made by the addition of Oxoid Ion Agar to Cambridge broth.

Isolation procedure. Pieces of lung from a pneumonic area when this was present (about 0.5 cm³) were triturated in 5 ml phosphate-buffered saline (pH 7.4) and serial ten-fold dilutions were prepared; 0.2 ml quantities were inoculated into 1.8 ml amounts of GS broth. The inoculated broths were incubated at 37°C for at least 3 wk and were examined at intervals for evidence of growth, as indicated by an acid change in pH. A drop of culture from each of the tubes in which such a colour change occurred was plated on GS agar, which was examined microscopically for colonies during a similar period of incubation. Solid media were incubated in a mixture of 5 per cent. CO₂ in nitrogen.

Staining methods. Films prepared from centrifuged deposits of broth cultures (25,000g for 30 min.) were fixed in methanol and stained for 2 hr at 56°C with Giemsa's fluid, used at a 1 in 15 dilution in tap water.

Colonies on solid media were stained and examined *in situ* by Dienes' method as described by Madoff (1960).

Satisfactory whole-colony preparations were obtained by the agar-fixation method of Klieneberger (Klieneberger-Nobel, 1962, p. 31), as adapted for routine use by the Mycoplasma Reference Laboratory (Bouin fixation, followed by staining with 1 in 50 Giemsa for 45 min. at 56°C).

Estimation of number of viable organisms. The titre of viable organisms was estimated by means of a colour-change test in which serial ten-fold dilutions of suspensions were prepared in 2-ml volumes of GS or Cambridge broth. The colour change end-point titre (CCT) was taken as the highest dilution of culture that gave a colour change when a volume of 0.2 ml was seeded into 1.8 ml of GS broth and tubes were incubated at 37°C for 3 wk.

Preparation of specific antiserum. Rabbit antiserum was prepared against a chosen prototype strain no. 462/2. This strain had been purified by 3 successive subcultures from single colonies (see *Results*). Organisms were grown in 300 ml of a modified GS liquid medium in which Hartley's digest broth and calf serum were replaced by rabbit meat infusion and rabbit serum respectively. After centrifugation at 40,000g for 20 min., the deposit was washed once in veronal-buffered saline and, after recentrifugation, suspended in the same buffer. One-quarter of this material was mixed with an equal volume of 4 per cent. sodium alginate containing 0.8 per cent. calcium gluconate (Medical Alginates Ltd, Wadsworth Road, Perivale, Middlesex, England) and inoculated intramuscularly at 2 different sites. One-third portions of the remainder of the suspension were inoculated intravenously, without adjuvant, 2, 3 and 4 wk later. Serum obtained 1 wk after the last injection was satisfactory for metabolic inhibition tests, but not for growth-inhibition tests. A further series of 3 intravenous injections of material prepared in a similar manner was given 3 mth later, and the serum subsequently obtained reacted strongly with the homologous strain no. 462/2 in growth-inhibition tests.

Growth-inhibiting antiserum for *M. bovirhinis* was prepared by a similar procedure. Antisera for other mycoplasmas were mainly those used routinely for identification of mycoplasmas in the Mycoplasma Reference Laboratory. The details relating to these sera are described elsewhere (Leach, to be published).

Serological identification of strains. Strains were examined serologically by the paper disk growth-inhibition (GI) technique (Clyde, 1964) on GS agar, and by the metabolic inhibition (MI) technique (Taylor-Robinson *et al.*, 1966) in GS broth.

Filtration. Two slightly different methods were used to estimate the order of size of the minimal viable particles by filtration through Millipore membrane filters (Millipore Filter Corp., Bedford, Mass., USA) of varying nominal pore diameters (p.d.).

(a) At Compton (experiment A), the organisms were grown in GS broth that had previously been filtered through a membrane of 220 nm p.d. Separate portions of each test culture were passed through filters of 650, 450 and 220 nm p.d. under a positive pressure of 5 lb. per sq. in. (c. 33 kN per m²). Ten-fold dilutions of the filtrates were titrated in GS broth.

(b) At the Mycoplasma Reference Laboratory (experiment B), the test organisms were first grown for several subcultures in Cambridge medium. Portions of cultures were passed through 800 and 450 nm p.d. membranes in Swinney-type adapters attached to hypodermic syringes operated manually. The conditions were partially standardised by adjustment of the rate of flow through the filter to about one drop per second and by discarding approximately the first 1 ml of filtrate. Titrations of filtrates were carried out in Cambridge broth.

Electron microscopy. Organisms were grown in 500 ml GS broth and then deposited by centrifugation at 25,000g for 30 min. The deposit was re-suspended in phosphate-buffered saline (pH 7.3) and re-centrifuged. The resulting pellet was prepared for electron-microscope studies as described by Anderson and Barile (1965) and was embedded in Araldite. Sections were cut with a Reichert OMU2 ultramicrotome, stained with uranyl acetate and lead citrate and examined with an A.E.I. EM6 electron microscope.

Deoxyribonucleic acid (DNA) base composition. The strain no. 462/2 was grown in 5 litres of GS broth containing pig serum instead of foetal calf serum, and the organisms were collected by centrifugation at 10,000g for 30 min. The DNA was extracted by the method of Marmur (1961) and its base composition was estimated from the "melting temperature" (T_m) in standard saline citrate (Marmur and Doty, 1962). Both the DNA extraction and base composition estimation were kindly carried out by Mr L. R. Hill.

RESULTS

Isolation of mycoplasmas from calf lung

Strains isolated by means of glucose calf-serum (GS) broth. Samples from 72 pneumonic calf lungs, 20 healthy calf lungs and 10 healthy adult cow lungs were inoculated into GS broth. Negative results were obtained with all samples from healthy animals, but 49 out of the 72 suspensions prepared from pneumonic lungs produced evidence of growth (acidification) in GS broth cultures (table I). Primary cultures showing colour change were subcultured on GS agar and also on blood, chocolate and MacConkey agar plates to exclude the presence of bacteria. No bacterial growth occurred on any of these media, but in each case minute colonies developed on GS agar. The colonies were of two distinct and easily recognisable types. One type (described as "typical" in table I) consisted of "fried-egg" colonies similar to those produced by many mycoplasma species. The 18 isolates that formed this type of colony were all identified as *M. bovirhinis*. The other type of colony (described as "atypical" in table I), which was cultivated from 36 samples, had a granular or sometimes "lacy" appearance and lacked a well-defined centre (figs. 1-3). The remainder of this paper is concerned mainly with strains producing this type of colony. These will be referred to as "atypical" strains.

"Atypical" strains were present in lung suspensions at titres (CCT) ranging from 10² to 10⁷ or greater, but in most cases the titres were between 10³ and 10⁵.

In 5 cases (table I) both "atypical" strains and *M. bovirhinis* were isolated from the same sample. *M. bovirhinis* generally grew to maximum titre within 3 days, but the "atypical" strains grew more slowly; when both were isolated from the same lung suspension the "atypical" strains were the more numerous

and, although overgrown by *M. bovirhinis* in broth inoculated with more concentrated lung suspensions, were easily isolated in pure culture from the broths inoculated with higher dilutions.

Most of the isolations shown in table I were confirmed by subsequent repetition of the cultures from lung material that had been stored at -70°C .

Isolation in medium without penicillin or thallium acetate

To eliminate the possibility that the "atypical" strains might be L-phase bacteria (L forms) induced by the presence of known bacterial inhibitors in the media, further portions of three lung emulsions from which these organisms

TABLE I
Isolation of mycoplasmas from bovine lungs by means of glucose-serum broth

Source	Number of lungs			
	examined	giving broths showing colour change	giving mycoplasma colony types classified as	
			typical	atypical
Pneumonic lungs of calves	72	49	18*	36*
Normal lungs of calves	20	0	0	0
Normal lungs of cows	10	0	0	0

* From 5 lungs both colony types were isolated.

had been isolated were cultured in GS medium from which the penicillin and thallium acetate had been omitted; similar organisms were again isolated. Each of these strains was subsequently subcultured several times in media free of these substances without any change in its cultural and colonial characteristics. The three strains were subsequently shown to be serologically indistinguishable from the chosen prototype "atypical" strain, no. 462/2 (see next section). The latter strain was itself subcultured for six passages in GS media free of penicillin and thallium acetate without evidence of reversion to bacterial forms. One of the three calves from which isolations were made in medium free from penicillin and thallium acetate had been treated earlier with neomycin, but the treatment had ended nearly 6 wk before the lung was examined. It was not certain whether the second of these calves had ever received antibiotics, but the third calf had been raised at Compton and was known not to have had antibiotic treatment at any stage of its life.

Properties of "atypical" strain

It was apparent at an early stage that all "atypical" strains were indistinguishable in their general cultural characteristics. Subsequently, one strain,

no. 462/2, was chosen as representative of the group for detailed examination. The following description applies specifically to this strain, but was confirmed where indicated by additional observations on other "atypical" strains.

Cultural and colonial characters. During primary isolation of "atypical" strains, growth was usually detectable in GS broth within 7-14 days (maximum range 4-23 days) of inoculation. Laboratory-adapted strains grew somewhat faster and growth from minimal inocula was usually detectable within 2 wk. Growth was indicated by an acid shift of pH accompanied by development of a slight, usually granular, deposit. "Atypical" strains grew poorly or not at all in conventional mycoplasma media. Plating of cultures on 5 per cent. ox-blood agar led after incubation to the production of an area of haemolysis under the inoculated area, but no growth was visible.

Early subcultures on GS agar produced mainly large colonies, up to 1/3 mm in diameter after 1-3 days' incubation. These colonies were roughly circular, although sometimes slightly uneven, and rather granular in appearance. They lacked the "centres" characteristic of most mycoplasmas (figs. 1 and 2) and did not penetrate the agar. They were friable in texture and disintegrated easily when touched. On Cambridge agar the colonies were generally similar, but had a more obviously "lacy" or reticulated appearance when viewed by transmitted light (fig. 3). Colonies of the above types could not be subcultured by means of a wire loop, but subcultures were successful when an agar block bearing colonies was transferred to broth or spread over agar medium. Usually a heavy inoculum was required for growth on the agar media, which were therefore unsuitable for titration of suspensions by means of colony counts. On the other hand, subculture from broth to broth was usually possible with inocula diluted at least to 10^{-7} .

After several passages of strain no. 462/2 in GS broth, the colonies on GS agar were found to have a different morphology, and at this stage a comparable broth culture produced many more colonies than previously. Colonies were then small, up to about 0.1 mm in diameter, and many possessed poorly defined centres which, nevertheless, penetrated the agar ("centre-forming colonies"; fig. 4). At this stage, 3 successive subcultures were made from single colonies. This cloned strain was used for the preparation of an antiserum in rabbits. Centre-forming colonies have not yet been observed on Cambridge agar.

The ability to produce small centre-forming colonies was temporarily lost after storage at -70°C for a few months, but reappeared after further passages of the strain in broth. Most other "atypical" strains also produced centre-forming colonies after several subcultures in broth and some were cloned in the same way as was strain no. 462/2. Other strains were cloned before they produced centre-forming colonies transferable with a wire loop; this was carried out by transferring into broth an agar block containing a single colony and repeating the procedure twice.

Microscopic examination. Both types of colony produced by the 462/2 strain on GS agar were stained blue *in situ* by Dienes' stain. When impression films of colonies on Cambridge agar were stained by the Giemsa method, they were seen to consist mainly of enlarged forms, giving the whole colony a

"vacuolated" texture (fig. 5), corresponding somewhat to the "lacy" appearance seen by direct observation. Under higher magnification the enlarged forms at the periphery of large colonies appeared to contain many particles, but these were more easily seen in small colonies as in fig. 6. As seen already with unstained preparations (fig. 2), colonies on GS agar stained with Giemsa had a less reticulate structure than those on Cambridge agar.

Smears were prepared from the deposit produced by centrifuging broth cultures of strain no. 462/2 at 25,000*g* for 30 min. When stained with Giemsa, they showed pleomorphic organisms, the majority of which were ring forms of varying diameter up to about 1500 nm when measured with an ocular micrometer, but there were also many signet-ring and bipolar forms (fig. 7). In some preparations there were also smaller granular forms and occasional short filaments.

Electron-microscopic examination. Thin sections were prepared from pellets of centrifuged cultures of strain no. 462/2 and two other cloned strains. When examined with the electron microscope at low magnification, the predominant forms were coccoid and varied in diameter between about 500 and 1500 nm (fig. 8). In addition, there were many somewhat smaller bodies and also some very small particles about 150–200 nm in diameter. The density of the internal structure varied according to the size of the particles, the smallest forms being the most dense. When viewed at higher magnification, the bodies were seen to possess a triple-layered cell membrane, but none showed any evidence of a cell wall (fig. 9). Some showed an outer "fringe" of amorphous material, somewhat similar to that seen in negatively stained electron micrographs of recognised mycoplasmas (Reuss *et al.*, 1967).

Metabolic characteristics. No. 462/2 and two other cloned strains were examined for their ability to grow without serum. Broth cultures were titrated in GS broth with and without serum and, after appropriate periods of incubation, the presence of growth was assessed by noting colour change in the broth cultures and by plating from appropriate tubes on GS agar. No growth was obtained at any dilution from the broths lacking serum, whereas growth occurred to a titre of 10^9 in the broths with serum. These strains also failed to grow on GS agar when serum was omitted from the medium.

In contrast with their ready growth at 33°–37°C, the same strains failed to grow when inoculated into GS fluid or on solid GS media and incubated at 22°C.

In a modified GS broth that contained no added glucose and in which glucose had been omitted from the Hanks' solution, no. 462/2 and the two other test strains grew well and produced a slight reduction in pH. There was no detectable utilisation of arginine or urea by the three test strains when they were grown in glucose-free GS medium to which 0.1 per cent. of either substrate had been added. These cultures also showed a slight acid shift of pH.

Filtration experiments. To determine the order of size of the minimal reproductive units of the "atypical" strains, cultures of no. 462/2 and two other cloned strains were filtered in parallel with a representative mycoplasma, *M. laidlawii* (PG8) and a representative bacterial L-phase organism, the stable L form of *Streptobacillus moniliformis* (L1).

NEW MYCOPLASMA FROM CALF LUNGS

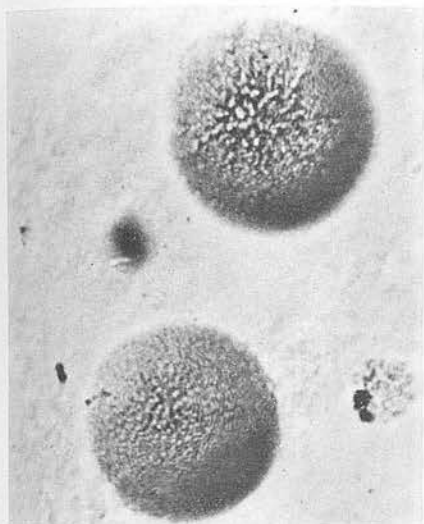


FIG. 1.—Colonies of recently isolated strain of "atypical" mycoplasma. Oblique transmitted light. Glucose calf-serum (GS) agar. Unstained. $\times 100$.

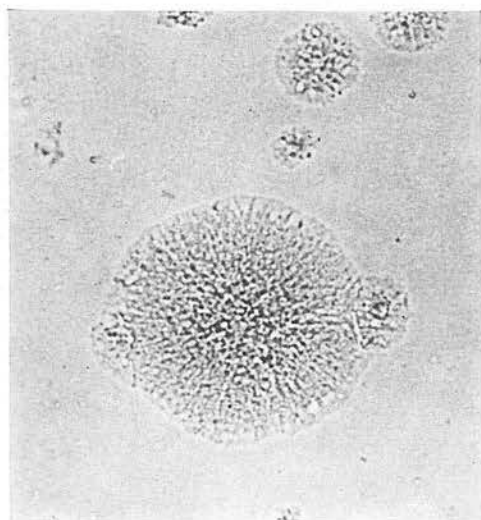


FIG. 2.—Colonies of recently isolated strain. Transmitted light. GS agar. Unstained. $\times 200$.

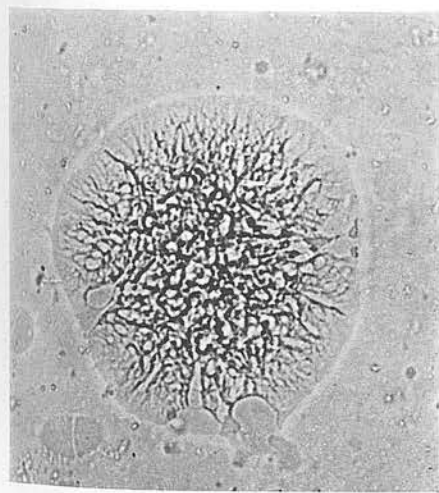


FIG. 3.—Colonies of recently isolated strain. Transmitted light. Cambridge agar. Unstained. $\times 180$.

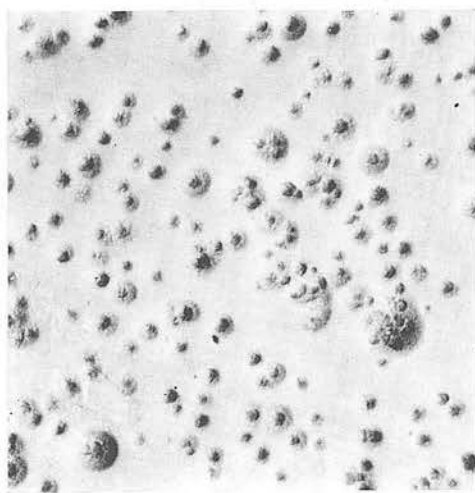


FIG. 4.—"Centre-forming" colonies. Oblique transmitted light. GS agar. Unstained. $\times 70$.

FIGS. 1-4.—The strain illustrated in these and subsequent figures is no. 462/2.

NEW MYCOPLASMA FROM CALF LUNGS

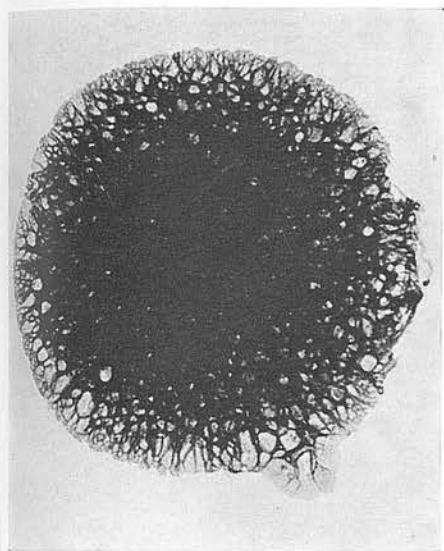


FIG. 5.—Large colony without "centre". Whole-colony preparation. Cambridge agar. Giemsa. $\times 120$.

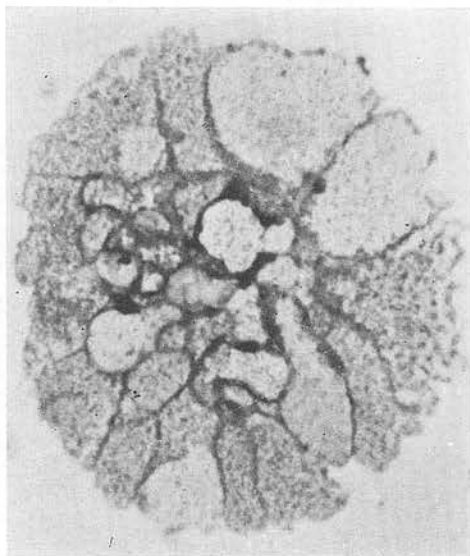


FIG. 6.—Small colony without "centre". Whole-colony preparation. Cambridge agar. Giemsa. $\times 1330$.

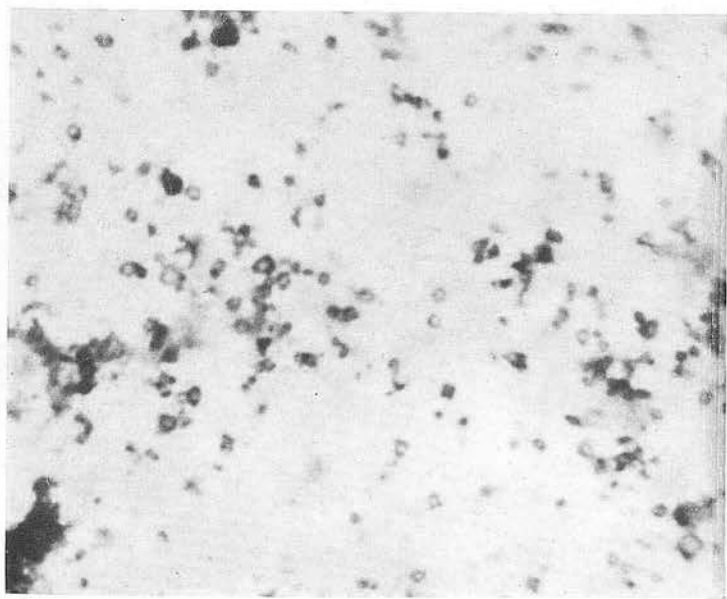


FIG. 7.—Light micrograph. Centrifuged deposit of culture in Cambridge broth. Giemsa. $\times 2000$.

NEW MYCOPLASMA FROM CALF LUNGS

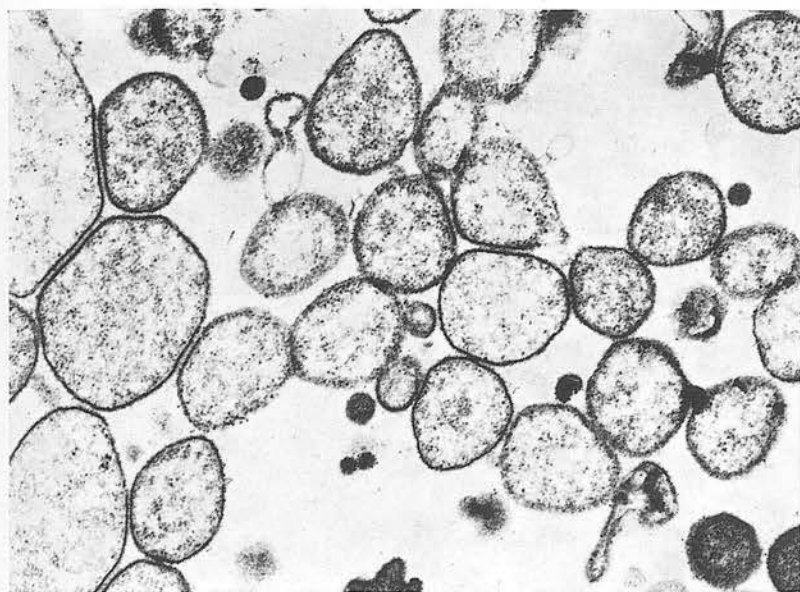


FIG. 8.—Electron micrograph. Section of centrifuged deposit of GS broth culture. $\times 23,000$.

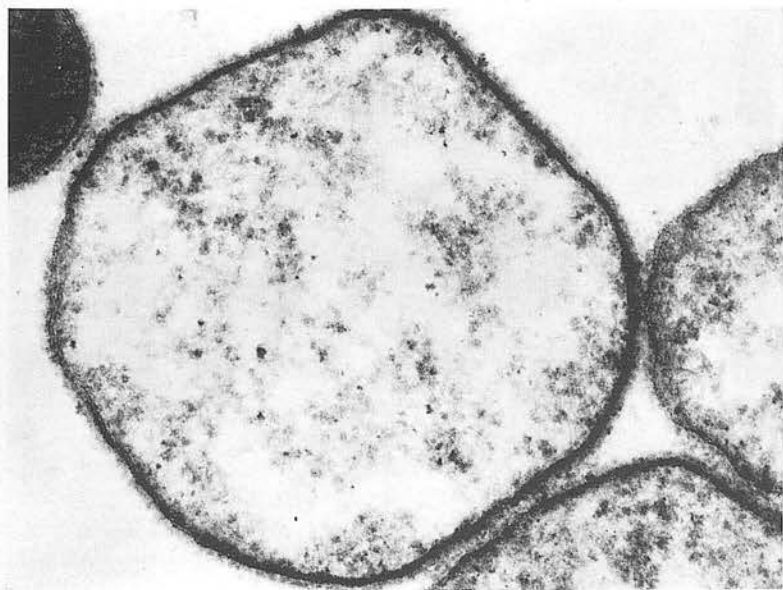


FIG. 9.—Electron micrograph. Section of centrifuged deposit of GS broth culture. $\times 83,700$.

Two experiments were carried out (see *Materials and methods*). In one (experiment A) the organisms were grown in GS medium and in the other (experiment B) the medium used was Cambridge broth. Cultures were filtered through membranes of varying p.d. In each experiment, all organisms were

TABLE II

Effect of filtration on the titres of viable organisms in broth cultures of "atypical" strains of mycoplasma and other organisms

Expt	Organism and number	Age of culture (days)	Log ₁₀ titre (CCT) before filtration	Reduction in log ₁₀ titre after filtration through membranes of p.d.			
				800 nm	650 nm	450 nm	220 nm
A*	Mycoplasma 462/2	†	8.5	...	2.5	5	>7.5
	V7/A/1	†	8.0	...	3	6	>7
	D2/A/3	†	7.5	...	3	5	>6.5
	<i>Mycoplasma laidlawii</i> PG8	†	10.0	...	0	0.5	6.5
	<i>Streptobacillus moniliformis</i> L phase (L1)	†	6.0	...	0.5	1.5	>5
B*	Mycoplasma 462/2	1	7	0	...	4	...
		2	7	2	...	>6	...
		4	8	2	...	7	...
		8	7	2	...	>6	...
	D2/A/3	2	7	1	...	>6	...
	<i>Mycoplasma laidlawii</i> PG8	1	8	0	...	0	...
		3	9	0.5	...	2	...
	<i>Streptobacillus moniliformis</i> L phase (L1)	2	7	0	...	2	...
		3	5	1	...	1	...

* For experimental details see *Materials and methods*.

† These GS cultures were filtered at a stage when a significant colour change had just appeared (usually 3–5 days).

grown, filtered and titrated in the same medium. The results of titrations of cultures before and after filtration are shown in table II.

In the case of the "atypical" strains no. 462/2 and D2/A/3, filtration through 800 nm p.d. membranes caused a fall in titre of a hundredfold or less (experiment B); after filtration through 650 nm p.d. the fall was between one hundred and one thousandfold (experiment A). In both experiments, filtration through the 450 nm membrane caused the titre to drop by 10⁴ or more. Less than one-millionth of the titre remained after filtration through filters of 220 nm p.d. (experiment A).

In contrast, the cultures of *M. laidlawii* and the L-phase organism passed the 800nm and 650nm filters with little loss of titre. The 450nm filter caused reduction in titres of about a hundredfold or less, whilst the quantitative effect of the 220nm filter was comparable with that obtained with the "atypical" strains.

With all these strains, filtration through membranes of decreasing nominal pore diameter caused a progressive diminution in titre. In this respect, the results were similar to those noted elsewhere for mycoplasmas and bacterial L forms, both of which are notably pleomorphic. Most viruses and bacteria, on the other hand, have a relatively uniform size, and show a much sharper "cut-out" effect around the limiting filter pore size (Van Boven, Ensering and Hijmans, 1968).

Serological studies. Antiserum prepared in rabbits against strain no. 462/2 was used in GI tests. The growth of 23 of the 36 available "atypical" strains was inhibited clearly by this antiserum and not by serum from an uninoculated rabbit. Fourteen of these strains, including no. 462/2, were also tested against *M. bovirhinis* antiserum with negative results. The strains reacting with no. 462/2 antiserum included cloned strains no. 462/2, D2/A/3 and V7/A/1. In addition to the above "atypical" strains, the three similar strains that had been isolated from lungs in medium free of penicillin and thallium acetate were also clearly inhibited by the no. 462/2 antiserum. The serological identity of the 13 remaining "atypical" strains has not yet been determined.

The reactivity in GI tests of the "atypical" strains could often be enhanced by rapid passage in GS broth before testing. For most recently isolated strains the zone of inhibition caused by the no. 462/2 antiserum was usually only slight or absent, and if present was seen only as a band of reduced colony size. After passage, a zone of definite inhibition was usually observed extending to 3-4 mm around the paper disk; this appeared to be correlated with the development of centre-forming colonies. The 13 unidentified strains are being examined and further passages may result in their being able to react with the no. 462/2 antiserum in the GI test.

The serological relationship between strains no. 462/2, D2/A/3 and V7/A/1 was confirmed in MI tests with antiserum to strain no. 462/2, although the heterologous strains did not react to full titre. The homologous titre for this antiserum varied between 1 in 80 and 1 in 320 in individual tests. Strain no. D2/A/3 reacted at either full or half titre; strain no. V7/A/1 reacted at one-half to one-eighth of the homologous titre. This degree of antigenic difference between serologically related strains was not unexpected and is consistent with our experience and that of other workers (Taylor-Robinson and Berry, 1969) using the MI test to examine a number of strains within a single species of *Mycoplasma*.

Further MI tests with strains no. 462/2 and D2/A/3 were carried out against antisera to the 8 recognised mycoplasmas found in cattle (Leach), and to 17 other mycoplasma species. With the partial exception outlined below, all gave negative results (table III), indicating that strains no. 462/2 and D2/A/3 can be distinguished from known bovine mycoplasma species and from most

of the commonly encountered mycoplasmas found in goats, pigs, dogs, rodents, poultry and man. The exceptional finding was that the two strains gave cross-reactions with antisera to one strain of *M. pulmonis* (no. 880), but this was not confirmed with antisera to two other strains of *M. pulmonis* (strains Kon and Negroni) nor by a reciprocal test of *M. pulmonis* (strain no. 880) against anti-serum to no. 462/2.

TABLE III

Metabolic inhibition (MI) tests with "atypical" strains against antisera to recognised bovine and other mycoplasma species

Species of <i>Mycoplasma</i> against which rabbit serum was produced	Titre of serum produced against stated strain in MI tests against		
	homologous organism	" atypical strain "	
		no. 462/2	no. D2/A/3
<i>Mycoplasma</i> , atypical strain (462/2)	80*	80*	80
<i>Mycoplasma mycoides</i> var. <i>mycoides</i> (PG1)	160	<10	<10
<i>M. bovirhinitis</i> (PG11)	2560	<10	<10
<i>M. laidlawii</i> (PG8)	80	<10	<10
<i>M. bovirhinitis</i> (5M331)	20,000	10	<10
<i>M. agalactiae</i> var. <i>bovis</i> (<i>M. bovimastitidis</i>) (Widanka)	20,000	<10	<10
<i>Mycoplasma</i> sp.; group 6 (Squire)	20,000	<10	<10
<i>Mycoplasma</i> sp.; group 7 (N39)	1280	<10	<10
<i>Mycoplasma</i> sp.; group 8 (D12)	80,000	10	<10
Other mycoplasma species†	All >160	<10	<10

* Homologous titres up to 320 were obtained in tests other than those used for this table.

† The following strains were examined: *M. pneumoniae* (FH), *M. fermentans* (PG18), *M. salivarium* (PG20), *M. orale* type 1 (CH19219), *M. orale* type 2 (CH20247), *M. hominis* (PG21), *M. arthritis* (PG6), *M. neurolyticum* (KSA), *M. gallisepticum* (X95), *M. pulmonis* (Kon; Negroni; see also text), *M. gallinarum* (PG16), *M. synoviae* (Lasswade), *M. canis* (PG14), *M. spumans* (PG13), *M. hyorhinitis* (GDL), *M. suisneumoniae* (J), *M. agalactiae* (PG2), *M. mycoides* var. *capri* (PG3).

Deoxyribonucleic acid composition. Mr L. R. Hill reported that the DNA base composition of strain no. 462/2, as determined from the "melting temperature" (T_m) and expressed as moles per cent. guanine + cytosine (per cent. GC), was 28.5 (T_m 81.0°C). A recognised *Mycoplasma* species (*M. mycoides* var. *capri*; PG3) which was tested by the same method gave a value of 24 per cent. GC (Owen, Lapage and Hill, 1969), which conforms with other published findings for this species (Hill, 1966).

DISCUSSION

Recent reports of the value of a special medium for the isolation of *M. suisneumoniae* prompted the use of a similar medium in attempts to isolate mycoplasmas from cattle. As a result, many exacting glucose-fermenting organisms were isolated from calf lungs in an enriched glucose calf-serum medium. These strains produced colonies on solid media that were somewhat

atypical in comparison with those of most recognised mycoplasmas. They grew very poorly in conventional mycoplasma media and were easily differentiated from known mycoplasma species, including *M. bovirhinis*, which was also isolated from some lungs by the same methods.

The majority of "atypical" strains isolated were shown to be antigenically related by means of the growth and metabolic inhibition tests. Preliminary studies with three of these strains (Gourlay, unpublished observations) suggest that they also give almost identical electrophoretic patterns when examined by the polyacrylamide gel technique as used by Razin and Rottem (1967). The remaining unidentified strains are still being studied serologically.

The new type of organism is distinct from bacteria in its size, pleomorphism and lack of a cell wall, as demonstrated by stained films and electron micrographs. It also differs from most bacteria by being resistant to both penicillin and thallium acetate and in showing growth inhibition by specific antiserum in the absence of complement. These properties, together with a dependence on serum for growth, suggested that the new organisms were either mycoplasmas or bacterial L-phase organisms (L forms). On the special agar media used, their granular or slightly "lacy" colonies were somewhat more reminiscent of L-forms than of mycoplasma colonies. This similarity was maintained in the failure of "atypical" strains to pass through Millipore filters of 450 nm p.d. without considerable loss of titre ($>10^5$). Other observations made under the same conditions (Andrews and Leach, unpublished observations) confirmed those of Klieneberger-Nobel (1962, pp. 63-68), in that cultures of recognised mycoplasma species usually passed the 450nm filter with little loss of viability, whereas the titres of several bacterial L forms suffered a much greater reduction (usually $>10^3$) than was observed for the L1 organism in the present tests. "Atypical" strains were actually *less* readily filtrable than the L-form when tested concurrently, but their filterability may be influenced to some extent by the tendency of the organisms to form clumps in the fluid media used.

Electron micrographs of no. 462/2 and of two other strains revealed numerous particles, approximately 150-200 nm in diameter, with many other roughly spherical forms up to about 1500 nm in diameter. The dimensions and general morphology of these particles are similar to those often reported for mycoplasmas. The microscopic appearance of "atypical" strains in Giemsa-stained broth deposits, with "ring" and other pleomorphic forms predominating, are also characteristic of many mycoplasmas.

The strongest evidence that the new organisms are mycoplasmas rather than L-phase bacteria is that three strains, serologically and otherwise indistinguishable from the prototype strain no. 462/2, were isolated without the use of antibacterial substances from the original lung material by inoculation directly into GS broth. One of these strains was from a calf that is known never to have been treated with antibiotics at any stage of life. In addition, none of these strains nor the prototype strain showed any evidence of reversion to bacterial forms after subculture in media free of bacterial inhibitors.

Further evidence in favour of the new isolates being mycoplasmas was provided by their DNA base composition. The figure of 28.5 per cent. GC

obtained for the DNA base ratio for the prototype strain no. 462/2 is outside the range quoted for *Pasteurella*, *Corynebacterium*, *Escherichia*, *Streptococcus* and *Staphylococcus* (Hill, 1966), the bacterial genera found in routine bacterial examinations of the lungs from which the "atypical" strains were also isolated. This figure is lower than those recorded for most bacteria (Hill) with the exception of the genus *Spirillum* and some *Clostridium* and *Vibrio* species, but is within the narrow range (26-32 per cent.) observed for recognised mycoplasma species so far tested (Bak and Black, 1968).

It is reasonable to conclude that, although they have some atypical characters, the organisms represented by strain no. 462/2 form a new mycoplasma species. The serological studies suggest that this species is distinct from other mycoplasmas hitherto isolated from cattle and from all other recognised mycoplasma species so far tested.

In our experience the cultural and colonial characteristics of the "atypical" calf strains are similar to those of the pig mycoplasma, *M. suipneumoniae*. Although serologically distinct from *M. suipneumoniae*, the new species grows satisfactorily on media developed for the pig mycoplasma and poorly on conventional mycoplasma media. In our hands, both organisms were difficult to grow from small inocula, suggesting that further study of their growth requirements is needed. We have also isolated numerous strains with somewhat similar cultural characteristics from the respiratory tract of sheep. The relationship between these three groups is currently being studied at Colindale, and it can be said at this stage that they do not show cross-reactions in growth inhibition tests. Further work may be necessary to establish a final classification for the new calf strains, especially in view of recent proposals to subdivide the Mycoplasmatales into further taxonomic groups (Furness, Pipes and McMurtrey, 1968; Edward and Freundt, 1969). However, we would at present regard the "atypical" calf strains as forming a single new species of the genus *Mycoplasma*, within the existing family Mycoplasmataceae. We propose the name of *Mycoplasma dispar* for the new species and designate strain no. 462/2 as the type strain. The specific epithet *dispar* was chosen to indicate the atypical characteristics of the species ("dispar": Latin adjective meaning "different" or "dissimilar"). A culture of strain no. 462/2 has been deposited in the National Collection of Type Cultures, Colindale, under the number NCTC10125.

The significance of the new mycoplasma species as a possible cause of calf pneumonia is unknown at present. Although these strains were isolated only from pneumonic and not from normal calf lungs, this was also true of *M. bovirhinis*. Moreover, many T-strain mycoplasmas were isolated from the same series of lungs on appropriate media (Gourlay, 1968). Experimental work on the pathogenicity of these mycoplasmas is in progress at Compton and will be reported separately.

SUMMARY

This paper describes the isolation and characterisation of a new mycoplasma species for which the name *Mycoplasma dispar* is proposed. Many strains of

this glucose-fermenting organism were isolated in an enriched glucose calf-serum medium from pneumonic lungs of calves, but not from healthy lungs of calves or cows. It could be isolated in medium free of known bacterial inhibitors, and when subcultured in this medium showed no evidence of reversion to bacterial forms.

The general characteristics of these strains were those of mycoplasmas; these included cultural and metabolic characters, morphological appearance in stained films and electron micrographs, growth inhibition by specific antiserum and a DNA base composition of 28.5 per cent. GC. However, the group showed some unusual features, including failure to pass readily through membrane filters of 450 nm pore diameter, poor growth in conventional mycoplasma media and production of atypical colonies on GS agar medium.

The serological identity of strains classified as *M. dispar* was demonstrated by growth and metabolic inhibition tests. They were serologically distinct from other recognised bovine mycoplasmas and from a wide range of mycoplasmas from other sources.

We wish to thank Mr L. R. Hill, National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, N.W.9, for carrying out the DNA extraction and base-composition estimation, Miss J. Bruce for the electron micrographs, Mr I. Jebbett for the photographs used in figs. 1 and 4 and Miss Sara Wyld and Mr P. G. Bradley for technical assistance. We are also very grateful to Dr B. E. Andrews for preparing and photographing many of the preparations, including those used in figs. 2, 3, 5, 6 and 7, and for his advice regarding some of the experimental work and during the preparation of the paper.

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**THE ISOLATION OF T-STRAINS OF MYCOPLASMA
FROM PNEUMONIC CALF LUNGS**

BY
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The Isolation of T-Strains of Mycoplasma from Pneumonic Calf Lungs

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SUMMARY. *T*-strains of mycoplasma were isolated from 9 out of 16 pneumonic calf lungs. Their role in the pathogenesis of pneumonia has not yet been investigated.

T-STRAINS of mycoplasma have been isolated from the human urogenital tract (Shepard, 1954; Ford, Rasmussen and Minken, 1962; Shepard, Alexander, Luncsford and Campbell, 1964) and oropharynx (Taylor-Robinson and Purcell, 1966), and the urogenital tract of cattle (Taylor-Robinson, Haig and Williams, 1967). This report describes the isolation of T-strains of mycoplasma from pneumonic lungs of calves.

METHODS

Media

The liquid medium containing 0.1% urea described by Taylor-Robinson *et al.* (1967) was used, and thallium acetate (1:4000) and penicillin (1000 i.u./ml.) or penicillin alone were included in most batches. Solid medium consisted of the broth, with the addition of 0.65% Agarose*.

Isolation Procedure

Pieces of pneumonic lung obtained from calves at autopsy were triturated in phosphate buffered saline (pH 7.4) and inoculated into broth with and without thallium acetate in 4 serial ten-fold dilutions.

Growth produced an alkaline shift of pH from metabolism of urea and was indicated by a colour change of phenol red in the broth. From the highest dilution in which this change occurred a drop of culture was placed on solid medium. When a similar colour change was observed in the solid medium, the surface of the medium was examined for T-strain mycoplasma colonies under a stereoscopic or conventional microscope at $\times 100$ magnification. Occasionally, the lung material was inoculated directly on to solid medium and although a colour change could be seen following incubation it was usually difficult to identify the T-strain colonies due to the debris from the inoculum. It was necessary in these cases to transfer an agar block from the solid medium into broth and to examine the resulting culture for T-strain colonies by sub-inoculation on to solid media.

All incubations were at 37°C. and solid medium plates were placed in a mixture of 5% CO₂ in nitrogen.

Isolation in the Absence of Bacterial Inhibitors

In two instances, triturated lung material was inoculated directly on to solid medium free of penicillin and thallium acetate. When a colour change was observed, a piece of solid medium showing the colour change, and as far as possible free from visible bacterial colonies, was removed and placed in broth containing penicillin and thallium acetate. When this broth

* Seravac Laboratories Ltd., Holyport, Maidenhead, Berks.

showed colour change a drop was placed on solid medium and examined for T-strain colonies after incubation.

RESULTS

T-strains of mycoplasma were isolated from 9 of 16 calf lungs examined. Seven of the isolates were from calves between 6 and 12 months of age and the remaining 2 were from calves 14 and 30 days old. All lungs showed macroscopic lesions of pneumonia generally involving more than one lobe.

In broth, colour changes were visible usually after 1 to 4 days, although occasionally as late as 10 days, after commencing incubation. The changes started at the lowest dilution and extended progressively to the highest dilution. Titrations of triturated lung material generally produced growth up to the 10^{-4} dilution, which was the highest dilution used in routine isolations. The colour change in the first tube of the titration was usually less intense than in the subsequent tubes. The presence of thallium acetate in the medium had an initial inhibitory effect indicated by a delay in growth in all dilutions when compared with the medium without thallium acetate. However, the final titre was usually as high with or without thallium acetate.

T-strain colonies on solid medium were minute, irregularly circular, convex or slightly conical, with a finely granular surface, a crenated edge and a small but well defined central nipple. The diameter of the colonies varied from 10–50 μ after 1 to 3 days growth, and it did not increase further in size on prolonged incubation.

Large-colony mycoplasmas were frequently isolated from the same lungs as the T-strains, also occasionally in the

same liquid and on the same solid medium. However, the large-colony mycoplasmas produced an acid and not an alkaline reaction, so they could be easily distinguished, although occasionally the initial colour change produced by growth of T-strain mycoplasmas from acid to alkali would later be reversed to acid, necessitating frequent examination of the media.

DISCUSSION

The organisms were identified as T-strain mycoplasmas on the morphology and size of their colonies, their ability to metabolize urea rapidly and because they could be isolated in the absence of bacterial inhibitors.

There is no evidence at present that T-strain mycoplasmas cause disease in either man or cattle, and we have no evidence that the T-strain mycoplasmas isolated from pneumonic lung play any part in the pathogenesis of pneumonia, but as they were isolated in large numbers this possibility will be investigated.

The less intense colour change, usually observed in the first tube of an isolation titration, perhaps indicates an inhibition due to cellular or humoral material in the inoculum, and suggests that the titration method of primary isolation may be more sensitive than a single tube method.

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The Isolation of Large Colony and T-strain Mycoplasmas from cases of Bovine Kerato-Conjunctivitis

Sir,—Infectious bovine kerato-conjunctivitis is common in cattle throughout the world and although numerous agents have been isolated from infected eyes including bacteria, viruses, rickettsia-like organisms and psittacosis agents, there is still no general agreement as to the aetiology of the condition (Wilcox, 1968). This report describes the isolation of large colony mycoplasmas and T-stain mycoplasmas (T-mycoplasmas) from cases of kerato-conjunctivitis (New Forest Eye) in cattle from three farms in Berkshire.

Two swabs were taken from infected eyes and were placed in glucose serum broth (Gourlay & Leach, in preparation) for the isolation of glucose-fermenting, large colony mycoplasmas and urea broth as described by Taylor-Robinson, Haig and Williams (1967) but with 1:4,000 thallium acetate, for the isolation of urea-fermenting T-mycoplasmas. The fluid in the swabs was expressed by means of sterile forceps and from this initial broth, designated 10^{-1} , serial ten-fold dilutions up to 10^{-6} were prepared in the same type of broth. From the highest dilution of broth showing growth (end point titre) after incubation, a drop of culture was placed on solid medium prepared from the corresponding broth by the

addition of 0.65 per cent. Agarose* and after incubation the surface was examined for colonies. In the case of the urea broths a further series of ten-fold dilutions were made prior to incubations onto solid medium. Incubations were at 37° C. and solid medium plates were placed in a mixture of 5 per cent. CO₂ in nitrogen. Large colony mycoplasmas were cloned by picking four single colonies and each one was passed three times on solid medium and the cloned organisms were examined by the disc growth inhibition (GI) test (Clyde, 1964).

Mycoplasmas were isolated from all 20 cases examined, details of which are given in Table 1. Large colony mycoplasmas were isolated from all cases except Nos. 4 and 20 and the mycoplasmas from 10 of these cases were examined by the disc growth inhibition test. From three of these cases *Mycoplasma bovirhinis* (see Leach, 1967) was isolated, from one case *M. laidlawii*, and from two cases both *M. bovirhinis* and *M. laidlawii*. From the remaining four cases mycoplasmas were isolated which preliminary GI tests indicate are probably not one of the recognised strains of bovine origin. T-mycoplasmas were isolated from all cases except No. 18. The high titres of mycoplasmas were generally obtained from severe cases and titres of T-mycoplasmas were generally higher than those for large colony mycoplasmas. No large colony mycoplasmas were isolated from the eyes of six healthy calves.

In addition to mycoplasmas, *Neisseria* sp. and *Moraxella bovis* were also isolated from 9 out of 10 cases (Nos. 11 to 20) examined bacteriologically. It is of interest that large colony mycoplasmas, T-mycoplasmas, *Neisseria* sp. and *M. bovis* were isolated from a number of cases which were refractory to topical treatment both with chloramphenicol/neomycin and with chlortetracycline.

There is no evidence at present that these mycoplasmas cause disease in animals, but the fact that they were frequently present in large numbers, particularly the T-mycoplasmas, in cases of kerato-conjunctivitis and not in healthy calves makes it possible that they play a rôle in this condition.

Further work is in progress which will be published later.

January 21st, 1969.

Yours faithfully,
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* Seravac Laboratories Ltd., Holyport, Maidenhead, Berkshire.

TABLE I
ISOLATION OF MYCOPLASMAS FROM CASES OF
KERATO-CONJUNCTIVITIS IN CALVES

Calf No.	Mycoplasmas isolated			
	Large colony mycoplasmas	Titre	T-mycoplasmas	Titre
1	+	ND	+	ND
2	+	ND	+	ND
3	+	ND	+	ND
4	0		+	ND
5	+	10^3	+	10^3
6	+	10^{6+}	+	10^2
7	+	10^3	+	10^3
8	+	10^3	+	10^4
9	+	10^3	+	10^4
10	+	10^4	+	10^5
11	+	10^1	+	10^1
12	+	10^1	+	10^5
13	+	10^1	+	10^{6+}
14	+	10^3	+	10^5
15	+	10^2	+	10^5
16	+	10^2	+	10^{6+}
17	+	10^1	+	10^{6+}
18	+	10^2	0	
19	+	10^2	+	10^5
20	0		+	10^5

ND Not done

* Highest dilution used

† *Mycoplasma bovirhinis* by growth inhibition test

‡ *Mycoplasma laidlawii* by growth inhibition test

§ Unidentified mycoplasma by growth inhibition test

The initial froth into which each eye swab was taken was designated 10^{-1} dilution.

STUDIES OF THE MICROBIOLOGY AND PATHOLOGY OF PNEUMONIC LUNGS OF CALVES

By

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INTRODUCTION

The aetiology of pneumonia in calves under intensive husbandry conditions is complex and numerous infectious agents have been recovered from the lungs and other tissues of calves suffering from respiratory infections. Comprehensive reviews of agents isolated from bovine lungs are those by Omar (1966) and Darbyshire and Roberts (1968). Many bacterial species have been isolated, but experimentally their significance as primary agents in pneumonia is not clear, although in field cases the lesions frequently appear to be attributable to infection with bacteria, especially *Pasteurella* spp. Viruses, particularly parainfluenza 3 virus (PI3), reovirus, adenovirus and infectious bovine rhinotracheitis virus have been shown to be capable of producing transient pneumonia in calves. The same applies to the chlamydia group of organisms. Mycoplasmas other than *Mycoplasma mycoides* var. *mycoides*, the causal organism of contagious bovine pleuropneumonia, have been isolated on numerous occasions from the respiratory tract of calves, but their role in respiratory disease is not clear. On the occasions that their pathogenicity has been tested in calves they have not usually produced any significant pneumonic lesions or illness.

As a preliminary study of pneumonia in calves, 65 lungs showing macroscopic areas of pneumonia were examined from calves under 1 year of age for the presence of bacteria, mycoplasmas, chlamydiae and viruses and observations on the pathology of the pneumonic areas were made. Earlier communications reported the isolation of T-mycoplasmas (Gourlay, 1968) and a new mycoplasma species, *Mycoplasma dispar* (Gourlay, 1969; Gourlay and Leach, 1970) from pneumonic calf lungs that were part of this survey. This paper gives full details of isolations made during the survey together with descriptions of lesions.

MATERIALS AND METHODS

Lung material. Pneumonic lung material was obtained from 2 different groups of calves between June 1967 and December 1968. The first and larger group comprised 45 apparently healthy 3-month old veal calves from an abattoir which collected calves for slaughter from an area extending from North Cornwall to Hampshire. The second group, from Berkshire farms, comprised 20 calves of varying ages which had died or had been killed in extremis and which at autopsy were found to have pneumonia. Severe illness or death may not have been due in all instances to pneumonia.

Mycoplasma media. Glucose serum broth (Gourlay and Leach, 1970) was used for

the isolation of glucose-fermenting mycoplasmas; for the isolation of T-mycoplasmas the liquid medium containing 0.1 per cent. urea described by Taylor-Robinson, Haig and Williams (1967) was used, including thallium acetate (1:4000) and penicillin 1000 i.u./ml. For the isolation of arginine-metabolizing mycoplasmas the T-strain medium was used except that 0.1 per cent. arginine replaced the urea and the pH was adjusted to 7.0. Solid medium consisted of the liquid medium with the addition of 0.65 per cent. agarose (Seravac Laboratories, Holyport, Berkshire).

Cell culture. Primary calf kidney and pig kidney monolayers were used as well as coverslip preparations of these cells. Lung explants were prepared using a modification of the method described by Rogers, Basnight, Gibbs and Gadjusek (1967).

Examination for mycoplasmas. Pieces of pneumonic lung (about 0.5 cm³.) were triturated in 5 ml. phosphate buffered saline, pH 7.4, and 0.2 ml. was inoculated into 1.8 ml. amounts of various liquid media, in serial ten-fold dilutions. Glucose-fermenting mycoplasmas were isolated and identified as described by Gourlay and Leach (1970). T-mycoplasmas were isolated and identified as described by Gourlay (1968). Isolation procedures were repeated in all instances on lung material that had meanwhile been stored at -70°C., for confirmation of positive or negative results.

Examination for bacteria. Triturated lung material was inoculated on to ox blood agar, 10 per cent. chocolate agar and MacConkey agar plates, and incubated aerobically at 37° C. A duplicate ox blood plate was incubated anaerobically and another chocolate agar plate was incubated in a mixture of 5 per cent. CO₂ in air. Colonies were identified by the methods of Cowan and Steel (1965). A few colonies of known non-pathogenic organisms were not considered significant and were not recorded.

Examination for viruses. Triturated lung material was added to 100 ml. modified Eagle's medium containing 5 per cent. foetal calf serum, penicillin 100 i.u./ml. streptomycin 100 µg./ml. kanamycin 100 µg./ml. and nystatin 25 units/ml. and after 1 hour was centrifuged at 2,000 r.p.m. for 5 min. and the supernatant fluid used for cell culture inoculations. Pieces of lung were also used for the preparation of "explants". A piece of pneumonic lung was chopped into small pieces and washed, a little Eagle's medium being added to facilitate chopping. With a Pasteur pipette small pieces of lung were removed and placed on to the side of a 1 oz. "medical flat" which was left to stand for 1 hour at room temperature and then 3 ml. of the Eagle's medium containing 5 per cent. foetal calf serum were added. The flask was placed on its side at 37°C. and examined after 2 to 3 days to see if cells, usually fibroblast in type, were growing. The medium was changed twice a week. Such explants could be kept alive for months, secondaries being prepared, by trypsinisation when necessary. Subinoculations from the explants were made on to monolayers. All preparations were examined for cytopathogenic effect and by haemadsorption tests, and supernatant fluids were used for haemagglutination tests. Lung material was passed through monolayers twice and often 3 times before being reported as negative.

Examination for chlamydiae. Triturated lung material was treated by the method of Wilson and Thomson (1968) with the following modifications. Streptomycin 2 mg./ml. kanamycin 0.5 mg./ml. and nystatin 100 units/ml. were added to the lung supernatant dilutions and 3 six day old chick embryos were inoculated into the yolk sac with each dilution. Embryos dying on or after the third day were examined for bacteria and for the presence of chlamydiae. Six passages were made with material from each lung before it was considered negative for chlamydiae.

Pathology and histopathology. A record by means of drawings was made of the extent of all the pneumonic areas. Representative portions of pneumonic lesions were taken from each calf at autopsy or at the abattoir and placed in neutral buffered 12 per cent. formalin for histopathological examination. Paraffin sections were prepared and stained by haematoxylin and eosin, azure eosinate, Giemsa, Gordon and Sweet's method for reticulin, Verhoeff-Van Gieson method for elastin and collagen and by

TABLE 1
PATHOLOGICAL LESIONS AND MICROORGANISMS ISOLATED FROM PNEUMONIC LUNGS OF 3 MONTH OLD APPARENTLY HEALTHY CALVES

Calf No.	Pathological lesions					Histopathological†				Microorganisms isolated			
	Macroscopic consolidation*	Alveolar collapse	Alveolar cells	Giant cells	Bronchiolitis	Broncho-pneumonia	Lymphoid hyperplasia	M. bovirhinis	M. dispar	T-Mycoplasmas	Bacteria		
1	+	-	+	-	++	++	+		10 ⁶	10 ²	Past. h.		
2	+	++	+	+	++	++	+		10 ⁵	10 ²	Past. h-l.		
3	+	++	+	+	++	++	+		10 ³	10 ³	Past. h.		
4	+	++	+	+	++	++	+		10 ⁴	10 ⁴	Past. h-l.		
5	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h.		
6	+	++	+	+	++	++	+		10 ⁴	10 ⁵	Past. h.		
7	+	++	+	+	++	++	+		10 ³	10 ²	Past. h.		
8	+	++	+	+	++	++	+		10 ³	10 ²	Past. h.		
9	+	++	+	+	++	++	+		10 ³	10 ²	Past. h-l.		
10	+	++	+	+	++	++	+		10 ³	10 ²	Past. h-l.		
11	+	++	+	+	++	++	+		10 ⁵	10 ⁶	Past. h-l.		
12	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
13	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
14	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
15	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
16	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
17	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
18	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
19	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
20	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
21	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
22	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
23	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
24	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
25	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
26	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
27	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
28	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		
29	+	++	+	+	++	++	+		10 ⁴	10 ⁶	Past. h-l.		

Table 1—continued

	<i>Past. h-l.</i>	<i>Past. h-l.</i> <i>S. epidermidis</i>	<i>Past. m.</i> <i>Past. m.</i> <i>Past. m.</i> <i>Past. h.</i> <i>C. pyogenes</i>	<i>S. epidermidis</i> <i>Past. h-l.</i> <i>Micrococcus sp.</i>
30	+	+	10 ⁵	10 ⁵
31	+	+	10 ⁴	10 ⁴
32	+	+	10 ⁵	10 ⁵
33	+	+	10 ⁴	10 ⁴
34	+	+	10 ⁵	10 ⁵
35	+	+	10 ⁴	10 ⁴
36	+	+	10 ⁵	10 ⁵
37	+	+	10 ⁴	10 ⁴
38	+	+	10 ⁵	10 ⁵
39	+	+	10 ⁴	10 ⁴
40	+	+	10 ⁵	10 ⁵
41	+	+	10 ⁴	10 ⁴
42	+	+	10 ⁵	10 ⁵
43	+	+	10 ⁴	10 ⁴
44	+	+	10 ⁵	10 ⁵
45	+	+	10 ⁴	10 ⁴

... depending on number of lobes involved.

† Graded — (negative) to + + + according to severity.

* Extent graded + to +++ depending on number. † Colour change units (0.2 ml.
 ‡ Graded - (negative) to +++ according to severity.
 Past. h. = *Pasteurella haemolytica* var. *haemolytica* Past. h-l. = *Pasteurella haemolytica* - like organisms
 Past. m. = *Pasteurella multocida*

Lendrum's phloxine tartrazine technique for the demonstration of inclusion bodies (Lendrum, 1949).

RESULTS

Examination of Pneumonic Lungs from Apparently Healthy Calves (Group 1)

Microorganisms isolated. Details of the microorganisms isolated from 45 pneumonic lungs from apparently healthy calves are given in Table 1.

No viruses were isolated from any of the 45 lungs, nor were chlamydiae isolated from a total of 14 of these lungs examined (Nos. 1, 6, 11, 12, 17, 19, 20, 21, 24, 31, 34, 42, 43, 45). *Pasteurella* spp. were isolated from 14 lungs (31 per cent.) As shown in Table 1, these organisms were identified as *Past. multocida* (indole positive), *Past. haemolytica* var. *haemolytica* (urease and indole negative), or *Past. haemolytica*-like organisms. The latter differed from *Past. haemolytica* var. *haemolytica* in requiring serum for growth. *Corynebacterium pyogenes* was isolated from one lung and non-haemolytic *Escherichia coli* was isolated from 2 lungs in significant numbers. A number of other bacteria of doubtful significance were also isolated details of which are given in Table 1. Mycoplasmas isolated from 31 lungs (69 per cent.) comprised 3 different species. Two of these were glucose-fermenting mycoplasmas, (Fig. 1) namely *Mycoplasma bovirhinis* (Leach, 1967) which was isolated from 5 lungs (11 per cent.) and *Mycoplasma dispar* (Gourlay and Leach, 1970) which was isolated from 27 lungs (60 per cent.). The other species was a urea-metabolizing T-mycoplasma which was isolated from 25 lungs (55 per cent.). No arginine-metabolizing mycoplasmas were isolated. Usually 2 and sometimes all 3 different mycoplasma strains were isolated from a single lung.

Pathology and histopathology. It can be seen (Table 1) that the right lung was affected in 44 out of the 45 cases (98 per cent.) whereas the left lung was only affected in 19 cases (42 per cent.). Macroscopic consolidation of lung tissue was frequently found to be associated with varying degrees of collapse or atelectasis. Collapsed alveoli appeared normal in other respects or they had a cellular reaction consisting of infiltration of the alveolar walls and alveolar exudate. Pulmonary collapse was assessed to be a significant feature in 33 out of 45 pneumonic lungs (73 per cent.). In 3 cases there were no other pathological changes in the sections of lung examined; more commonly pulmonary collapse was accompanied by lesions of acute bronchiolitis or by peribronchial lymphoid hyperplasia with stenosis of the bronchi. It was not possible to correlate collapse consistently with bronchial obstruction due to these various lesions although compression collapse associated with extensive lymphoreticular hyperplasia was sometimes evident.

In most of the sections of pneumonic lungs examined there was an alveolar exudate that contained large alveolar epithelial type cells as well as varying numbers of other cells, neutrophils, eosinophils and small round cells. Multi-nucleate cells or giant cells occurred in 27 (64 per cent.) of the 42 lungs (93 per cent.) showing alveolar cell exudate and in 9 cases the formation of giant cell syncytia in alveoli or in bronchioles was a pronounced feature. Bronchiolitis was seen in 34 out of 45 lungs (75.5 per cent.) either as acute bronchiolitis with the lumen of the airway plugged with neutrophils or less frequently as subacute

TABLE 2
PATHOLOGICAL LESIONS AND MICROORGANISMS ISOLATED FROM PNEUMONIC LUNGS OF CALVES THAT HAD DIED
OR BEEN DESTROYED IN EXTREMIS

Calf No.	Age (months)	Macroscopic consolidation*				Histopathological†				Microorganisms isolated			
		Left lung	Right lung	Alveolar collapse	Alveolar cells	Giant cells	Bronchiolitis	Broncho-pneumonia	Lymphoid hyperplasia	Mycoplasmas			Bacteria
										M. bovirhinis	M. dispar	T-strain Mycoplasmas	
1	8	+	+	+	+	+	+	+	+	10 ⁵	10 ⁶	10 ⁴	
2	6	+	+	+	+	+	+	+	+	10 ⁶	10 ⁶	> 10 ³	<i>N. catarrhalis</i>
3	6	+	+	+	+	+	+	+	+	10 ⁶			<i>Past. h.</i>
4	6	+	+	+	+	+	+	+	+				<i>Past. h.</i>
5	5	+	+	+	+	+	+	+	+	10 ⁴	10 ⁴	10 ³	<i>N. catarrhalis</i>
6	3	+	+	+	+	+	+	+	+	10 ³	10 ³	10 ³	<i>Past. h.</i>
7	9	+	+	+	+	+	+	+	+	10 ⁵	10 ⁵	10 ⁴	<i>N. catarrhalis</i>
8	6	+	+	+	+	+	+	+	+	10 ⁵	10 ⁵	10 ⁴	<i>N. catarrhalis</i>
9	1 1/2	+	+	+	+	+	+	+	+	> 10 ⁷		10 ²	<i>Past. h.</i>
10	7	+	+	+	+	+	+	+	+			10 ²	<i>Bacteroides</i> sp.
11	2	+	+	+	+	+	+	+	+	10 ⁴	10 ⁴	10 ⁵	<i>Past. m.</i>
12	9	+	+	+	+	+	+	+	+	10 ⁷	10 ⁷	10 ³	Gram negative bacillus
13	7	+	+	+	+	+	+	+	+	10 ⁶	10 ⁶	10 ²	<i>S. dublin</i>
14		+	+	+	+	+	+	+	+				<i>E. coli</i>
15	11/2	C	+	+	+	+	+	+	+	10 ⁴	10 ⁴	10 ³	<i>Past. m.</i>
16	3	+	+	+	+	+	+	+	+			10 ⁵	<i>C. pyogenes</i>
17	1	C	C	+	+	+	+	+	+				<i>Past. h.</i>
18	3	C	C	+	+	+	+	+	+				<i>S. dublin</i>
19	1	C	C	+	+	+	+	+	+				<i>E. coli</i>
20	3	C	C	+	+	+	+	+	+				

Footnotes as in Table 1. C = Congested lung.

bronchiolitis, in which the lumen contained organising exudate or bronchial ingrowths having a reticulin and collagen framework with neutrophil and round cell reaction; the bronchial wall showed similar inflammatory changes. Transformation of bronchial epithelia to form giant cell granulomas was occasionally seen. Varying degrees of alveolar involvement accompanied bronchial changes, but in only a small number of cases was there extensive fibrinous or purulent alveolitis. On the basis of alveolar or bronchial reactions 28 out of 45 cases (62 per cent.) were classified as showing purulent bronchopneumonia.

Peribronchial lymphoid hyperplasia was found in 34 out of the 45 cases (75.5 per cent.), the remainder being either negative or having only a minimal amount of lymphoreticular tissue in the sections examined. About 50 per cent. of the pneumonic lungs in the series had pronounced follicular lymphoid hyperplasia as seen in "cuffing" pneumonia (Jarrett, McIntyre and Urquhart, 1953). The lesions were not accompanied by corresponding increases in collagenous connective tissue and the reticulin network did not extend into the germinal centres of lymphoid follicles. Examination of at least one section stained by Lendrum's method from each of the pneumonic lungs failed to reveal phloxinophilic inclusion bodies in any of the tissues.

Examination of Pneumonic Lungs from Calves which had Died or been Killed in Extremis (Group 2)

Microorganisms isolated. Details of the microorganisms isolated from 20 pneumonic lungs from calves which had died or been killed in extremis are given in Table 2. No viruses were isolated from any of the lungs nor were any chlamydiae isolated from the 6 lungs examined (Nos. 11, 13, 15, 16, 17 and 19). *Past. haemolytica* var. *haemolytica* and *Past. multocida* were isolated from 4 and 2 lungs respectively. *Neisseria catarrhalis* was isolated from 3 lungs (15 per cent.) and *Salmonella dublin*, *C. pyogenes*, *E. coli* and a *Bacteroides* sp. each from 1 lung. An unidentified gram negative bacillus was also isolated from 1 lung. Mycoplasmas were isolated from 18 lungs (90 per cent.) and they comprised the same 3 mycoplasma species as were found in the lesions from the healthy calves. *M. bovirhinis* was isolated from 10 lungs (50 per cent.), *M. dispar* from 6 lungs (30 per cent.) and T-mycoplasmas from 13 lungs (65 per cent.). No arginine metabolizing mycoplasmas were isolated.

Pathology and histopathology. Some of the lungs showed no areas of consolidation but the whole lung was congested, and these are marked with a C in Table 2. Both lungs were usually equally affected in this group of calves. A summary of the main histopathological findings in the second group is recorded in Table 2. Pulmonary collapse was assessed to be significant in 11 of the 20 cases (55 per cent.), and in one animal this was the only apparent abnormality apart from hyperaemia and intrapulmonary haemorrhage. With this one exception all lungs showed alveolar epithelial cell exudate and in several instances there was symplasma formation. In 11 out of 20 cases (55 per cent.) there was significant giant cell formation in alveoli and in 4 animals there was interstitial pneumonia with eosinophilic or hyaline membrane formation and alveolar epithelialisation. In 6 (30 per cent.) of the cases (Nos. 3, 6, 10, 13, 17 and 18) there was fibrinous or

necrotizing alveolitis of the type usually associated with infection by *Pasteurella* spp. There was bronchiolitis in 17 out of 20 lungs (85 per cent.), and lesions of subacute bronchiolitis or bronchiolitis obliterans occurred in addition in 6 animals. Thirteen animals (65 per cent.) were recorded as having purulent bronchopneumonia, an incidence similar to that in the first series. Significant peribronchial lymphoid hyperplasia was found in only one animal, this being the only case classified as "cuffing" pneumonia. No phloxinophilic inclusion bodies were found in sections of lung stained by Lendrum's method.

DISCUSSION

It is not possible to attempt any exact correlation between laboratory observations and the presence or absence of clinical disease or death, because the 2 groups of calves differed in many additional respects. *Pasteurella* spp. were isolated from a similar percentage of calves in both groups, 31 and 30 per cent. respectively. *Neisseria catarrhalis* was only isolated from the calves of group 2 (15 per cent.). Mycoplasmas were isolated, frequently in high titre, from 69 per cent. of the apparently healthy calves and from 90 per cent. of the calves that died. *M. bovirhinis* was isolated from 11 per cent. of the healthy calves and 50 per cent. of the calves that died, possibly indicating a role as a secondary or opportunist invader. *M. dispar* was isolated from 60 per cent. of the apparently healthy calves and from only 30 per cent. of the dead calves. However, *M. bovirhinis* and *M. dispar* were isolated in the same medium and as *M. bovirhinis* grows quicker than *M. dispar* on primary isolation (Gourlay and Leach, 1970), it can mask the presence of *M. dispar* unless the latter grows to a higher dilution and, therefore, the figures for *M. dispar* particularly in the group of calves that died and from which *M. bovirhinis* isolations were more common, may be unduly low. T-mycoplasmas were isolated from 55 and 65 per cent. of the healthy and dead groups respectively.

No viruses were isolated from either group by the techniques used. Some lung specimens were stored at -70°C . before virus isolations were attempted and only calf kidney cells were used initially. Later attempts to isolate viruses on pig kidney cells were instituted using the stored lung specimens. It is unfortunate that no serology was possible to confirm or refute the negative virus isolation results. There was, however, no evidence of PI3 or adenovirus inclusions in sections of infected lungs. As no virus was isolated it was decided to attempt the isolation of chlamydiae from 20 selected lung specimens. Those selected included many of the lungs from which no bacteria or mycoplasmas were isolated. However, no chlamydiae were isolated by the method used. Once again the lung specimens had been stored at -70°C . The results would seem to indicate either that viruses and chlamydiae were not involved or that they had disappeared by the time examination was carried out.

Peribronchial lymphoid hyperplasia was found in 75.5 per cent. of the group 1 calves and in about 50 per cent. of these cases it was sufficiently pronounced to be termed "cuffing pneumonia". In the second group significant peribronchial lymphoid hyperplasia was found in only 1 animal (5 per cent.). In the calves that

died 6 cases (30 per cent.) showed a fibrinous or necrotizing alveolitis of the type usually associated with *Pasteurella* spp. infection; this lesion was not observed in the healthy calves. However, there was a poor correlation between actual isolation of *Pasteurella* spp. and occurrence of histological lesions normally associated with this type of infection. It was of interest that the degree and incidence of purulent broncho-pneumonia was not significantly different between the 2 groups. The common occurrence of peribronchial lymphoid hyperplasia and "cuffing pneumonia" in the group of apparently healthy calves and the very low incidence in the group that died, and which had a very high incidence of mycoplasma isolations, may indicate that lymphoid hyperplasia and mycoplasma infection are not connected, though other explanations are possible.

It would seem that *Pasteurella* spp. play some part in the disease process and warrant further study. *Neisseria catarrhalis* and other bacteria were far less common and are probably not significant pathogens in the general pneumonic syndrome. *Haemophilus* spp. were not isolated, but the isolation techniques used may not have been sensitive enough to recover small numbers of these organisms amongst other faster growing organisms. The isolation figures for mycoplasmas were surprisingly high (75.4 per cent. of all the lungs) and these organisms must surely be important in the disease process. T-mycoplasmas were the most common (58.5 per cent. of all lungs) and *M. dispar* was the next most common (50.8 per cent. of all lungs): these two organisms obviously warrant further study. *M. bovirhinis* was the least common mycoplasma (23.1 per cent. of all lungs).

In earlier work we had not found *M. bovirhinis* nor *M. dispar* in lung tissue of macroscopically normal lungs of 20 calves and 10 cows (Gourlay and Leach, 1970), neither did we isolate T-mycoplasmas nor arginine-metabolizing mycoplasmas from the the same lungs (Gourlay, unpublished data).

SUMMARY

Microbiological and pathological observations were made on 65 pneumonic calf lungs from 2 groups of calves. The first group contained 45 clinically healthy 3 month old veal calves and the second group contained 20 calves that had died or been killed in extremis. Mycoplasmas were isolated from 75.4 per cent. of all lungs and comprised T-mycoplasmas from 58.5 per cent., *Mycoplasma dispar* from 50.8 per cent. and *Mycoplasma bovirhinis* from 23.1 per cent. *Pasteurella* spp were isolated from 30.8 per cent. of lungs. No viruses were isolated. No chlamydiae were isolated from 20 lungs examined. No search for serological evidence of virus infection was carried out.

Apart from pulmonary collapse the main pathological lesions observed were peribronchial lymphoid hyperplasia in 75.5 per cent. of the lungs in the first group, but in only 5 per cent. of the second group, acute and subacute bronchiolitis, purulent bronchopneumonia, fibrinous alveolitis, alveolar giant cell reaction and interstitial pneumonitis. Assessment of pathological changes in relation to isolation of micro-organisms failed to show consistent or characteristic patterns of association in this series.

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SUMMARY

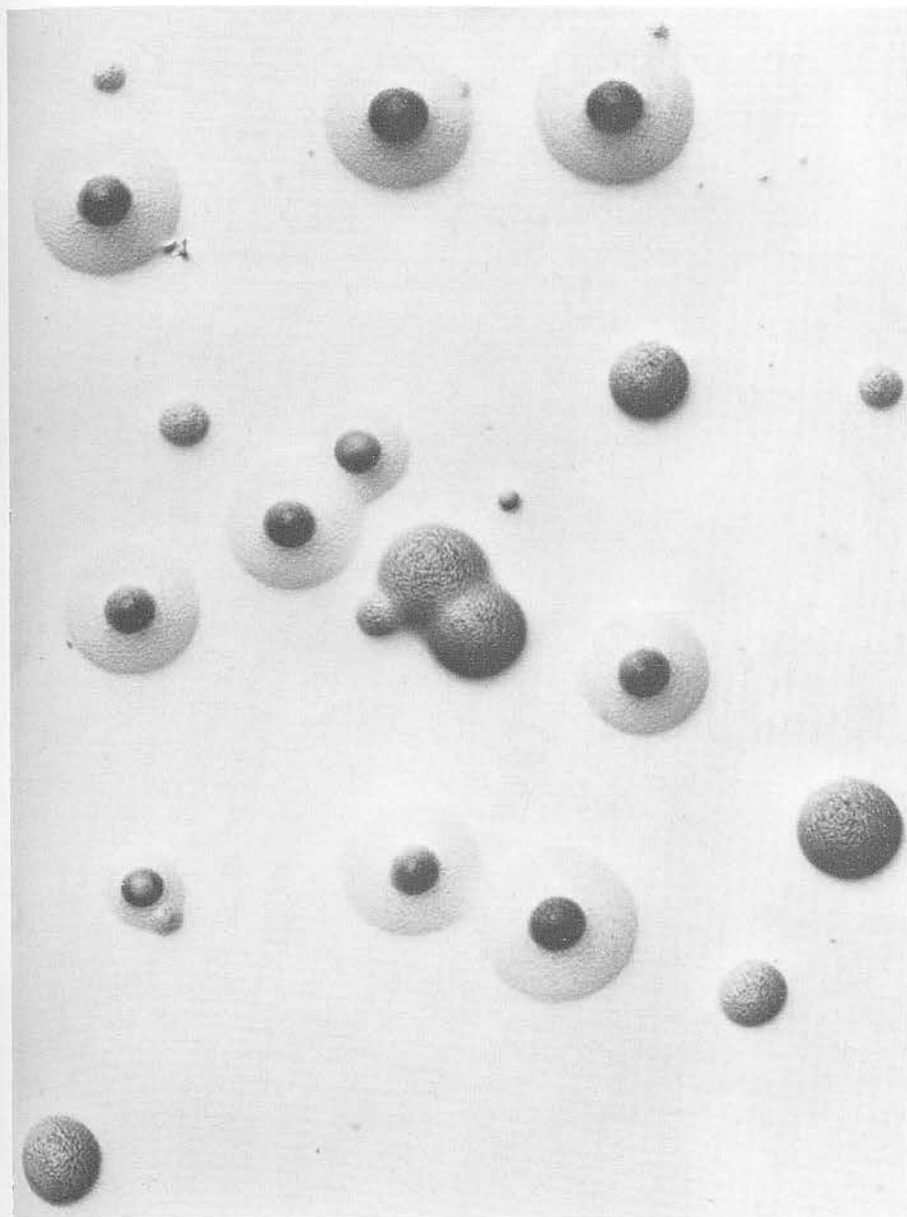


Fig. 1. Colonies of *Mycoplasma bovirhinis* (large with centres) and *Mycoplasma dispar* (smaller with no centres). Oblique transmitted light. Glucose calf-serum agar unstained. $\times 90$.

THE EXPERIMENTAL PRODUCTION OF PNEUMONIA IN CALVES BY THE ENDOBRONCHIAL INOCULATION OF T-MYCOPLASMAS

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INTRODUCTION

Leach (1967) divided the mycoplasmas of bovine origin into 8 serotypes or groups and members of 5 of these groups have been isolated from the respiratory tract of cattle. Since then other bovine mycoplasmas have been reported that do not fall into any of these 8 groups, namely T-mycoplasmas isolated from the uro-genital (Taylor-Robinson, Haig and Williams, 1967) and respiratory tracts (Gourlay 1968) and *Mycoplasma dispar* (Gourlay and Leach, 1970) from the respiratory tract.

Apart from work with *Mycoplasma mycoides* var. *mycoides*, the causal organism of contagious bovine pleuropneumonia and the type species of the genus *Mycoplasma*, little experimental work has been done on the role of mycoplasmas in bovine respiratory infections. Strains subsequently placed by Leach (1967) in group 3 (*Mycoplasma laidlawii*), group 4 (*Mycoplasma bovirhinis*) and group 6 have been inoculated into calves by the intranasal and/or intratracheal routes by various workers. Hamdy, Trapp, Gale and King (1963) inoculated *M. laidlawii*, Langer and Carmichael (1963) inoculated group 6 and Hamdy, Gale and King (1958), Langer and Carmichael (1963) and Dawson, Stuart, Darbyshire, Parker and McCrea (1966) inoculated *M. bovirhinis* into calves. They all reported that clinical disease was not produced. Later, Hamdy and Trapp (1967) inoculated *M. bovirhinis* into calves by the intranasal and intratracheal routes and into the prescapular lymph gland. Clinical signs of respiratory illness were not observed in any of the calves, but 2 killed 1 week after inoculation had pericarditis while calves killed after 4 weeks showed small scattered pneumonic lesions in the right anterior and/or cardiac lobes of the lung.

In a recent survey of microorganisms isolated from pneumonic lungs of calves in South-West England, Gourlay, Mackenzie and Cooper (1970) reported the isolation of T-mycoplasmas from 38 out of 65 lungs examined (58.5 per cent.). This high incidence of T-mycoplasma isolations raised the question of their role in calf pneumonia, and this paper reports the experimental production of pneumonia in experiments in which T-mycoplasmas were inoculated endobronchially into calves. A preliminary report of this work was published recently (Gourlay and Thomas, 1969).

MATERIALS AND METHODS

Experimental calves. Twenty-five colostrum-deprived Friesian and Channel Island and colostrum-fed Channel Island bull calves between 3 and 4 weeks of age were divided into 2 large groups, a T-mycoplasma group containing 16 calves and a control group containing 9 calves. In the absence of gnotobiotic calves, the experimental animals were obtained from different farms. Calves in the T-mycoplasma and control

groups were subjected to conventional methods of raising. They were housed and reared in an identical manner and were placed in groups at random. The groups were each further subdivided into 3 smaller groups depending on breed and whether they received colostrum or not. The T-mycoplasma groups were numbered 1, 2 and 3 and the control groups were numbered 4, 5 and 6. Groups 1 and 4 contained similar, colostrum-deprived, Friesian calves, as did groups 2 and 5, colostrum-deprived Channel Island calves, and groups 3 and 6, colostrum-fed Channel Island calves.

The 16 T-mycoplasma calves were inoculated with broth cultures of T-mycoplasmas and 7 of the 9 control calves with sterile broth. The remaining 2 control calves (1 and 361) were inoculated with T-mycoplasma culture which had been incubated at 37°C. for a long period and in which viable organisms could no longer be demonstrated. Details of the different groups are given in Table 1. Following inoculation, calves were observed daily and morning temperatures were recorded. Blood for serological and for haematological examination was obtained before inoculation and at slaughter. All calves were killed 4 weeks after inoculation except for calf 81 which was killed after 6 weeks. At autopsy the lungs were examined for macroscopic lesions and representative pieces of pneumonic lung or a piece of the right apical lobe, if no pneumonia was evident, were taken for isolation of bacteria, viruses, chlamydiae and mycoplasmas and also for histological examination.

Endobronchial inoculation. Calves were anaesthetised with intravenous methohexitone sodium (Brietal Sodium, Elanco Products Ltd.) at 25 mg./4.5 kg. body weight, which produced deep anaesthesia for about 15 minutes which was ideal for this operation. Recovery was quiet and uneventful and calves were generally standing and eating within one hour. The animals were placed on their left side and the trachea exposed by a skin incision in the mid-tracheal region of the neck. A trochar and cannula were inserted between the tracheal rings, withdrawn and a preinoculation swab was taken. The swabbing device, consisting of a Portex polythene tube (Portex PP280) into which a small swab on a long piece of wire was threaded, was passed through the cannula into the trachea and down into the lower respiratory tract to impact in a bronchiole. The swab was pushed out and withdrawn again into the tube, and the whole tube plus the swab withdrawn from the cannula. A second Portex tube was introduced into the lower respiratory tract by the same method and 8 to 10 ml. of broth culture were passed down the tubing by means of a syringe, followed by 8 to 10 ml. of sterile broth and 10 ml. of air. The tubing and cannula were withdrawn and the skin incision closed with nylon sutures. Procaine penicillin, 1,500,000 i.u. was given intramuscularly immediately after inoculation.

Strains of mycoplasma. Eight calves were inoculated over a period of 8 months from January to August, 1969 with the M9/T/4 strain of T-mycoplasmas isolated from pneumonic calf lung and 8 with the A417/T/2 strain obtained from calf 417 experimentally inoculated with the M9/T/4 strain. (see Table 1). M9/T/4 and A417/T/2 are presumably variants of the same strain. On primary isolation the M9/T/4 strain was obtained from medium free from penicillin and thallium acetate, it was then passed once more in this medium before being transferred on to medium containing penicillin and thallium acetate. All subsequent passages of this and all passages of the A417/T/2 strain were in medium containing these microbial inhibitors. No virus was isolated from the calf lung from which the M9/T/4 strain was obtained, but a *Pasteurella multocida* strain was cultured in moderate numbers together with *Mycoplasma dispar*. Serum from this calf possessed no detectable antibodies to parainfluenza 3 virus, adenovirus nor chlamydia by the tests used, but had a titre of 1/40 to reovirus by the haemagglutination inhibition (HI) test. As attempts to clone the M9/T/4 and A417/T/2 strains of T-mycoplasmas on solid medium were unsatisfactory they were cloned by the terminal dilution method which entailed passage in broth using 10-fold dilutions and sub-inoculating from the highest dilution showing growth into further broth dilutions. This process was performed 6 times, checking each time for bacterial or other mycoplasma contaminants. Early T-mycoplasma calves were inoculated with

uncloned organisms whereas cloned organisms were used for later calves. Partially purified cultures represented a dilution of the original lung material from which the organisms were isolated of at least 10^{-13} for the M9 strain and 10^{-6} and 10^{-7} for the A417 strain. Cloned cultures represent a dilution of original lung of at least 10^{-28} and 10^{-32} for the M9 strain and 10^{-22} and 10^{-25} for the A417 strain (Table 1). Aliquots of inocula were titrated after inoculation in the T-mycoplasma and glucose-fermenting mycoplasma broths referred to under "isolation and propagation of microorganisms".

Two calves were also inoculated with a strain (V2/B/3) of mycoplasma belonging to group 4 (*Mycoplasma bovirhinis*) isolated from a pneumonic calf lung. This strain was cloned by picking a single colony 3 times and the resulting culture checked by the disc growth inhibition (GI) test (Clyde, 1964) against *M. bovirhinis* antiserum.

Isolation and propagation of microorganisms. Pieces of pneumonic or normal lung obtained at autopsy were triturated in phosphate buffered saline (PBS) at a pH 7.3 and this suspension was the inoculum in various isolation procedures. Mycoplasmas were isolated using the media of and identifying by the methods described by Gourlay (1968) for T-mycoplasmas and by Gourlay and Leach (1970) for glucose-fermenting mycoplasmas. The preinoculation swabs from the lower respiratory tract were placed in the broth used for isolating glucose-fermenting mycoplasmas and this broth suspension was used for inoculum in the isolation and identification procedures as referred to above. For bacterial isolation ox blood agar, 10 per cent. chocolate agar and MacConkey agar plates were inoculated and incubated aerobically at 37°C. Ox blood agar and chocolate agar plates were also incubated anaerobically and in a mixture of 5 per cent. CO₂ in air.

Primary calf kidney monolayers in tubes and on coverslips were used for virus isolation as well as lung tissue 'explants' prepared from the lung being examined, by a modification of the method of Rogers, Basnight, Gibbs and Gajdusek (1967) described by Gourlay *et al.* (1970). Material from negative results was passaged 3 times before being discarded. The supernatant fluids of inoculated cell cultures were used for the performance of haemagglutination and haemadsorption tests.

Haematology. Pre- and post-inoculation blood samples were taken in ethylene diamine tetra-acetic acid (EDTA) for the estimation of haemoglobin and for leucocyte and differential leucocyte counts.

Histopathology. Representative portions of lung taken from each calf at autopsy were fixed in neutral buffered formalin. Paraffin sections were stained by haematoxylin and eosin, Giemsa and phloxine tartrazine (Lendrum, 1949).

Serology. Sera of calves were examined for T-mycoplasma antibodies by the metabolic inhibition (MI) test (Purcell, Taylor-Robinson, Wong and Chanock, 1966), for *M. bovirhinis* antibodies by the latex agglutination (LA) test (Morton, 1966), for antibodies to adenovirus and chlamydia by the complement fixation (CF) test by the method of Bradstreet and Taylor (1962) using 'Wellcome' adenovirus and psittacosis viral antigens (C.F.T.) (Burroughs Wellcome & Co., London), and for parainfluenza 3 virus (PI3) and reovirus by the haemagglutination inhibition (HI) test (Rosen, 1960) with minor modifications. Some of the sera were also examined by Mr. J. I. Phillip, Central Veterinary Laboratory, Weybridge, for antibodies to adenovirus by the agar gel diffusion test and for reovirus I and II by the serum neutralization test.

RESULTS

Examination of Preinoculation Swabs for Mycoplasmas

T-mycoplasmas, *M. bovirhinis* and *M. dispar* were isolated alone or in combination from the preinoculation swabs from the lower respiratory tract of 15 of the 21 calves examined, T-mycoplasmas were isolated from calves 1, 908 and 915,

M. bovirhinis from 1, 310, 921, 922, 924, 926, and 933 and *M. dispar* from 1, 309, 310, 361, 904, 906, 908, 910, 915, 922, and 923. Preinoculation swabs were not obtained from 81, 412, 416, 417, 421 or 723.

Examination of Experimental Calves after Inoculation with T-mycoplasmas Sterile Medium, Dead Culture or M. bovirhinis

Clinical observations. None of the 9 calves inoculated with sterile medium or dead culture showed any evidence of clinical illness except calf 1 which showed signs of dyspnoea. Of the 2 calves inoculated with *M. bovirhinis* one (723) showed pyrexia (over 40°C.) but no other signs of illness. Amongst the 16 inoculated with T-mycoplasma cultures the 3 colostrum-deprived Channel Island calves in group 2 showed marked signs of clinical disease, the salient features being coughing, pyrexia and dyspnoea. Persistent coughing was recorded for

TABLE 1
DETAILS OF CALVES INOCULATED ENDOBRONCHIALY

Inocula used, presence of pneumonic lesions at autopsy and mycoplasma isolated from the lungs of calves inoculated endobronchially with T-mycoplasmas, sterile medium and mycoplasma bovirhinis

Group	Breed	Colostrum	Inoculum			Calf No.	Gross lesion	Titre* of Mycoplasmas isolated		
			Strain	Titre*	Dilution†			T-mycoplasma	M. bovirhinis	M. dispar
1	F	—	M9	10 ⁵	10 ⁻¹³	81	+	>10 ¹⁰	0	10 ²
			"	"	"	412	+	10 ⁵	>10 ⁷	0
			"	"	"	416	+	>10 ¹⁰	10 ⁶	0
			"	"	"	417	+	>10 ¹⁰	>10 ⁷	0
			"	>10 ¹⁰	10 ⁻²⁸	53	—	10 ¹	10 ¹	0
			"	"	"	56	+	10 ²	0	0
2	CI	—	A417	"	10 ⁻²²	55	+	0	0	10 ⁴
			A417	10 ⁴	10 ⁻⁶	904	+	>10 ¹⁰	0	10 ⁴
			"	"	"	906	+	10 ⁸	0	10 ⁴
			"	"	"	910	+	>10 ¹⁰	0	10 ⁵
3	CI	+	A417	10 ⁴	10 ⁻⁷	908	+	10 ⁷	0	10 ⁶
			"	>10 ¹⁰	10 ⁻²⁵	915	+	>10 ¹⁰	10 ⁴	0
			"	"	"	923	+	0	0	0
			"	"	"	924	+	>10 ¹⁰	0	0
			M9	10 ⁹	10 ⁻³²	921	+	10 ⁸	0	0
			"	10 ⁸	"	922	—	0	0	0
4	F	—	Sterile medium			309	—	0	0	0
			" "			310	—	0	0	0
			Dead culture			1	±	0	0	10 ¹
			" "			361	—	0	0	0
5	CI	—	Sterile medium			929	—	0	0	0
			" "			931	—	0	0	0
6	CI	+	"	"	"	925	—	0	0	0
			"	"	"	926	—	0	0	0
			"	"	"	933	+	0	0	0
7	F	—	<i>M. bovirhinis</i> culture			421	±	0	0	0
			10 ⁶			723	+	0	0	0

* Colour change units/0.2 ml

† Dilution of original lung suspension

F Friesian

CI Channel Island

these 3 calves and was especially severe in calf 906 which subsequently was shown to have extensive lung lesions. This calf showed obvious dyspnoea, with a respiratory rate of over 60 per minute and auscultation of the thorax revealed harsh inspiratory sounds over the bronchial area. Among the other calves inoculated with T-mycoplasmas, 417 showed obvious dyspnoea and a significant temperature rise of over 40°C. was recorded in 3 (81, 412 and 417). This rise did not occur at any particular period and was of variable duration.

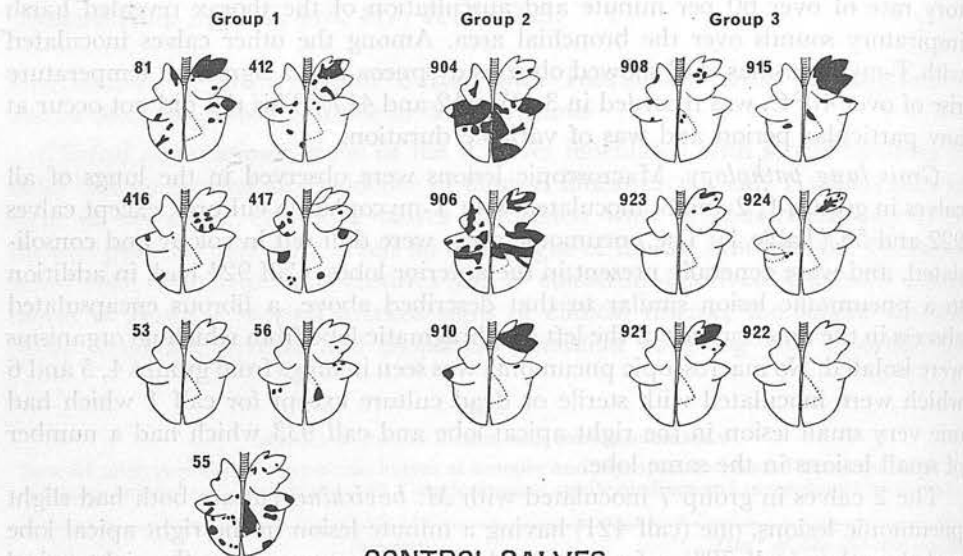
Gross lung pathology. Macroscopic lesions were observed in the lungs of all calves in groups 1, 2 and 3, inoculated with T-mycoplasma cultures, except calves 922 and 53 (Table 1). The pneumonic areas were dull red in colour and consolidated, and were generally present in the anterior lobes. Calf 924 had, in addition to a pneumonic lesion similar to that described above, a fibrous encapsulated abscess in the anterior part of the left diaphragmatic lobe from which no organisms were isolated. No macroscopic pneumonia was seen in lungs from groups 4, 5 and 6 which were inoculated with sterile or dead culture except for calf 1 which had one very small lesion in the right apical lobe and calf 933 which had a number of small lesions in the same lobe.

The 2 calves in group 7 inoculated with *M. bovirhinis* culture both had slight pneumonic lesions, one (calf 421) having a minute lesion in the right apical lobe and the other (calf 723) a few scattered areas of pneumonia in the right apical and cardiac and left cardiac lobes. The extent of the macroscopic lesions is shown diagrammatically in Fig. 1, while photographs of the lungs of calves 915 and 921 are shown in Figs. 2 and 3.

Histopathological examination of lung lesions. Histopathological examination of lung sections from the 9 control calves revealed minor cellular infiltrations. However, the 2 lungs (calves 1 and 933) that possessed macroscopic pneumonic lesions showed lobules of distinct alveolar collapse, but no changes in related bronchioles. One other control lung (calf 929) also revealed a small area of collapse. In 2 other lungs (calves 926, 361) there was mild broncholitis, but no luminal plugging of bronchioles or bronchi with inflammatory exudate was observed. Mild interstitial round cell infiltration was recorded in the lungs of 3 calves (1, 925 and 929) and there was a distinct peribronchiolar lymphoreticular hyperplasia in calf 926.

The lungs from the 16 calves inoculated with T-mycoplasmas generally showed more pronounced histopathological changes which were mainly pulmonary collapse and acute bronchiolitis. Thus there was distinct alveolar collapse, not usually accompanied by any alveolar reaction, in the 14 lungs that possessed macroscopic pneumonic lesions. All these lungs, except from calf 923, had distinct acute bronchiolitis associated with the collapsed areas and plugging of the bronchi and bronchioles with inflammatory exudate (Fig. 4). Three of these lungs (calves 417, 904, 924) also showed evidence of bronchopneumonia. Lymphoreticular hyperplasia was observed (Fig. 5) in 3 out of the 16 lungs (908, 915, 921) and, as in the controls, there was non-specific round cell infiltration in other lungs. Only one lung (calf 921) showed distinct peribronchiolar lymphoid hyperplasia ('cuffing'). Proliferation of alveolar epithelial cells or the formation of multinucleate giant cells was not seen.

T-MYCOPLASMA CALVES



CONTROL CALVES

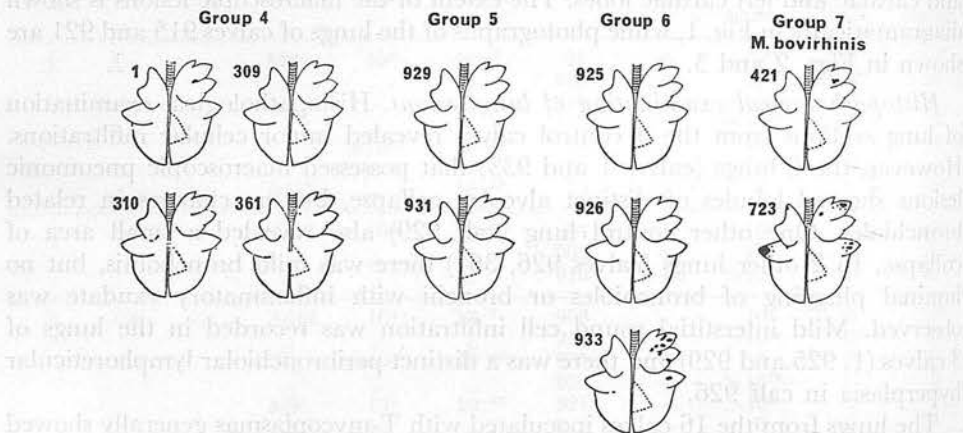


Fig. 1. Diagrammatic representation of pneumonic lesions following inoculation of T-mycoplasmas and sterile medium into calves. Inoculated calves: group 1, Friesian—colostrum deprived; group 2, Channel Island—colostrum deprived; group 3, Channel Island—colostrum fed. Control calves: group 4, Friesian—colostrum deprived, sterile medium or dead culture; group 5, Channel Island—colostrum deprived, sterile medium; group 6, Channel Island colostrum fed, sterile medium; group 7, Channel Island—colostrum deprived, *M. bovirhinis* culture.

Isolation of microorganisms from lungs of inoculated calves. T-mycoplasmas were isolated from the lungs of 13 of the 16 calves inoculated with T-mycoplasm broth cultures. The titres were usually high and frequently reaching 10^{10} colour change units (c.c.u./0.2 ml.) which was the highest titre obtainable in our titration system. The 3 calves from which no T-mycoplasmas were isolated were from the

7 inoculated with high dilution organisms. One of these (922) also received a low titre inoculum (10^3 c.c.u./0.2 ml.) In addition to T-mycoplasmas, *M. bovirhinis* and *M. dispar* were isolated from 5 and 6 lungs respectively. Bacteria which were isolated from a few of the lungs, were usually of non-pathogenic varieties and in very small numbers. In 2 cases, however, bacteria of more potential importance were isolated, namely, *Corynebacterium pyogenes* from calf 910 and calf 55 and a *Pasteurella multocida* strain from calf 915. Mycoplasmas and bacteria were not isolated from the lungs of any of the 9 calves inoculated with sterile medium except for *M. dispar* at very low titre (10^1 c.c.u./0.2 ml.) from calf 1. Similarly, no mycoplasmas were isolated from the lungs of the 2 calves inoculated with *M. bovirhinis*. Details of the calves and mycoplasmas isolated from the lungs are given in Table 1. Viruses were not isolated from any of the lungs.

Serology. Serum samples obtained before inoculation and at slaughter were examined for antibodies to T-mycoplasmas by the MI test and a four-fold or higher rise in titre was observed in the paired sera of all calves inoculated with T-mycoplasmas except for calves 53 and 56, whereas the sera of the 9 control calves showed no rise at all except for calf 933 which had a preinoculation titre of $<\frac{1}{2}$ and a titre at slaughter of $\frac{1}{8}$. The sera of the 2 calves inoculated with *M. bovirhinis* (421 and 723) showed an eight-fold rise in titre using the LA test for *M. bovirhinis*.

The same serum samples were examined for antibodies to PI3, reovirus, adenovirus and chlamydiae. None of the paired sera possessed any detectable antibodies to these agents, except 923, a calf inoculated with T-mycoplasmas, which had a preinoculation titre of $\frac{1}{8}$ and a titre at slaughter of $\frac{1}{4}$ to chlamydia. Only the colostrum-fed calves possessed antibodies to PI3 and none showed any significant rise in titre.

Haematology. No significant variation from the normal blood picture for the age of calf under experiment was detected.

DISCUSSION

The endobronchial method of inoculation proved a most reliable means of producing experimental contagious bovine pleuropneumonia in cattle using the causal agent *Mycoplasma mycoides* var. *mycoides* (Campbell, 1938; Brown, 1964). The use of this route in calves has enabled us to show that under our system T-mycoplasma cultures produced macroscopic pneumonic lesions in 14 out of 16 calves compared with 2 out of 9 that showed no more than small lesions following inoculation with control medium ($P < 0.01$).

The two control calves that possessed lesions on autopsy were 1 and 933. The clinical signs in one calf and the revelation of only a minute lesion on autopsy, and in another the larger lesion at autopsy from which no microorganisms were isolated, would suggest that a transient virus or chlamydia infection was responsible. Although the serological evidence does not support this hypothesis in that paired sera did not indicate any intercurrent chlamydial or viral infection by the viruses under test, it is possible that some unknown virus was responsible. On the other hand, a rise in titre to T-mycoplasmas was observed in the sera of one calf

and although these organisms were not isolated from the pneumonic lesions at autopsy, it is possible that they might have been responsible.

Pneumonic lesions were produced in 5 out of 7 calves by cultures of T-mycoplasmas in which the original lung material from which they had been isolated had been diluted to 10^{-22} and beyond and in one case to 10^{-32} . In 3 of the 7 calves, T-mycoplasmas were not reisolated from the lung at autopsy and bacteria were not found either except in one case when *C. pyogenes* was isolated. A further 3 calves revealed both pneumonic lesions and T-mycoplasmas on autopsy and in all cases no other bacteria were isolated. One calf (no. 53) was unusual since, although no macroscopic pneumonic lesion was visible, both T-mycoplasmas and *M. bovirhinis* were isolated in very low titre from the right apical lobe. The production of pneumonic lesions with these high dilution cultures particularly in the 3 calves from which we were able to reisolate T-mycoplasmas eliminates the possibility of a virus from the original lung being involved in the pathogenesis of these lesions. This dilution step was necessary even though no virus was isolated from the original lung by the technique used. The failure to reisolate T-mycoplasmas from the lung lesions in 3 cases and the complete failure to produce any lesions at all in the case of two calves although one was inoculated with a low titre culture might indicate that some attenuation of the organisms had occurred on subculture in broth. It is apparent that T-mycoplasmas multiplied in the pneumonic lungs since in a number of cases a considerable increase in titre of organisms was obtained from the small piece of lung triturated at autopsy as compared with the inoculum.

M. bovirhinis and *M. dispar* were frequently isolated at autopsy from the lungs of calves inoculated with T-mycoplasma cultures. In earlier work we had not found *M. dispar* nor *M. bovirhinis* in lung tissue of macroscopically normal lungs of 20 calves and 10 cows (Gourlay and Leach, 1970) nor did we isolate T-mycoplasmas from the same lung specimens (Gourlay *et al.*, 1970). However, from the examination of preinoculation endobronchial swabs it was apparent that *M. bovirhinis*, *M. dispar* and T-mycoplasmas can be found in the respiratory tract of some apparently normal calves.

Ideally, work of this nature should be carried out in gnotobiotic calves and when the opportunity arises we intend to do this. Under the conditions available to us the presence of the different mycoplasma species in the respiratory tract of the calves prior to inoculation appeared to depend mainly on the calf groupings during rearing, as those reared together usually possessed the same mycoplasma species. There appeared to be little correlation, however, between the isolation of a specific mycoplasma species from the preinoculation swabs and the subsequent development of lung lesions or the reisolation of the same mycoplasma from the pneumonic lesions at autopsy. Examples of this are that *M. dispar* was isolated from preinoculation swabs of two calves; yet no pneumonic lesions developed and *M. dispar* was not reisolated from the lungs. Again *M. dispar* was isolated from the preinoculation swab from one calf but not from the pneumonic lung at autopsy, and *M. bovirhinis* was isolated from the preinoculation swab of another but not from the pneumonic lesion at autopsy. The isolation techniques are probably not sufficiently sensitive to detect a few mycoplasmas of one species among a large number of mycoplasmas of another species and this may explain some of these

anomalies. It is our impression, however, that various mycoplasma species can be present in small numbers in the upper and lower respiratory tract of apparently normal calves and are of no significance unless they find their way into the lung tissue and even then they apparently require to be in relatively large numbers to cause any lesions. There are a number of other examples of microorganisms which form part of the flora of the normal respiratory tract and are potentially virulent; the pneumococcus is a good example. So far as *M. bovirhinis* is concerned it is probably weakly pathogenic and is an opportunist invader. The pathogenicity of *M. dispar* is under investigation at present and the results will be reported later. We have shown, however, that this organism may produce pneumonia in calves under experimental conditions (Gourlay and Thomas, 1969).

C. pyogenes was isolated from 2 pneumonic lungs and a *Pasteurella multocida* strain from one. The role that these organisms played in the production of these pneumonic lesions is not known, but the pathological and histopathological picture of the lesions did not differ significantly from the other pneumonic lungs. Since bacterial agents recovered from animals with respiratory disease, including *C. pyogenes* and *Pasteurella multocida* species, have not been conclusively proved to be of primary aetiological importance (Omar, 1966), it would seem that, like *M. bovirhinis*, they were present in the upper respiratory tract and were opportunist invaders.

The histopathological picture of an acute bronchiolitis associated with collapse and plugging of the bronchi and bronchioles with inflammatory exudate is consistent with the picture seen in other mycoplasma respiratory infections (Goodwin and Whittlestone, 1963; Brennan, Fritz and Flynn, 1969; Hodges, Betts and Jennings, 1969) and may be associated with the tendency for mycoplasma to localise on the epithelium of the small bronchioles (Switzer, 1967; Brennan *et al.*, 1969). This reaction could initiate events leading to later peribronchiolar lymphocytic 'cuffing'. The relative absence of the 'cuffing' described in mycoplasma infections may be explained by the relatively short duration of the experimental disease—about 4 weeks.

SUMMARY

T-mycoplasmas isolated from pneumonic calf lungs were inoculated endo-bronchially into calves 3 weeks of age and the calves were killed 4 weeks later. Clinical signs of pneumonia were observed in 6 out of 16 before slaughter and pneumonic lesions were found in 14 of them. T-mycoplasmas were reisolated from 13 of the 16 lungs. In nine control calves inoculated with sterile medium, clinical symptoms of pneumonia were observed in one and a slight pneumonic lesion was found in its lung at autopsy; another displayed pneumonic lesions at autopsy. No T-mycoplasmas were isolated from either calf. *M. bovirhinis* was inoculated by the same route into 2 calves and only slight pneumonic lesions were observed on autopsy and the organism was not reisolated.

ACKNOWLEDGMENTS

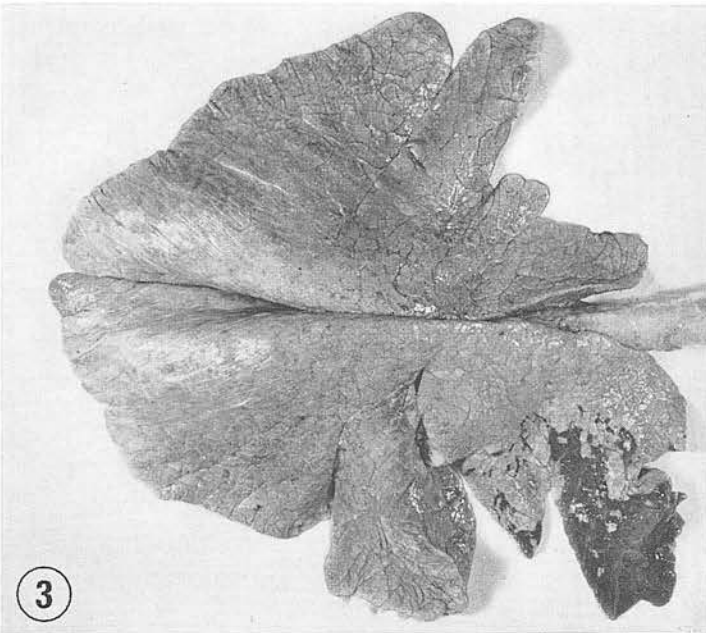
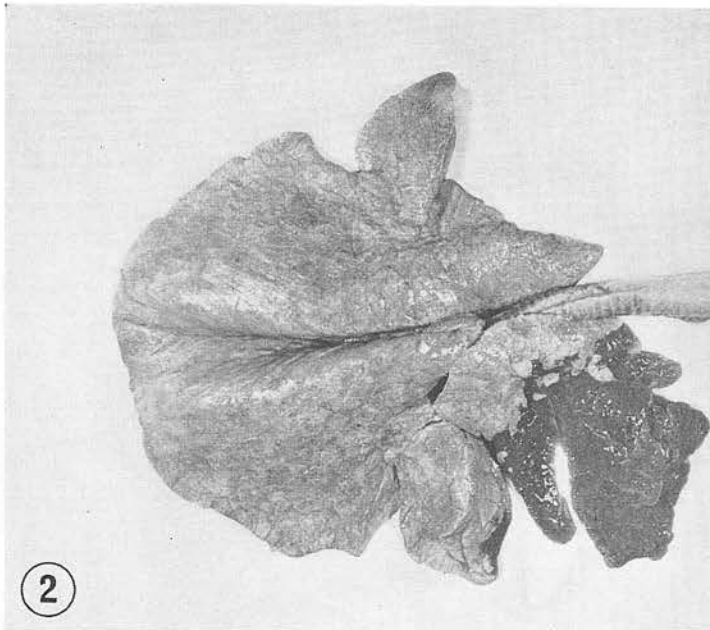
We would like to thank Dr. D. A. Haig and Mr. J. E. Cooper for the attempted virus isolations and for examining the sera for parainfluenza 3 virus and

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- Fig. 2. Lungs of calf 915 inoculated with T-mycoplasmas at 10^{-7} dilution showing pneumonic areas in right apical and cardiac lobes. The other pneumonic areas shown diagrammatically in Fig. 1 are on the ventral surface of the lungs and so are not visible.
- Fig. 3. Lungs of calf 921, inoculated with T-mycoplasmas at high dilution (10^{-32}), showing pneumonic areas in the right apical lobe.

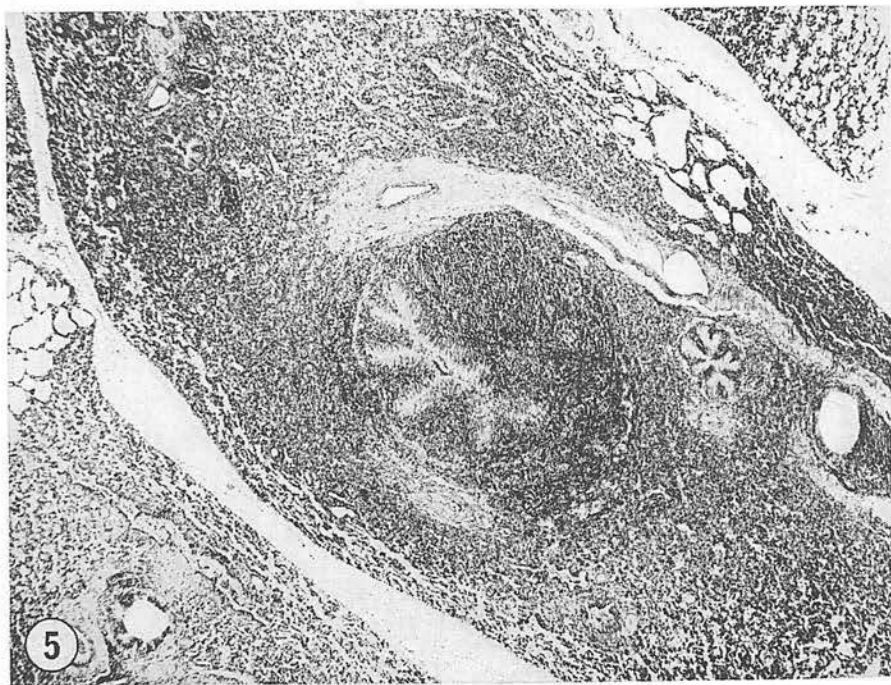
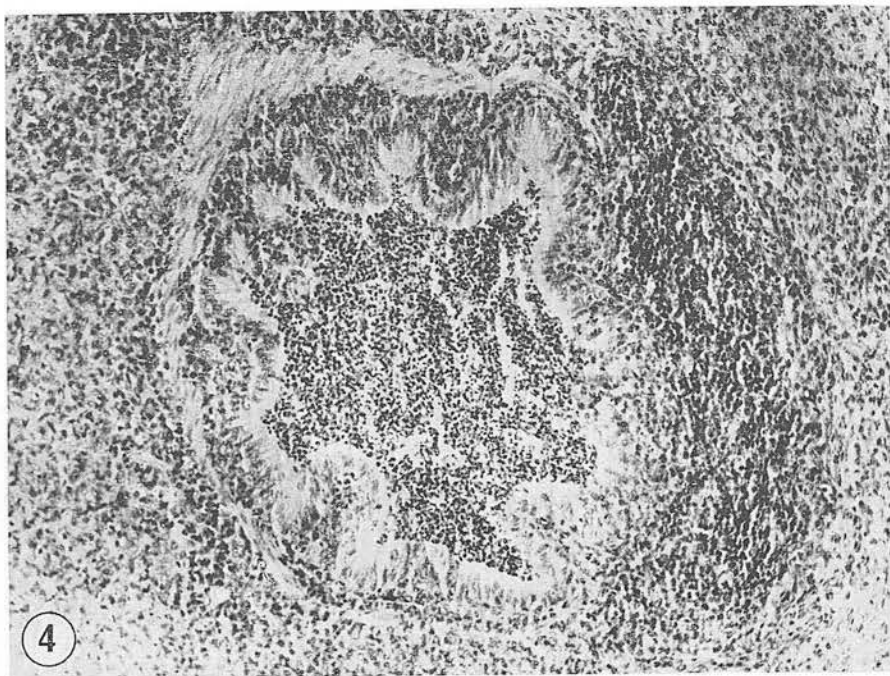


Fig. 4. Acute bronchiolitis and plugging of the bronchiole with inflammatory exudate (calf 417). H. & E. $\times 75$.

Fig. 5. Peribronchiolar round cell infiltration and collapse of the bronchiole with adjacent alveolar atelectasis (calf 915). H. & E. $\times 18$.

The production of mastitis in cows by the intramammary inoculation of T-mycoplasmas

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SUMMARY

Six milking cows were inoculated with bovine and human T-mycoplasmas and control materials into the udder via the teat canal. Control materials produced only a slight transient cell response in the milk. Bovine T-mycoplasmas produced clinical mastitis in nine out of ten quarters inoculated. Seven developed clinical mastitis together with visible changes in the milk, excretion of T-mycoplasmas and greatly increased cell counts in the milk. In three of these quarters, in two different cows, milk secretion ceased completely. Two quarters in a different cow showed visible milk changes, excretion of T-mycoplasmas and increased cell counts. Two quarters were inoculated with human T-mycoplasmas and neither produced any signs of mastitis.

Infection of the udder with T-mycoplasmas did not stimulate high-titre serum antibody levels as measured by the metabolic inhibition test, but whey samples gave high titres in two of the cows that were able to control and resolve the infection.

INTRODUCTION

T-mycoplasmas were isolated first by Shepard (1954) from the human urogenital tract. Since then they have been isolated from the human oropharynx (Taylor-Robinson & Purcell, 1966), from the urogenital tract of cattle (Taylor-Robinson, Haig & Williams, 1967), from pneumonic calf lungs (Gourlay, 1968), from eyes in cases of infectious bovine keratoconjunctivitis (Gourlay & Thomas, 1969), from the throats of cats (Tan & Markham, 1971), from the genital tract of dogs and the throats of squirrel monkeys (Taylor-Robinson, Martin-Bourgon, Watanabe & Addey, 1971).

The role of T-mycoplasmas in disease is not clear: they have been incriminated in non-specific urethritis in man (Shepard, 1969), abortions and premature births in women (Knudsen, Driscoll & Ming, 1967) and pneumonia in calves (Gourlay & Thomas, 1970). In the last instance pneumonic lesions were produced in calves following the endobronchial inoculation of T-mycoplasmas.

In view, however, of the difficulty of obtaining calves of an appropriate gnotobiotic specification for further experimental work on pneumonia, and the difficulty of monitoring the progress of pneumonia, we decided to investigate whether the bovine mammary gland was an alternative site for studies of T-mycoplasma

infection. An advantage of the bovine udder is that it comprises four separate quarters; all of which are accessible for clinical examination, easy to sample and have a limited and readily determined bacterial flora. This paper reports on the production of mastitis in cows by the intramammary inoculation of T-mycoplasmas and on the potential value of the udder for studying the pathogenesis of bovine T-mycoplasma infections and the immune response of the host.

MATERIALS AND METHODS

Cows

Six Ayrshire or Friesian Cross Ayrshire cows, 3-5 years of age, were used. They were each giving about 3 gal. of milk a day at the time of inoculation, except cow M 153 which was giving only 1½ gal. Total cell counts were performed on samples of milk from each quarter for a few days before inoculation and only quarters that had a cell count of less than $10^{5.2}$ cells/ml. were used. Milk smears were stained by the 'Single Dip' method of Broadhurst & Paley (1939) and cells were counted by the technique described by Pattison & Holman (1951). By cultural methods no large-colony or T-mycoplasmas were demonstrated in the pre-inoculation milk samples.

After the afternoon milking the cows were inoculated in each quarter with 10 ml. of either T-mycoplasma broth culture or control broth material. The inoculum was inserted via the teat canal with a syringe. Sixteen hours after inoculation, and at daily intervals thereafter, milk samples were examined from each quarter for abnormal appearance, T-mycoplasmas, cells and bacteria. The quarters were also examined clinically for signs of mastitis.

Strains of T-mycoplasma

Four strains of T-mycoplasmas were used: the A417 strain that had been isolated from a pneumonic calf lung (Gourlay & Thomas, 1970), the D32 strain that had also been isolated from a pneumonic calf lung (calf 24, table 1, Gourlay, Mackenzie & Cooper, 1970), the REOW strain isolated from the human urogenital tract and supplied by Dr D. Taylor-Robinson, and the M 126/68 strain also isolated from the human urogenital tract and supplied by Dr B. E. Andrews. Two different substrains of the A 417 strain were used; the first was at the 10^{-11} dilution from the original lung tissue while the second - 'cloned' - had undergone six subcultures in broth of which three had been from the terminal dilution, as a means of purification, and was at a 10^{-25} dilution from the original lung. The D32 strain had undergone eight subinoculations, three of them from the terminal dilution, and was at a 10^{-23} dilution from the original lung.

The T-mycoplasmas were grown in U-broth. This was similar to GS broth (Gourlay & Leach, 1970) except that it also contained 0.1 % urea, 5 % yeast extract (Difco) and 0.05 M Hepes (Sigma). Glucose, lactalbumin hydrolysate and DNA were omitted and the pH adjusted to 6.0. The titre of viable organisms in cultures or milk was estimated by making duplicate serial tenfold dilutions in U-broth. Growth produced an alkaline shift of pH as a result of the metabolism of urea and was indicated by a colour change of phenol red in the medium. Viability was esti-

mated as colour change units (c.c.u.). The titre was the highest dilution that produced a colour change after incubation at 37° C.

Control inocula were either uninoculated broth or broth culture of *T-mycoplasmas* which had been incubated at 37° C. until viable organisms could no longer be demonstrated (dead organisms). The pH was then adjusted to 7.0.

Histopathology

Representative portions of mammary tissue, taken at autopsy, were fixed in neutral buffered formalin. Paraffin sections were stained by haematoxylin and eosin.

Serology

Sera and whey were examined for antibodies to *T-mycoplasma* (A 417 strain) by the metabolic inhibition (MI) test of Purcell, Taylor-Robinson, Wong & Chanock (1966). Titres are given as the reciprocal of the highest dilution of serum that inhibited growth.

Sera were also examined for the presence of haemoglobin reactive protein (HRP) as described by Spooner & Miller (1971).

Bacteriology

The number of bacteria was estimated in milk samples by spreading 0.1 ml. of milk over the surface of an ox-blood-agar plate and counting the number of colonies after incubation at 37° C. for 24 hr. The bacterial count in milk was not considered significant if there were fewer than 1000 colony-forming units per ml. and there was an absence of obvious mastitis pathogens.

RESULTS

Udder response

Details of the inocula used for the six cows are given in Table 1. The results obtained from cows A 5, L 629 and L 686 are given in Figs. 1-3. All three cows developed mastitis in the quarters inoculated with 10⁶ or more viable A 417 *T-mycoplasmas*, as indicated by an increase in milk cells, and visible milk and udder abnormalities. *T-mycoplasmas* were also isolated from these quarters. Plate 1A shows the changes in appearance of milk from the LF quarter of cow L 629. Injection of control materials into the udder produced only a transient cell response in milk. Uninoculated broth gave only a slight cell response but the injection of dead organisms produced a greater though still transient cell response. Cow L 686 reacted slowly and less severely than the others and eventually overcame the infection. The RH and LH quarters of this cow developed only transient cell responses. *T-mycoplasmas* were not isolated at any time from the milk from the RH quarter even though the control 'dead' inoculum unfortunately contained 10³ viable organisms. *T-mycoplasmas* were isolated from milk of the LH quarter (inoculated with REOW organisms) only on the day after inoculation.

Cow L 91 reacted only slightly to the intramammary inoculation of mycoplasmas and only the LF quarter developed more than a transient cell response. It is of

Table 1. *Details of inocula*

Cow	Quarter	Inoculum
A 5	LF	10 ⁶ A 417
	LH	Uninoculated broth
	RF	NI
	RH	NI
L 629	LF	10 ⁷ A 417
	LH	Dead A 417
	RF	10 ⁷ A 417
	RH	Uninoculated broth
L 686	LF	10 ⁷ A 417 'cloned'
	LH	10 ⁷ REOW
	RF	10 ⁶ A 417
	RH	10 ⁸ 'dead' A 417 'cloned'
L 91	LF	10 ⁷ A 417 'cloned'
	LH	10 ⁶ M 126/68
	RF	10 ⁶ A 417
	RH	Dead A 417
M 153	LF	10 ⁸ A 417
	LH	NI
	RF	NI
	RH	NI
M 626	LF	NI
	LH	10 ⁶ D 32
	RF	NI
	RH	10 ⁶ A 417 'cloned'

NI = not inoculated.

particular interest that the RF quarter did not develop mastitis, considering the results with the inoculation of A 417 organisms into the first three cows. The milk from the LF quarter had an initial cell response, in which T-mycoplasmas were isolated, but the cell response had returned to normal by the 5th day. On the 6th day, a second response occurred which became moderately severe and persistent. T-mycoplasmas were still being excreted in the milk at a low titre after 41 days. Milk and udder abnormalities were observed at the height of the second response.

Cows M 153 and M 626 were killed before the full extent of their infection could be assessed. The LF quarter of cow M 153 reacted severely and, at slaughter on the 4th day after inoculation, the T-mycoplasma titre was 10⁶/ml. and the cell count was 10^{7.7} cells/ml. Udder abnormalities were evident, manifest by induration and a drop in milk yield, while the milk became watery and separated. Cow M 626 was killed 9 days after inoculation. The two inoculated quarters both reacted; the RH one more severely than the LH one. T-mycoplasmas were isolated from both quarters up to the time the cow was killed. The maximum titres were 10⁶ organisms/ml. in the RH quarter and 10⁵ organisms/ml. in the LH quarter. The cell counts in the RH quarter reached a maximum of 10^{8.2} cells/ml. and in the LH quarter 10^{7.3} cells/ml. Both quarters produced abnormal milk but showed no obvious udder abnormalities. The milk from the RH quarter became watery and separated whereas the milk from the LH quarter contained only clots. Small

A 5

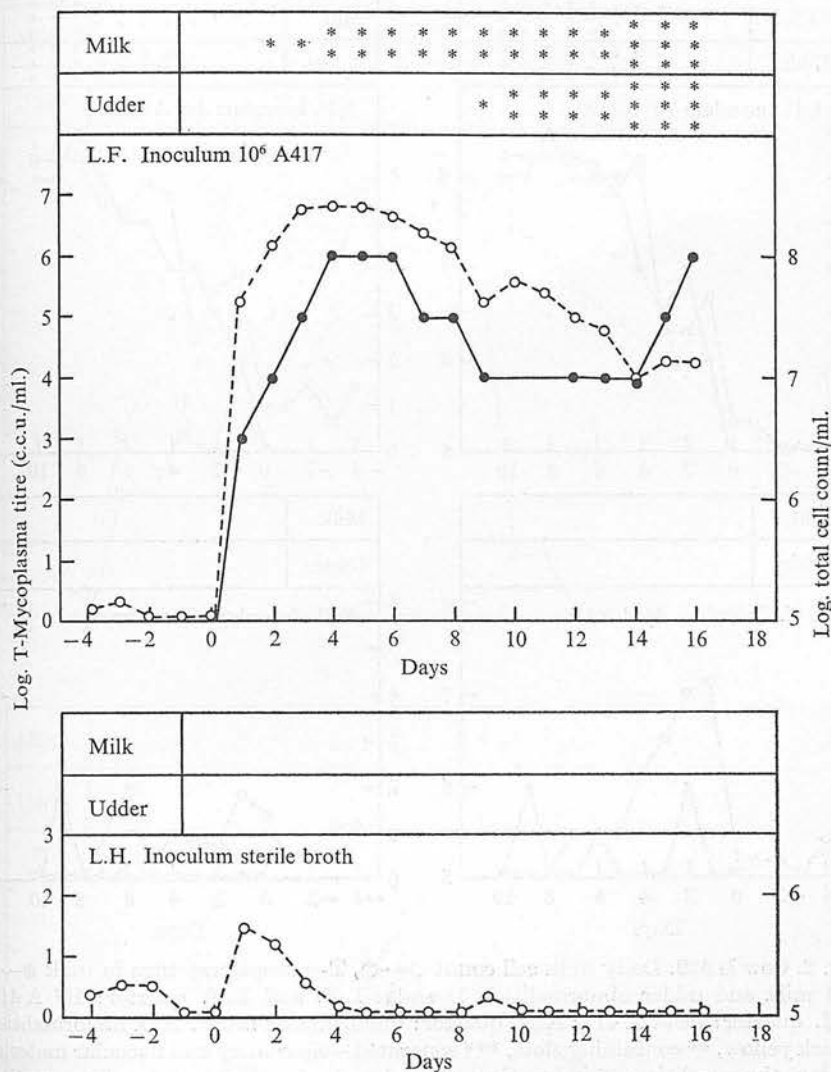


Fig. 1. Cow A 5. Daily milk cell count \bigcirc — \bigcirc , T-mycoplasma titre in milk \bullet — \bullet , and milk and udder abnormalities. Inocula: L.F. quarter, 10^6 A 417; L.H. quarter, uninoculated broth. Milk abnormalities: * dark yellow, ** containing clots, *** separated—clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

pieces of udder tissue were taken from these latter two cows for histopathological examination.

Bacteriology

Bacterial counts in milk were monitored throughout the course of mycoplasma infections, as a guard against the possibility of a concurrent bacterial infection causing the mastitis, coincident with high mycoplasma titres. There was no increase in the bacterial count in any of the experiments reported. In fact, the

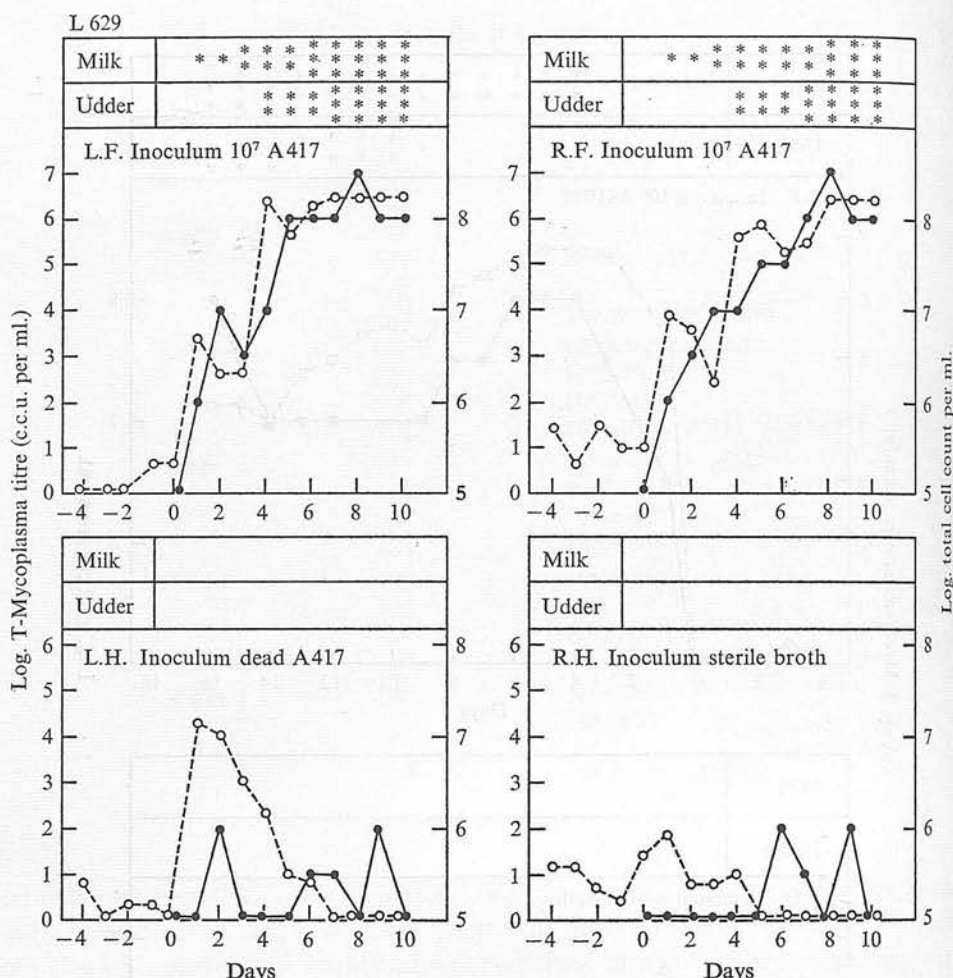


Fig. 2. Cow L 629. Daily milk cell count \bigcirc — \bigcirc , T-mycoplasma titre in milk \bullet — \bullet , and milk and udder abnormalities. Inocula: L.F. and R.F. quarter: 10^7 A 417; L.H. quarter: dead A 417; R.H. quarter: uninoculated broth. Milk abnormalities: * dark yellow, ** containing clots, *** separated—clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

bacterial count noticeably decreased in quarters with high mycoplasma titres and, in many instances, the milk appeared bacteriologically sterile.

Serology

No serum samples were obtained from cow M 153 as it was slaughtered after only 4 days. All the other serum samples tested before inoculation had an antibody titre of < 10 . MI antibody titres of 20 were found in the sera of three of the five cows after infection. These were cows A 5, L 629 and L 686.

All whey samples taken before inoculation showed MI antibody titres of < 10 . The whey from cow L 91 appeared to stimulate the growth of the T-mycoplasmas. After inoculation, MI antibody (titre > 640) was found in whey samples from all

L 686

Log. total cell count per ml.

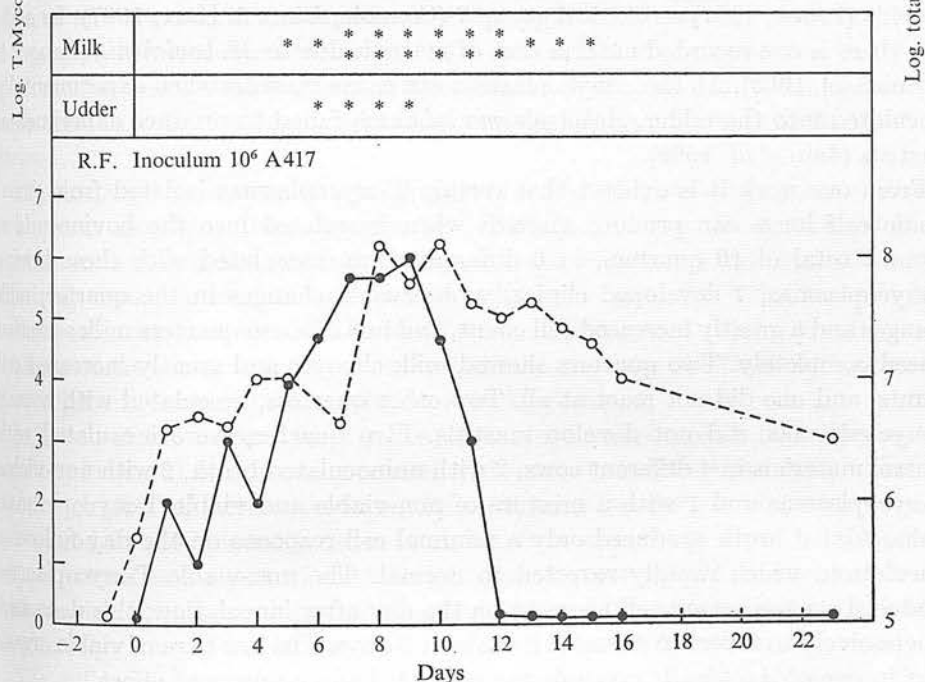
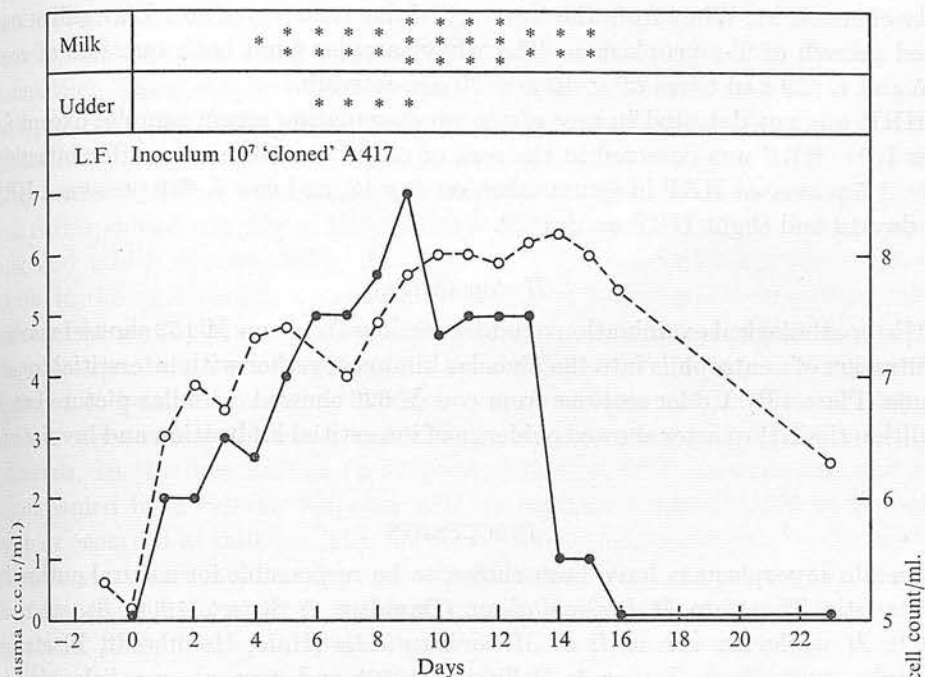


Fig. 3. Cow L 686. Daily milk cell count $\bigcirc-\bigcirc$, T-mycoplasma titre in milk $\bullet-\bullet$, and milk and udder abnormalities. Inocula: L.F. quarter, 10^7 'cloned' A 417; R.F. quarter, 10^6 A 417. Milk abnormalities: * dark yellow, ** containing clots, *** separated - clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

inoculated quarters of cows L 686 and L 626 and in the whey from the LF quarter only of cow L 91. Whey from the three remaining quarters of cow L 91 still stimulated growth of T-mycoplasmas. The whey samples from both quarters of cows A 5 and L 629 had titres of < 10 and 20 respectively.

HRP was not detected in any of the pre-inoculation serum samples except for cow L 91. HRP was detected in the sera of only two of the cows after infection. Cow A 5 possessed HRP in serum taken on day 13, and cow L 629 possessed HRP on day 10 and slight HRP on day 20.

Histopathology

Histopathological examination of udder sections from cow M 153 showed marked infiltration of neutrophils into the alveolar lumina together with interstitial hyperaemia (Plate 1B). Udder sections from cow M 626 showed a similar picture but in addition the RH quarter showed evidence of interstitial infiltration and involution.

DISCUSSION

Certain mycoplasmas have been shown to be responsible for natural outbreaks of mastitis. These are *M. bovis genitalium* (Davidson & Stuart, 1960; Stuart *et al.* 1963), *M. agalactiae* var. *bovis* or *M. bovimastitidis* (Hale, Helmboldt, Plastringe & Stula, 1962; Jain, Jasper & Dellinger, 1969) and mycoplasma belonging to Leach's (Leach, 1967) serological group 7 (Connole, Laws & Hart, 1967). In addition there is one recorded natural case of mastitis due to *M. bovirhinis* (Langer & Carmichael, 1963). All these mycoplasmas can cause mastitis when experimentally inoculated into the udder. *Acholeplasma laidlawii* failed to produce experimental mastitis (Jain *et al.* 1969).

From our work it is evident that certain T-mycoplasmas isolated from pneumonic calf lungs can produce mastitis when inoculated into the bovine udder. From a total of 10 quarters, in 6 different cows, inoculated with these bovine T-mycoplasmas, 7 developed clinically observable changes in the quarter, milk changes and a greatly increased cell count, and in 3 of these quarters milk secretion ceased completely. Two quarters showed milk changes and greatly increased cell counts, and one did not react at all. Two other quarters, inoculated with human T-mycoplasmas, did not develop mastitis. Five quarters were inoculated with control materials in 4 different cows, 2 with uninoculated broth, 2 with non-viable T-mycoplasmas and 1 with a mixture of non-viable and viable T-mycoplasmas. Uninoculated broth produced only a minimal cell response on the day following inoculation, which rapidly reverted to normal. The non-viable T-mycoplasmas produced a more severe cell response on the day after inoculation; this decreased progressively to revert to normal after about 5 days. The low titre of viable organisms in one of the 'dead' mycoplasma controls had no apparent effect.

T-mycoplasmas were detected in milk from all quarters that were inoculated with the bovine T-mycoplasmas. From one quarter (that which did not develop any signs of mastitis) these organisms were only detected at a very low titre on the day after inoculation. In all the other infected quarters mycoplasmas were

detected daily until either the quarter went dry, the animal was killed or the infection was resolved. However, there was one exception, when *T-mycoplasmas* were not detected, this being from the LF quarter of cow L 91 on the 5th day after inoculation. There can be no doubt that the *T-mycoplasmas* actually multiplied in the quarters, considering the large volume of milk produced by the cows throughout the infection period and the consistently high titres of mycoplasmas recorded in the milk. The titre of mycoplasmas excreted in the milk increased to a peak that corresponded roughly to the maximum cell response and also the maximum milk and udder abnormalities. The titre of *T-mycoplasmas* and the total cell counts in the milk usually corresponded closely and indicated a close relationship between the mycoplasmas and cells. Following the inoculation of human *T-mycoplasmas*, organisms were detected only on the day following inoculation and this appeared to be due to survival of the inoculum. Except in one animal, *T-mycoplasmas* were not detected in the milk from quarters inoculated with control materials. In this one animal (L 629) the detection of *T-mycoplasmas* was not accompanied by a cellular response and we presume contamination of the milk samples occurred at milking. This failure to detect *T-mycoplasmas* in the control quarters would indicate a lack of spread of infection between quarters, in contrast to what occurs with other mastitis-producing mycoplasmas (Stuart *et al.* 1963; Hale *et al.* 1962; Jain *et al.* 1969).

There was some variation in response of these cows to inoculation of *T-mycoplasmas* unrelated to inoculum titre. Cows A 5, L 629 and M 153 reacted quickly and severely while the remainder (L 686, L 91 and M 626) reacted more slowly and on the whole less severely. It would appear that these latter three cows possessed some resistance to *T-mycoplasma* infection, the nature of which is unknown at present.

The failure of the human *T-mycoplasmas* to produce mastitis may be indicative of a species specificity, but only the REOW strain result can be considered at all significant as the M 126/68 strain was perhaps inoculated at too low a titre in a relatively resistant animal. It is, however, possible that the REOW strain had become attenuated or was an avirulent strain originally.

The evidence from cow M 626 may indicate that the A 417 strain of bovine *T-mycoplasma* is more pathogenic than the D 32 strain.

Infection of the udder with *T-mycoplasmas* did not stimulate high titre serum antibody levels. It is interesting to note that in the two cases, cows A 5 and L 629, where high titre MI antibody was not found in whey, the infected quarters eventually ceased to produce milk. In cows L 686, L 91 and M 626 whey samples gave high MI titres as a result of the infection and in the case of L 91 and L 686 this was associated with an ability of the animals to control and resolve the infection respectively. Animal M 626 was killed before the infection had run its course.

The milk and udder abnormalities together with the histopathological findings indicate that *T-mycoplasmas* produce an inflammatory reaction in the udder and furthermore the demonstration of HRP in the sera of two cows indicates that this may sometimes be of an acute nature.

We have no evidence at all that *T-mycoplasmas* are responsible for natural cases

of mastitis in cattle, although from the experimental results it is not inconceivable that they might be. From our point of view, however, the main conclusion to be drawn from this work is that the bovine udder appears to be a suitable model for studying the pathogenesis and immunity of bovine T-mycoplasma infections.

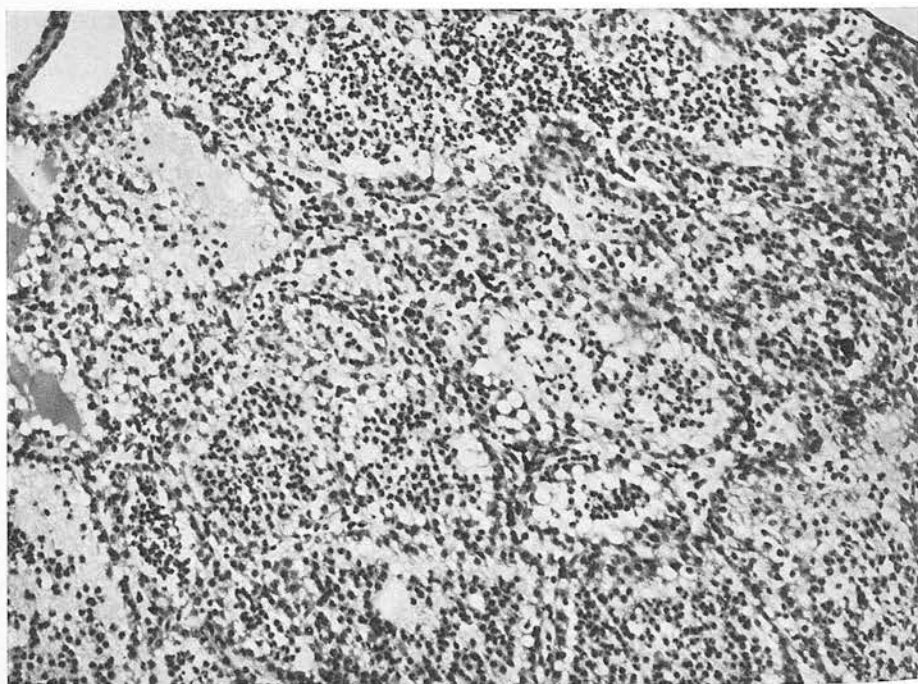
We wish to thank Miss J. Wren and Miss M. Admans for technical assistance.

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A



B

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EXPLANATION OF PLATE

- (A) Changes in the appearance of milk from the L.F. quarter of cow L 629 following the intramammary inoculation of 10⁷ uncloned A 417 T-mycoplasma culture. Left, day 0; centre, day 5; right, day 7.
- (B) Cow M 153. L.F. quarter. Histological section showing neutrophil infiltration of the alveoli, and slight interstitial hyperaemia. H and E. $\times 147$.

SEROLOGY OF BOVINE T-MYCOPLASMAS

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SUMMARY

T-mycoplasmas isolated from pneumonic calf lungs have been found to form a serologically heterogeneous group in which antigenic cross reactions between strains are common. Some strains isolated from lungs contain antigens which are also present in strains isolated from the bovine urogenital tract, eye or nose. Some bovine strains also cross react serologically with human, simian and canine strains.

INTRODUCTION

Following an earlier finding that T-mycoplasmas could be isolated from 58 per cent of pneumonic lungs, Gourlay & Thomas (1970) inoculated them endo-bronchially into calves. This resulted in the formation of pneumonic lesions from which T-mycoplasmas could be reisolated. Bovine T-mycoplasmas can also cause experimental mastitis in cows following their injection into the udder via the teat canal (Gourlay, Howard & Brownlie, 1972). They are therefore potentially pathogenic for cattle. Although T-mycoplasmas have been incriminated in urogenital tract infections of man, there is still doubt regarding their role in these conditions (Purcell, Chanock & Taylor-Robinson, 1969).

In contrast to species of classical large-colony-forming mycoplasmas, which are serologically distinct when examined by the metabolic and growth inhibition tests, human T-mycoplasmas are serologically heterogeneous. Thirteen strains isolated from man were compared by Purcell *et al.* (1969). None of the organisms were identical but all were related, sometimes by one-way reactions only. Strains which cross-reacted were isolated from both the urogenital tract and oral cavity and from clinically normal and diseased people. The only information available on the antigenic structure of bovine T-mycoplasmas is that strains isolated from the urogenital tract are not serologically identical (Taylor-Robinson, Martin-Bourgon, Watanabe & Addey, 1971).

This paper reports the results of serological studies with bovine T-mycoplasmas isolated from various sites and T-mycoplasmas isolated from other species.

MATERIALS AND METHODS

The bovine T-mycoplasma isolates examined and listed in Table I were obtained from pneumonic calf lungs, the urogenital tract of cows, the nose of a calf and the eye of a cow with keratoconjunctivitis. Strains were purified as

previously described (Gourlay *et al.*, 1972). T-mycoplasmas isolated from the urogenital tract and oral cavity of man and strains of canine and simian origin were provided by Dr D. Taylor-Robinson.

TABLE I

RELATION OF BOVINE T-MYCOPLASMAS TO EACH OTHER AND STRAINS FROM OTHER SPECIES MEASURED BY THE METABOLIC INHIBITION TEST

Strain No.	Source	Antisera raised against				Simian T	Canine T
		A417	D20	Vic 9	013		
A417	BL	++	-	-	-	-	-
D20	BL	-	++	++	-	-	-
Vic 9	BL	-	+	++	+	-	-
Gra X	BL	++	++	-	+	-	+
Mmb 137	BL	-	+	+	-	+	-
Gra 383	BL	-	-	-	-	-	-
Mmb 167	BL	-	-	-	-	-	-
F 312	BN	++	-	-	-	-	-
O 13	BE	-	++	-	++	-	-
M 525	BU	++	-	-	-	-	-
Bu 2	BU	±	-	-	-	-	-
B 101	BU	-	-	-	-	-	-
CD 573	HU	++	++	++	++	+	++
CD 343	HO	++	++	++	++	++	++
CD 408	HU	+	+	++	±	-	++
M 126	HU	±	-	+	+	-	-
REOW	HU	+	-	+	+	-	-
Simian T		++	+	++	+	++	-
Canine T		+	-	+	-	-	++

- = titre < 20
 ± = titre 20-40
 + = titre 80-160
 ++ = titre > 320

Source of strains:
 BL = bovine lung
 BU = bovine urogenital tract
 BE = bovine eye
 BN = bovine nose
 HU = human urogenital tract
 HO = human oral cavity

Antisera were raised against bovine T-mycoplasma strains grown in U broth without Hepes (Gourlay *et al.*, 1972). The antigen was harvested by centrifugation at 20,000 *g* for 30 minutes and washed three times in 0.15 M NaCl. Rabbits were given an intramuscular injection of antigen in adjuvant (4 per cent sodium alginate with 0.67 per cent calcium gluconate—Medical Alginates Ltd.) followed by a series of three intravenous injections, without adjuvant, over the next two weeks. Animals were bled four and seven days after the final injection. Antisera against the simian and canine T-mycoplasmas were provided by Dr D. Taylor-Robinson. All antisera were incubated with an equal volume of U broth for 60 minutes at room temperature prior to use in order to neutralize antibody against medium components. Antisera were inactivated by heating at 56° for 30 minutes.

With minor modifications the metabolic inhibition (MI) test (Purcell *et al.*,

1969) was used to compare strains. Doubling dilutions of antiserum 25- μ l volumes, were made in complement fixation test diluent (Oxoid) containing 5 per cent v/v guineapig serum. Equal volumes of antigen (actively growing T-mycoplasma cultures adjusted to pH 7) were added to the antisera. After 60 minutes incubation at 37° a further 100- μ l of U broth was added to the wells and the trays reincubated at 37°. The titre was taken as the reciprocal of the highest dilution of serum that prevented a change in colour of the medium compared with a control well which did not contain antiserum.

RESULTS AND DISCUSSION

The titres of the antisera against the various strains are presented in Table I. The titre of normal rabbit serum was < 20. It is recognized that apparent cross-reactions between mycoplasma strains can be caused by antibody to medium acting on medium components absorbed onto the organism. As the titres of the antisera, which had previously been incubated with medium, were found to vary from < 20 to > 1280 against the different strains, and as only some strains were affected by some of the sera, it seems unlikely that antibody against medium was responsible for the cross-reactions observed.

From our results it appears that isolates from pneumonic calf lungs form a serologically heterogeneous group and cross reactions between these strains in the MI test indicate that many of them possess antigens in common. T-mycoplasmas which cross-react serologically with the lung strains have also been isolated from the urogenital tract of cows, the eye of a cow with keratoconjunctivitis and the nose of a calf. If T-mycoplasmas were the cause of the lesions in the lung from which some of these strains were isolated, then pathogenicity does not appear to be limited to a particular serotype. The serological similarity between some strains from the urogenital tract of cows and the respiratory tract of calves is in accord with the possibility that calves become infected at parturition as they pass down the birth canal. Besides the finding that strains isolated from different sites of cattle may possess antigens in common, strains isolated from different species may also cross react in the MI test.

Species differentiation amongst the Mycoplasmatales is based largely on serological tests. Certain of these, for example the metabolic and growth inhibition tests, are highly specific and clearly delineate species. On the other hand, T-mycoplasmas are recognized as a distinct group by their small colony size, urease activity, inability to ferment carbohydrates, optimum pH for growth of 6 and their selective inhibition by erythromycin, hydroxyurea, iododeoxyuridine and certain concentrations of thallium acetate. The proteins of strains of T-mycoplasmas isolated from different species have also been found to give similar patterns in polyacrylamide gel electrophoresis (Taylor-Robinson *et al.*, 1971). It would therefore appear from our results and those of other workers that T-mycoplasmas are a serologically heterogeneous group of micro-organisms which are probably not going to be divided as precisely into species by serological methods as are the classical mycoplasmas.

ACKNOWLEDGEMENTS

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The virulence of T-mycoplasmas, isolated from various animal species, assayed by intramammary inoculation in cattle

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SUMMARY

The virulence of T-mycoplasmas for cattle was tested by examining their ability to produce mastitis in cows. It was found that both virulent and avirulent strains of T-mycoplasmas can be isolated from cattle. All of four strains from pneumonic calf lungs and a strain from a case of bovine kerato-conjunctivitis caused mastitis but only two of four strains isolated from the urogenital tract of cows were virulent. None of the human, simian or canine T-mycoplasmas examined were able to cause mastitis in cattle. However, a bovine strain was found to be capable of causing mastitis in goats. Virulent and avirulent strains from the same and different species contain common antigens detected by the metabolic inhibition test. Pathogenicity could not be shown to be characteristic of any particular serotype. The possibility is raised of some species barrier being responsible for the inability of non-bovine strains to infect cattle.

INTRODUCTION

T-mycoplasmas have been isolated from the urogenital tract of cattle (Taylor-Robinson, Haig & Williams, 1967), pneumonic calf lungs (Gourlay, Mackenzie & Cooper, 1970) and from cases of bovine kerato-conjunctivitis (Gourlay & Thomas, 1969).

The virulence for cattle of two strains of T-mycoplasmas from pneumonic lesions of calves has been demonstrated by the findings that calves inoculated endo-bronchially developed pneumonia and cows inoculated via the teat canal developed experimental mastitis. However two human T-mycoplasma strains included in these tests did not cause mastitis in cows (Gourlay & Thomas, 1970; Gourlay, Howard & Brownlie, 1972).

An explanation for these findings could be that all human T-mycoplasmas are non-pathogenic. However, since both the bovine strains examined were isolated from the lung whereas the human strains came from the urogenital tract, pathogenicity might be related to the anatomical site of colonization. Furthermore, one of the cows challenged with a human strain (animal L91, Gourlay *et al.* 1972) appeared to be partially resistant to infection with the bovine strain and there may have been some variation in susceptibility among the experimental animals. Another explanation for the failure of human strains to cause mastitis in cows is that there may be an effective species barrier.

Table 1. *Strains used and their sources*

Strain no.		Source
A417	Pneumonic calf lung	Compton
D32	Pneumonic calf lung	Compton
D20	Pneumonic calf lung	Compton
Vic9	Pneumonic calf lung	Compton
Bu2	Bovine urogenital tract	Compton
B101	Bovine urogenital tract	Compton
M525	Bovine urogenital tract	Compton
U12	Bovine urogenital tract	Compton
O13	Bovine eye	Compton
REOW	Human urogenital tract	Dr D. Taylor-Robinson
CD408	Human urogenital tract	Dr D. Taylor-Robinson
CD573	Human urogenital tract	Dr D. Taylor-Robinson
CD342	Human urogenital tract	Dr D. Taylor-Robinson
M126	Human urogenital tract	Dr B. E. Andrews
CD343	Human oral cavity	Dr D. Taylor-Robinson
Simian	Simian throat	Dr D. Taylor-Robinson
Canine	Canine urogenital tract	Dr D. Taylor-Robinson

The antigenic structure of sixteen of the seventeen strains of T-mycoplasmas used here has been reported previously (Howard & Gourlay, 1972). Strains were examined by a slight modification of the metabolic inhibition (MI) test of Purcell, Taylor-Robinson, Wong & Chanock (1966). The strains were serologically heterogeneous and the possibility existed that virulence might be a characteristic of particular serotypes. The virulence of strains of T-mycoplasmas from various anatomical sites and species was studied by intramammary inoculation of cattle to answer some of the specific questions raised concerning the pathogenicity of T-mycoplasmas.

MATERIALS AND METHODS

T-mycoplasma strains

Strains A417 and D32 were isolated from pneumonic calf lungs and have been described previously (Gourlay *et al.* 1972). Strains Vic9 and D20 were also isolated from the lungs of calves with pneumonia. O13 was isolated from the eye of a cow with kerato-conjunctivitis (Gourlay & Thomas, 1969). Strains Bu2, M525, U12 and B101 were all isolated from the urogenital tract of cows. The bovine strains were purified as previously described (Gourlay *et al.* 1972). All the other strains were obtained from Dr D. Taylor-Robinson except strain M126 (Table 1). The canine and simian strains as well as strains CD343 (Johnson) and REOW have been described by Taylor-Robinson, Martin-Bourgon, Watanabe & Addey (1971). The human strains were isolated from the urogenital tract except strain CD343 which originated from the oral cavity. The strains and their sources are listed in Table 1.

All strains were grown in U-broth without Hepes (Gourlay *et al.* 1972).

Inoculation of animals

Cows and goats in milk were inoculated via the teat canal with 10 ml of actively growing mycoplasma cultures. The number of T-mycoplasmas and the number of

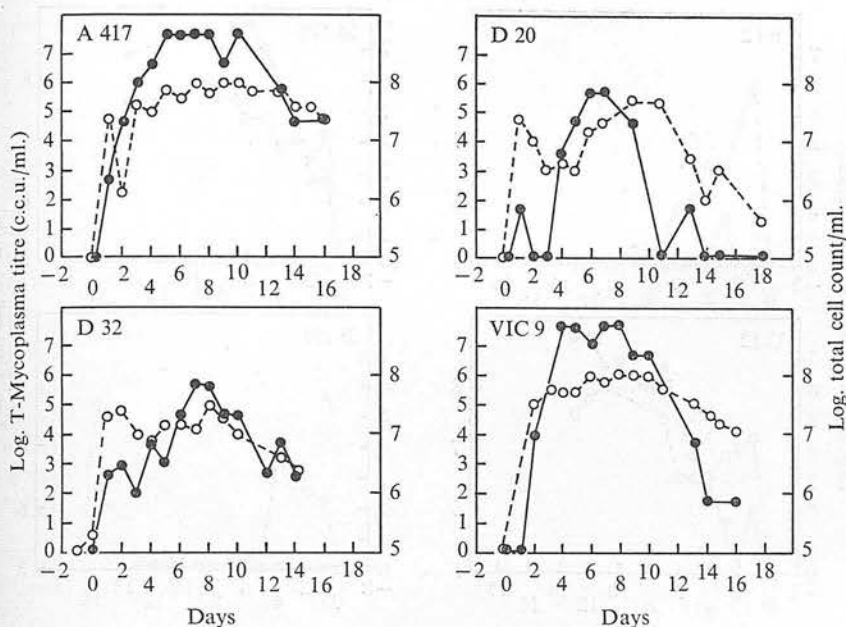


Fig. 1. Number of *T-mycoplasmas* and cells in the milk of cows inoculated with four strains from pneumonic calf lungs. Inocula: A417, $10^{5.7}$; D20, $10^{5.7}$; D32, $10^{5.7}$ and Vic9, $10^{5.7}$ c.c.u./ml. ●—● *T-mycoplasmas*; ○—○ cells.

cells present in milk were measured as previously described (Gourlay *et al.* 1972) except that the *T-mycoplasma* titre was recorded as the 50% endpoint (Gourlay & Domermuth, 1967). The criteria used to determine whether strains produced mastitis following intramammary inoculation were the continued increase in the number of cells in milk associated with the consistent reisolation of *T-mycoplasmas* from milk. Strains which did not cause infection were tested in at least two animals. Each animal was inoculated at the same time in another quarter with strain A417 as a control. The possibility of a concurrent bacterial mastitis occurring was excluded by spreading blood agar plates with milk and examining them for bacterial colonies.

RESULTS

Strains from calf lungs

Of 20 cows inoculated with strain A417, 19 developed mastitis. In one case the animal appeared to be partially immune to infection (animal L91, Gourlay *et al.* 1972). Another cow was found to be refractory to infection with strain A417, although it was susceptible to infection with the bovine urogenital strain U12. A typical response to strain A417 is shown in Fig. 1. The maximum *T-mycoplasma* titre and number of cells in the milk occurred about 5–10 days after injection. *Mycoplasmas* have been found to be excreted for as long as 6 months after inoculation, the longest time studied. The lowest dose of actively growing *T-mycoplasmas* that has been inoculated was 10^4 /ml. strain A417, and this caused mastitis.

The response of cows to inoculation with three other *T-mycoplasma* strains isolated from cases of calf pneumonia is shown in Fig. 1. All of these strains caused

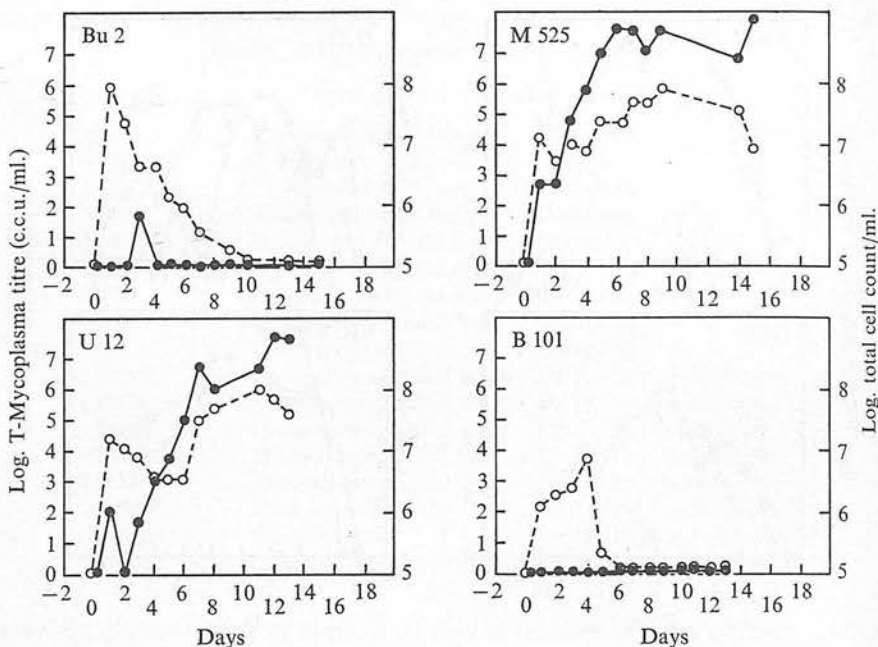


Fig. 2. Number of T-mycoplasmas and cells in the milk of cows inoculated with four strains from the urogenital tract of cows. Inocula: Bu2, $10^{6.7}$; M525, $10^{6.7}$; U12, 10^7 and B101, $10^{6.7}$ c.c.u./ml. ●—● T-mycoplasmas; ○—○ cells.

mastitis, but they varied in their ability to persist and multiply in the udder. Infection with strain D20 was resolved rapidly compared to infection with A417. Infection with all of these strains, except D20, caused the milk to become yellow and produced clots and could thus be considered to have caused clinical mastitis. Strain D20 caused only subclinical mastitis.

Strains from the urogenital tract of cows

Four strains isolated from the urogenital tract of cattle were tested for virulence. Two of these four strains, U12 and M525, proved virulent on intramammary inoculation and caused the milk to become yellow and produced clots. The number of milk cells and T-mycoplasmas found in the milk after inoculation is shown in Fig. 2. The infection was as severe as that caused by strains isolated from the lung. Two of the urogenital strains examined, B101 and Bu2, were avirulent, the response to their injection was essentially similar to that produced by non-viable cells of strain A417 (Gourlay *et al.* 1972). Inoculation of B101 and Bu2 caused a transient cell response in the milk (Fig. 2).

A T-mycoplasma strain from the eye of a cow

The results of inoculation with strain O13 are shown in Fig. 3. This strain caused clinical mastitis as evidenced by the yellow milk produced subsequent to injection.

T-mycoplasmas isolated from man

Six human T-mycoplasmas listed in table 1 have been tested for their ability to cause mastitis in cows. Five of the strains were from the urogenital tract and one

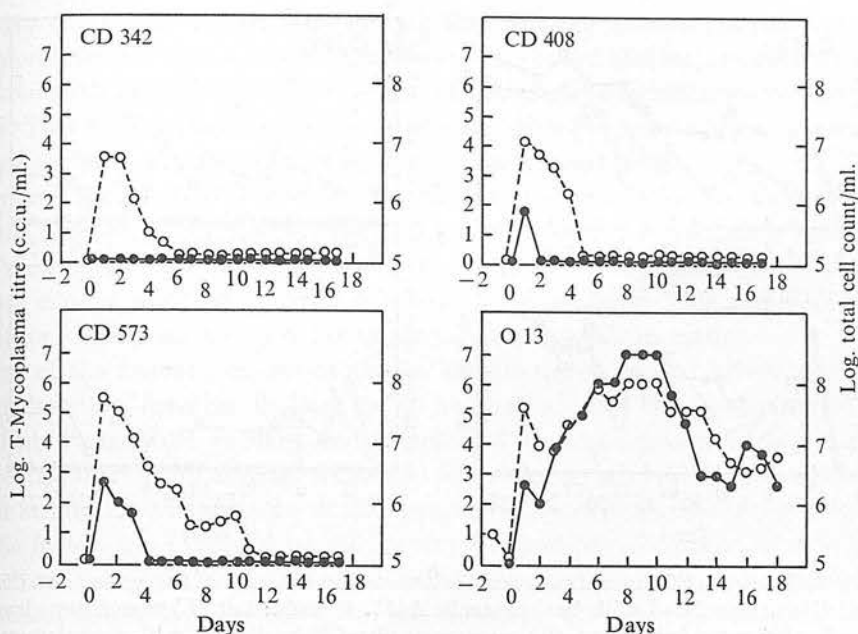


Fig. 3. Number of *T-mycoplasmas* and cells in the milk of cows inoculated with three human strains and a strain (O13) from the eye of a cow. Inocula: CD342, $10^{6.7}$; CD408, 10^6 ; CD573, $10^{6.7}$ and O13, $10^{5.7}$ c.c.u./ml. ●—● *T-mycoplasmas*; ○—○ cells.

was from the oral cavity (CD343). All the strains were inoculated into at least two different cows. None of the human strains was virulent for cattle. The response induced by three of the human *T-mycoplasmas* is shown in Fig. 3. In all cases on the day after injection a cell response was found in the milk but the high level of cells did not persist. *T-mycoplasmas* were sometimes isolated on the first or even second and third day after inoculation but this was considered to be due to the persistence of the inoculum. No gross milk changes or udder abnormalities were observed following inoculation with human *T-mycoplasmas*. The response of cows to human strains was essentially the same as that produced by inactivated bovine *T-mycoplasma* strain A417 (Gourlay *et al.* 1972) and by the avirulent bovine strains.

Simian and canine T-mycoplasmas

Neither of these strains caused mastitis in cows. The type of response produced by their inoculation was identical with that of human *T-mycoplasmas* and the avirulent bovine strains. Neither of the strains multiplied in the udder and they were not re-isolated from the milk. The cell response induced was maximal on day one after injection and thereafter the number of leucocytes in the milk gradually declined. No gross milk or udder abnormalities were observed.

Infection of goats with bovine T-mycoplasma

Goats were challenged in the same way as cows with strain A417. An infection with clinical signs of mastitis was produced in four out of four animals. An increase

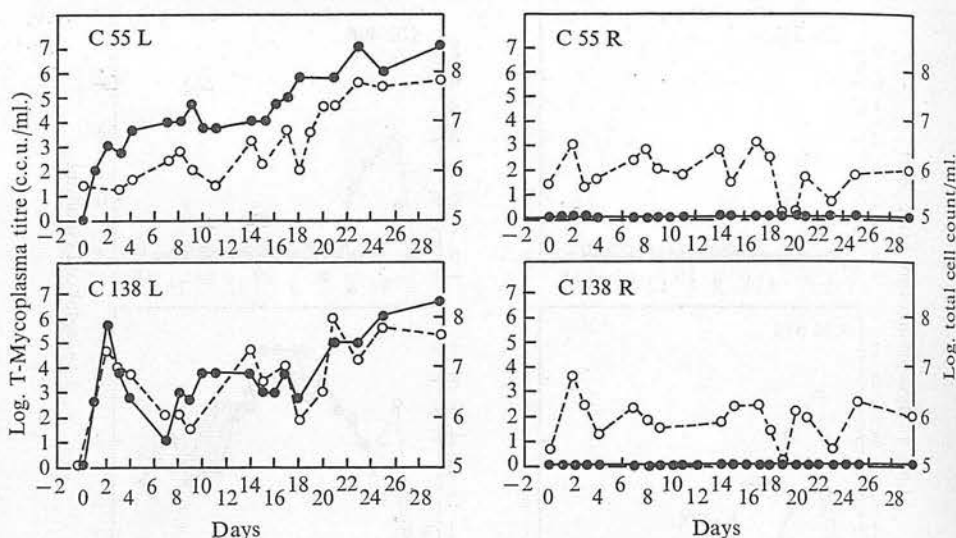


Fig. 4. Number of T-mycoplasmas and cells found in the milk of two goats, nos. C55 and C138, inoculated with bovine strain A417. Inocula: left (L) mammary gland A417, $10^{4.7}$ c.c.u./ml.; right (R) mammary gland U-broth. ●—● T-mycoplasmas; ○—○ cells.

in the number of leucocytes in the milk was observed and T-mycoplasmas were reisolated from the milk samples (Fig. 4). The number of cells found in control glands injected with U-broth was higher than in cows. The infections caused an increase in the number of cells present and gross milk changes were apparent.

DISCUSSION

All four T-mycoplasmas isolated from pneumonic calf lungs and the strain from a case of bovine kerato-conjunctivitis were found to be virulent for cattle. However, both virulent and avirulent T-mycoplasmas have been isolated from the urogenital tract of cows. Pathogenicity is not therefore a specific feature of bovine strains isolated from a particular site.

It is possible that the urogenital tract of cows acts as a reservoir of T-mycoplasmas. The upper respiratory tract could become infected as calves pass down the birth canal. Klein, Buckland & Finland (1969) considered that the oral cavity of babies can become infected with T-mycoplasmas during parturition.

Although T-mycoplasmas have been incriminated in urogenital tract infections of man, there is still doubt regarding their role in these conditions (Shepard, 1969; Taylor-Robinson, 1971; Ford, 1970) and it has been suggested that T-mycoplasmas may usually be commensals in man (Klein *et al.* 1969; Biberfeld, 1971) and in the urogenital tract of bulls (Taylor-Robinson, Thomas & Dawson, 1969).

The possibility that T-mycoplasmas may be of aetiological significance in calf pneumonia has been suggested by their isolation from 58% of pneumonic calf lungs (Gourlay *et al.* 1970) but not from non-pneumonic lungs (Thomas & Smith, 1972). Furthermore, they cause pneumonia in calves inoculated endobronchially

(Gourlay & Thomas, 1970). The finding that all four strains isolated from calf pneumonia were virulent, unlike the bovine urogenital strains, is consistent with the possibility that *T-mycoplasmas* are of aetiological significance in calf pneumonia. This finding may be the result of a selective pressure being present in the respiratory tract which is not present in the urogenital tract.

Taylor-Robinson (1971) reported that the inoculation of a bovine *T-mycoplasma* into the urethra of a Caesarian-derived pathogen-free bull-calf caused infection but failed to produce disease. However, bovine *T-mycoplasmas* are capable of causing clinical mastitis in cows infected experimentally. This group of micro-organisms should not be regarded as merely commensals in cattle.

None of the human, canine or simian strains tested caused infection in cows. An explanation for this finding could be that all the strains examined were avirulent. Since both virulent and avirulent *T-mycoplasmas* have been isolated from cattle there is by analogy no reason for assuming that all human, simian and canine strains are avirulent *per se* and an alternative explanation is that some host specific factors are involved which prevent the non-bovine strains from infecting cows. However, since the bovine A417 strain was capable of causing experimental mastitis in goats, host specificity is not absolute, although specific strains may only be able to infect a limited range of animals. The findings reported by Taylor-Robinson *et al.* (1971) that only human *T-mycoplasmas* adsorbed to HeLa cells, not bovine, simian or canine strains, and only simian strains adsorbed to chicken erythrocytes indicates that some specificity exists in cell adsorption by *T-mycoplasmas*.

Human and bovine *T-mycoplasmas* have been found to be serologically heterogeneous. Strains which contain common antigens can be isolated from various anatomical sites and from normal or diseased conditions (Ford, 1967; Purcell, Chanock & Taylor-Robinson, 1969; Taylor-Robinson *et al.* 1969; Howard & Gourlay, 1972). Furthermore, strains from different animal species have been found to cross react in the MI test (Howard & Gourlay, 1972).

Since strains that had been reisolated from milk reacted with the antisera in the same way as they did before injection, the antigenic structure of the organisms is apparently a stable characteristic.

Sixteen of the strains tested for virulence have been examined by the MI test for cross-reacting antigens using antisera raised against six of the strains (Howard & Gourlay, 1972).

Both virulent and avirulent bovine *T-mycoplasmas* possess common antigens. Moreover, strains from other species which contain antigens present in virulent bovine *T-mycoplasmas* are avirulent for cows. Judging from the results presented here, no particular serotype, as indicated by the MI test, appears to be characteristically pathogenic.

The results reported by Taylor-Robinson *et al.* (1971), noted above, indicated differences in the cell adsorptive properties of strains from different species. Our results indicate that important differences between strains from the same and different animal species exist which affect the virulence of strains for a particular species of animal.

We would like to thank Dr D. Taylor-Robinson of the M.R.C. Clinical Research Centre, Harrow and Dr B. E. Andrews of the Mycoplasma Reference Laboratory, Colindale for providing strains, and Miss J. Wren, Miss S. Wyld and Miss M. Admans for excellent technical assistance.

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March 21, 1970)

Isolation of a Virus infecting a Strain of *Mycoplasma laidlawii*

MYCOPLASMAS are a group of microorganisms which can grow on cell-free medium and which are characterized by pleomorphism, for they are bound by a triple layered unit membrane instead of a rigid cell wall. The smallest reproductive forms are about 100 to 150 nm in size. They are the smallest known free-living organisms comparable in size with the myxoviruses, to which they show some morphological resemblance. The largest mycoplasma cell can exceed 1,000 nm in size.

In view of the heterogeneity and wide host range of viruses, it seemed most unlikely that the biological characteristics of the mycoplasma cell would make the whole group resistant to infection with these agents. It was decided to investigate the susceptibility of mycoplasmas isolated from bovine nasal passages to virus attack. The bovine nasal passage was thought to be a favourable site for the multiplication and interaction of various mycoplasma species and strains. Virus screening was carried out by adding filtrates from other mycoplasma isolates to test mycoplasma. This report describes the isolation of a virus which infects a strain of *Mycoplasma laidlawii*.

Broth cultures of different isolates of mycoplasmas were prepared. These were not necessarily pure cultures, for many were recent isolates and not yet cloned. Aliquots of these were filtered through 'Millipore' 200 nm membranes. The filtrates were tested by adding a drop to solid medium plates seeded with each of the other mycoplasma cultures. The plates were examined after incubation for 24-48 h at 37° C, and showed a confluent lawn of host mycoplasmas; there was one exception where a distinct, clear zone of inhibition or lysis occurred at the site of application of the drop of filtrate. This zone, which covered the same area as the filtrate drop, was made up of a central partially clear area surrounded by a clear periphery with a partially clear halo on the outside.

The filtrate containing the inhibitory or lytic agent was completely inactivated on exposure to a temperature of 100° C (but not 60° C) for 30 min and ultraviolet light. It was sensitive to 5 per cent chloroform for 10 min but insensitive to 20 per cent ethylether at 4° C for 18 h. The agent, harvested from lawns of the host mycoplasma and diluted in phosphate buffered saline (PBS), pH 7.3, containing 10 per cent foetal calf serum, was serially filtrated through 'Millipore' membrane filters of decreasing pore size. It passed readily through filters of 10 nm pore size.

The agent in the initial filtrate was titrated in PBS in

ten-fold dilutions and produced single isolated areas or plaques about 2-3 mm in diameter at high dilutions (titre of filtrate 2.5×10^9 plaque forming units/ml.) on a lawn of the host mycoplasma. At lower dilutions these discrete areas coalesced to form one large zone (Fig. 1). Addition of a high dilution of the agent to broth cultures of growing mycoplasmas resulted in a 4-5 log increase in titre of the agent. Furthermore, a culture containing the agent in which the mycoplasmas had been inactivated by heating at 60° C for 30 min was subcultured on the host mycoplasma on solid medium 8 times. Each time a plaque was subinoculated into PBS and heated at 60° C for 30 min; the final subculture was equivalent to at least a 10^{-33}

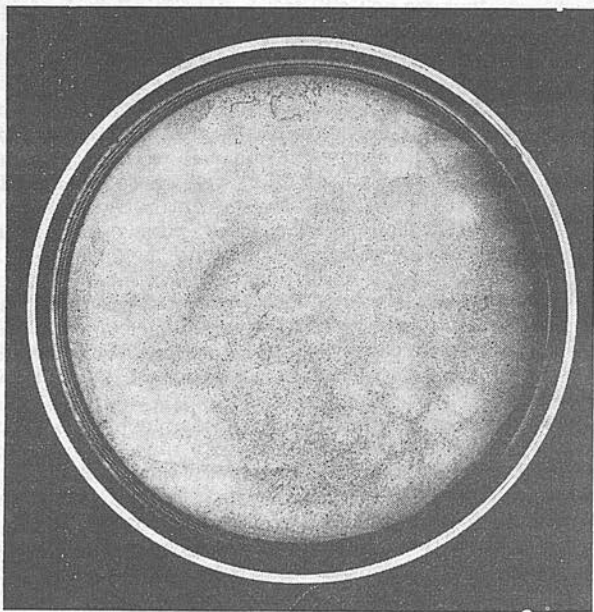


Fig. 1. Plaques formed by serial ten-fold dilutions of virus on *Mycoplasma laidlawii* strain BN1 culture plates. Stained neutral red.

dilution of the original mycoplasma culture. There was no evidence of replication of the agent in the absence of actively growing cells of the specific mycoplasma. It therefore seems that this agent possesses properties consistent with those of a virus.

The culture responsible for the host mycoplasma lawn was inoculated on to solid medium. It was purified by picking a single colony three times. The cloned culture (strain BN1) continued to receive the virus. This was apparent on a lawn of the cloned strain from the formation of plaques at the site of virus application. The cloned culture was able to grow at 22° C in serum-free medium and in the growth inhibition test¹ it was inhibited by

antiserum to *M. laidlawii*.

This is the first report of the isolation of a virus which infects mycoplasmas. There has been a previous report², however, of small electron-dense bodies about 25-30 nm resembling intracellular bacteriophage in electron micrographs of the EAY strain of human mycoplasma, but, as far as is known, no virus was isolated then or subsequently. Evidence from the filtration experiments indicates that a viable unit of this virus can be exceedingly small.

I thank Mr I. Jebbett for the photograph and Miss Sara Wyld for technical assistance.

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¹ Clyde, W. A., *J. Immunol.*, **92**, 958 (1964).

² Swartzendruber, D. C., Clark, J., and Murphy, W. H., *Bact. Proc.*, 151 (1967).

Characterization of Mycoplasmatales Virus *laidlawii* 1

A RECENT report¹ described for the first time the isolation of a virus that infects a species of mycoplasma. This agent, now designated MVL1, has been purified, examined in the electron microscope and its nucleic acid type determined. Cultures of *M. laidlawii* strain BN1 (ref. 1), grown in glucose serum broth or agar², were inoculated with virus during their log phase and incubated at 37° C for 18-24 h. On solid medium, plaques were observed in the mycoplasmal lawn from which virus was harvested by flooding the plate with phosphate buffered saline (PBS), pH 7.3, for 3-5 h. The titre of the virus was estimated as previously reported¹.

Because the virus adsorbs strongly to disrupted host cells (unpublished observation), the crude virus suspension was treated with 0.4% 'Nonidet P40' to solubilize the lipoprotein membranes. The virus was then pelleted through 10% sucrose by centrifugation at 230,000g for 2 h, resuspended in PBS and incubated with 100 µg trypsin/ml. for 1 h at 25° C. This step in the purification procedure significantly decreases the contamination of the virus by cellular and serum proteins. The trypsin digestion was terminated by the addition of 100 µg of soybean trypsin inhibitor per ml. and CsCl added to raise the density to 1.37 g cm⁻³. The density gradient formed after centrifugation at 192,500g for 17 h was fractionated into 50 aliquots and the virus was located by infectivity assay and optical absorbance at 260 and 280 nm. The fractions containing the virus were pooled and recentrifuged to produce a second CsCl gradient. The virus was located as before and the single fraction containing the highest infectivity was examined by electron microscopy. The purified virus preparation, supported on carbon-collodion coated grids, fixed with

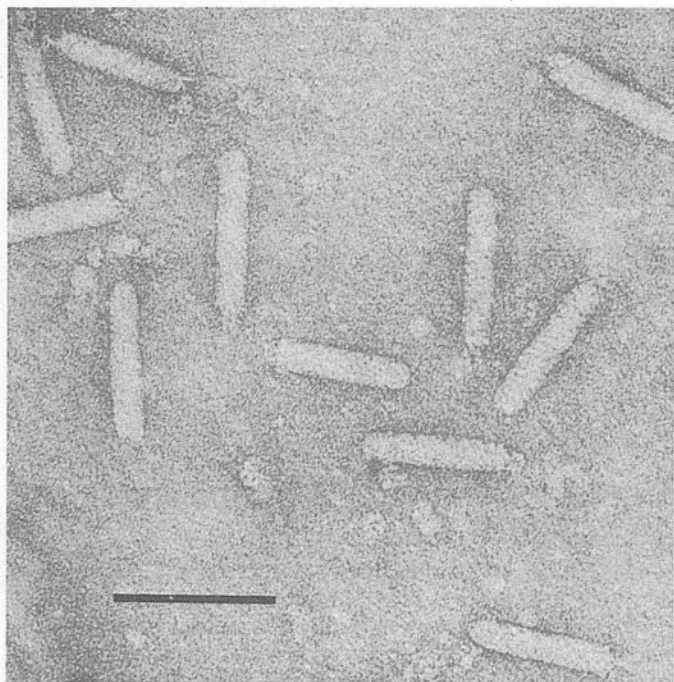


Fig. 1 Purified virus preparation, negatively stained with uranyl acetate. $\times 209,000$. Bar equals $0.1 \mu\text{m}$.

3% glutaraldehyde in 0.1 M phosphate buffer and stained with 1.5% uranyl acetate, was examined in an AEI EM6 electron microscope. The only structures visible were numerous short rigid rods with rounded ends and occasionally a hollow centre (Fig. 1). The dimensions of 40 single rods were measured on the photographic plate relative to the lattice spacing of beef liver catalase crystals fixed in 3% glutaraldehyde. The mean diameter was 14.6 nm (s.d. 1.9 nm) and mean length 89.8 nm (s.d. 10.0 nm), with a few particles up to 400 nm in length. In appearance, these rods closely resembled those seen attached to cellular membranes or lipoprotein strands in crude preparations of infected *M. laidlawii*. No similar particles have been seen in lysed uninfected control *M. laidlawii* cultures. Earlier filtration studies¹ indicated that the virus was small enough to pass easily through membrane filters of 10 nm mean pore size. But since that time the manufacturers (Millipore Filter Corp.) have stated that the pore size of this filter is in fact 25 nm.

To determine the type of nucleic acid present in the particles, virus was grown as described above on medium containing either ^3H -thymidine or ^3H -uridine (each at a specific activity of 1,000 mCi/mmol and a concentration of 10 μM). DNA was not added to the medium containing ^3H -thymidine. Following purification, the fractions from the second CsCl density gradient were analysed for their tritium content with a liquid scintillation counter. Radioactivity was found only in association with virus grown in the presence of labelled thymidine, suggesting that the virus contains DNA. To rule out the possibility that this was from free mycoplasmal DNA of the same buoyant density as the virus, the fraction containing the greatest amount of virus was incubated with deoxyribonuclease, at a concentration of 10 $\mu\text{g/ml}$. for 1 h at 37° C. Following this treatment no decrease in virus infectivity nor acid-precipitable radioactivity was found, showing that the DNA was protected from enzymic digestion, probably within the virion.

Certain viruses have a bacilliform morphology³ but MVL1 differs from these in that those known to contain DNA—some of the arthropod viruses—are surrounded by clearly visible membranes from which the nucleoprotein is readily separable. We have seen no structures resembling viral membranes surrounding the MVL1 virions and the particle morphology does not change after purification or treatment with detergents and trypsin. It is therefore of interest not only that this virus infects a mycoplasma but also that it occupies a position hitherto unfilled within the scheme of virus classification based primarily on morphology and nucleic acid type.

We thank Miss Sara Wyld for technical assistance.

Mycoplasma laidlawii has recently been renamed *Acholeplasma laidlawii*.

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¹ Gourlay, R. N., *Nature*, **225**, 1165 (1970).

² Gourlay, R. N., and Leach, R. H., *J. Med. Microbiol.*, **3**, 111 (1970).

³ Mattern, C. F. T., in *The Biochemistry of Viruses* (edit. by Levy, H. B.), 55 (Marcel Dekker, New York and London, 1969).

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Typical symptoms caused by mycoplasmas in plants are witches' broom growth, greening of flowers and yellowing of leaves. Nothing is known how the plant metabolism is influenced after infection by mycoplasmas. They are transmitted from plant to plant by biting insects, by grafting and by cuscuta, a parasitic plant.

Ultrastructural Studies of *Mycoplasmatales* Viruses. R. N. GOURLAY, Institute for Research on Animal Diseases, Compton, Newbury, Berkshire (U.K.).

We have isolated 2 fundamentally different *Mycoplasmatales* viruses. *Mycoplasmatales* Virus-laidlawii 1 (MV-L 1) [Nature New Biology 229, 118 (1971)], produces plaques on 11 out of 19 strains of *Acholeplasma laidlawii* and serologically similar virus has been isolated from 4 different strains of *A. laidlawii* including BN 1. MV-L 2 [J. gen. Virol. 12, 65 (1971)] produces plaques on 6 out of 19 strains of *A. laidlawii* and a serologically similar virus has been isolated from 2 strains of *A. laidlawii*. Neither virus produces plaques on any other *Mycoplasma* species tested. The production of turbid plaques and clones of *A. laidlawii* resistant to both viruses, indicate, that lysogeny occurs.

Electronmicroscopic examination of MV-L 1 (in preparation) shows straight or slightly curved rods with one end rounded and the other end either rounded, flat or possessing short protuberances. Complete rods average 90 nm in length; others 80 nm. Mean diameter is 16 nm. Some virions appear hollow. Long rods exceeding 1 μ m in length are sometimes seen: these have a diameter of 16 nm but rods of 30 nm diameter are occasionally seen.

Electronmicroscopic examination of unpurified MV-L 2 shows roughly spherical apparently enveloped bodies about 50–120 nm in diameter [J. gen. Virol. 12, 65 (1971)].

The sensitivity of MV-L 2 to detergent and ether and the resistance of MV-L 1 to these agents supports the EM evidence for the presence and absence of an envelope on MV-L 2 and MV-L 1, respectively.

Properties of *Mycoplasmatales* Viruses. J. MANLOFF and A. LISS, Departments of Microbiology and of Radiation Biology and Biophysics, University of Rochester Medical Center, Rochester, New York 14642 (U.S.A.).

Fifteen *Mycoplasmatales* viruses have been isolated; nine from *A. laidlawii* strains, one from an *M. goat* strain, one from an *M. pneumoniae* strain, two from rabbit anti-*Mycoplasma* sera, one from leafhoppers carrying corn stunt disease agent, and one from an *A. strain* isolated from stunt diseased corn. Three viruses were characterized and found to be different: these are MVL 1, the original isolate of Gourlay; MVL 52, isolated from *A. laidlawii* B; and MVL 51, from *M. sp.* strain 14 (goat). The three viruses follow one-hit UV inactivation kinetics and each has a different inactivation cross-section. The one-step growth kinetics of the viruses were different: for MVL 1, the burst time is 60 min and the burst size is 3; for MVL 52, the time is 180 min and size is 80; and for MVL 51, 150 min and 5. Using seven different *laidlawii* strains, the host-range for each virus was determined. MVL 1 and MVL 51 have a similar range, which is different from MVL 52. Electron microscopic studies of five viruses show them all to be bullet-shaped particles. Optical analysis of the micrographs indicates that the viral structure units are arranged with helical symmetry. Viral plaques on some hosts are turbid, and on others are clear. Two virus-resistant clones have been isolated; in each the resistance mechanism seems to be different. Streptomycin resistance is acquired along with virus resistance. It is probable that both lytic and lysogenic viral infections occur.

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Ultrastructure of Mycoplasmatales Virus laidlawii 1

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(Accepted 12 April 1972)

SUMMARY

The morphology and ultrastructure of Mycoplasmatales virus laidlawii 1, a new helical DNA virus, were studied by electron microscopy and optical diffractometry. Numerous small unenveloped rod-like particles and a few longer forms were seen in purified, concentrated preparations of infective virus, and associated with virus-infected mycoplasmal cells in culture. Observations on these virus particles are discussed in relation to their structure.

INTRODUCTION

A recent report (Gourlay, 1970) described the isolation of a virus, subsequently named Mycoplasmatales virus laidlawii 1 (MV-L1), which infects *Acholeplasma laidlawii*, a member of the Mycoplasmatales. A later communication (Gourlay, Bruce & Garwes, 1971) described the purification of the virus which was found to be a short rod probably containing DNA. This paper presents further morphological studies on the virus both in purified preparations and in association with mycoplasmal cells.

METHODS

The host, *A. laidlawii* strain BNI, was grown in modified glucose serum broth or agar (Gourlay & Wyld, 1972). To recover and purify virus, solid medium cultures of infected cells were incubated at 37° for 24 hr and then washed with phosphate buffered saline. The suspension obtained was treated with Nonidet P40 to dissolve host membranes before concentration through two caesium chloride density gradients (Gourlay *et al.* 1971).

Mycoplasma cells were grown in broth. They were infected during the early log phase of growth and incubated at 37° for up to 10 hr. Cells were harvested by centrifuging at low speed and, to prevent lysis, were fixed for 3 to 5 min. with 3 % glutaraldehyde in 0.1 M-sodium cacodylate buffer solution at pH 7.0.

For electron microscopy, purified virus and infected cells were applied to carbon-collodion coated grids, washed thoroughly with 0.1 M-sodium cacodylate or 2 % ammonium acetate at pH 7.0 before negatively staining for a few seconds with either 2 % potassium phosphotungstate at pH 6.5 or 2 % uranyl acetate at approximately pH 4.5. A 1 % solution of uranyl formate at pH 3.5 was sometimes used for studies at high resolution. The dimensions of the virus particle were measured from photographic enlargements of accurate magnification, calibrated with reference to the lattice spacing of crystalline beef liver catalase (Koch Light Laboratories Ltd), fixed in 3 % glutaraldehyde and applied to the grid with the specimen.

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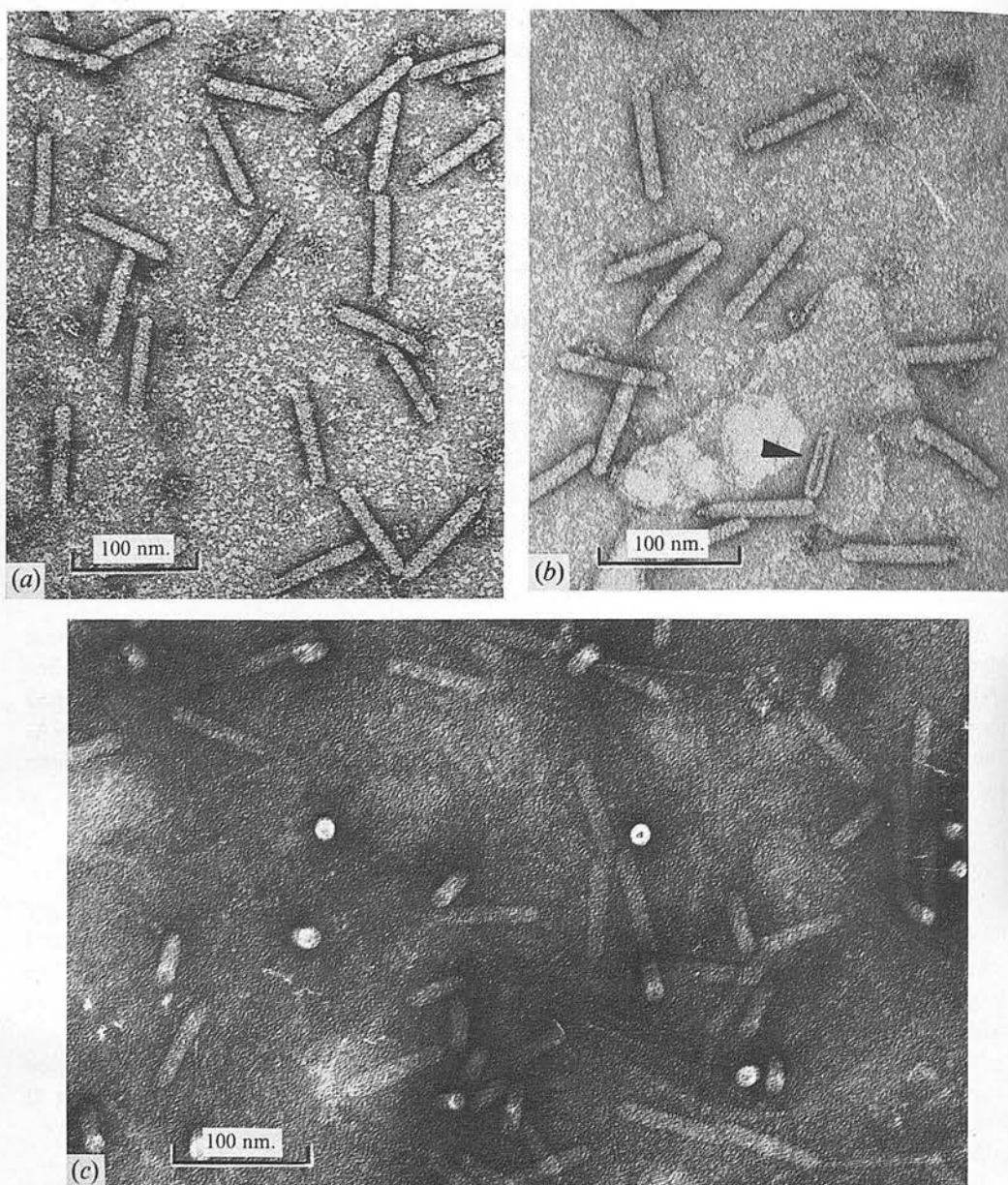


Fig. 1. Purified preparations of MV-L1, negatively stained with 2 % uranyl acetate. (a) showing degradation at one end of some particles. (b) arrow indicates particle penetrated by stain. (c) upright particles, some showing stain penetration into centre.

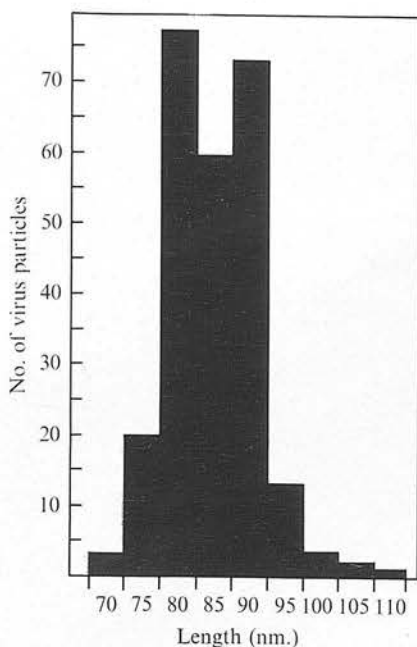


Fig. 2. Histogram of lengths of 320 rods, with peaks at approximately 80 and 90 nm.

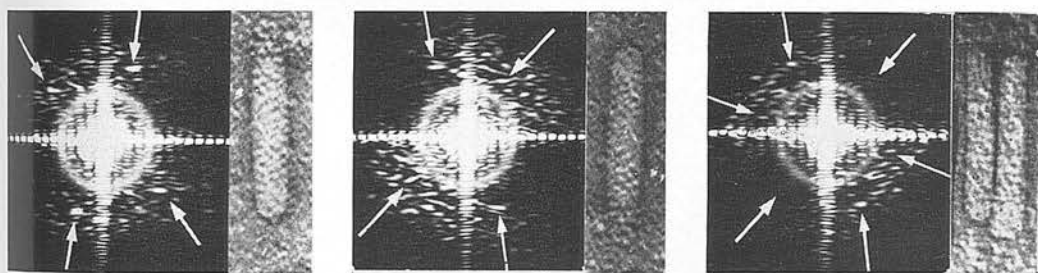


Fig. 3. Optical transforms from electron micrographs of MV-L1, negatively stained with 2% uranyl acetate. Arrows indicate the spots found consistently in diffraction patterns.

Examinations were made with an AEI EM6 electron microscope at an accelerating voltage of 80 kV and instrumental magnification of 20,000 to 100,000 \times .

Optical transforms of electron micrographs were made as described by Markham (1968).

RESULTS

Negatively stained preparations of purified, concentrated virus showed numerous straight or slightly curved rods (Fig. 1*a, b, c*). Nearly all such particles were rounded at one end; usually the other end was rounded also but was sometimes flat or visibly degraded to one or two short protuberances. Measurements of particles showed two modal lengths: the average length of complete rods was 90 nm. and of the others 80 nm. (Fig. 2). The mean end-on diameter of particles was 16 nm. as measured from upright particles (Fig. 1*c*). However, rods lying flat on the mounting film showed a mean diameter of 14.5 nm. This discrepancy is probably due to overlapping stain which masked the diameter of flat particles. Available

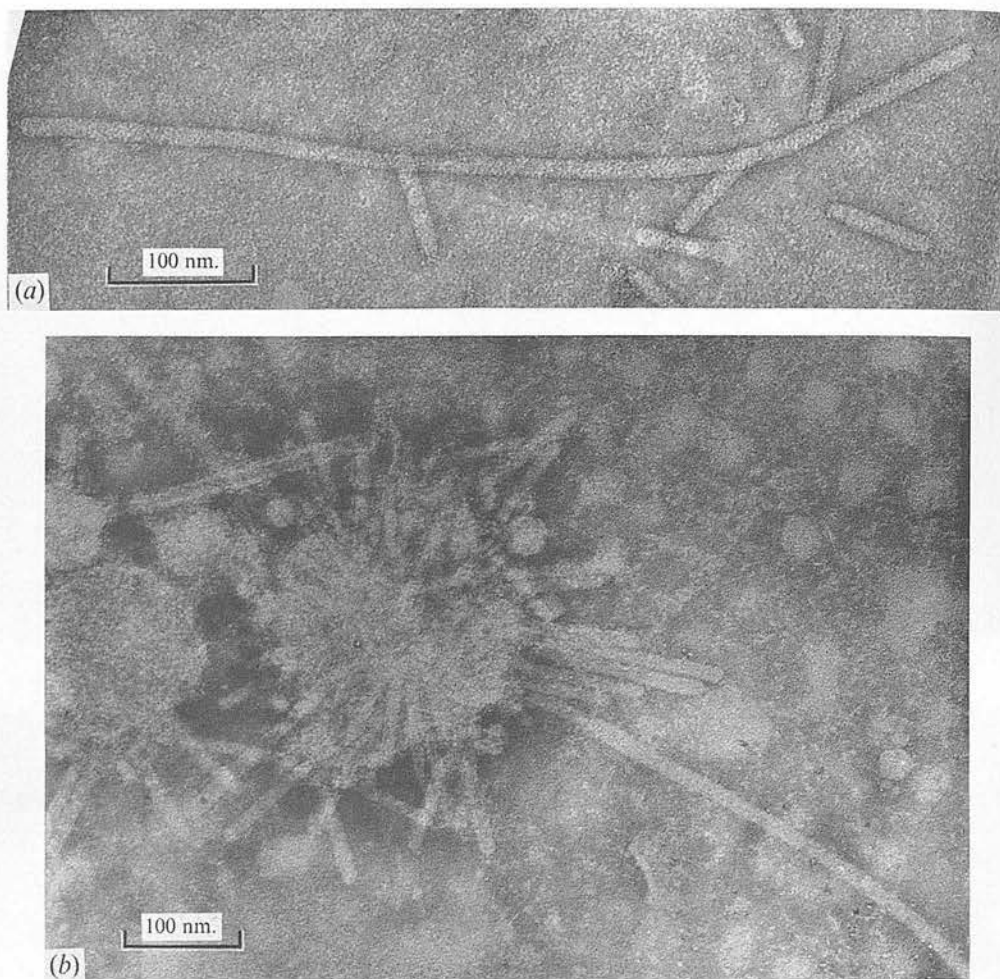


Fig. 4. (a) Purified preparation of virus showing particles of normal morphology and one long form. Negatively stained with 2 % uranyl acetate. (b) Mycoplasma cell showing attached virus; one apparently hollow rod is shown. Negatively stained with 2 % potassium phosphotungstate.

concentrations of virus were insufficiently high for measurement of centre to centre spacings in close packed arrays of particles.

When stained with uranyl salts, especially uranyl formate, some particles were penetrated by stain (Fig. 1*b*), thus indicating hollow centres and ends normally sealed; wall thickness measurements were from 5.2 to 6.2 nm.

Difficulties were experienced in obtaining clear optical diffraction patterns from electron micrographs of particles. This was probably due to the small size of the particles and the limited repetition of structure. However, certain intense spots were found consistently (Fig. 3) and indicated diffracting elements arranged in a hexagonal lattice forming a helix of pitch angle about 20° . Using catalase as a standard for measurement, the lattice spacing was about 4.8 nm.

Long curved rods, exceeding 500 nm. in length, appeared occasionally in purified cultures (Fig. 4*a*). Their diameters and optical diffraction patterns were similar to those of the short

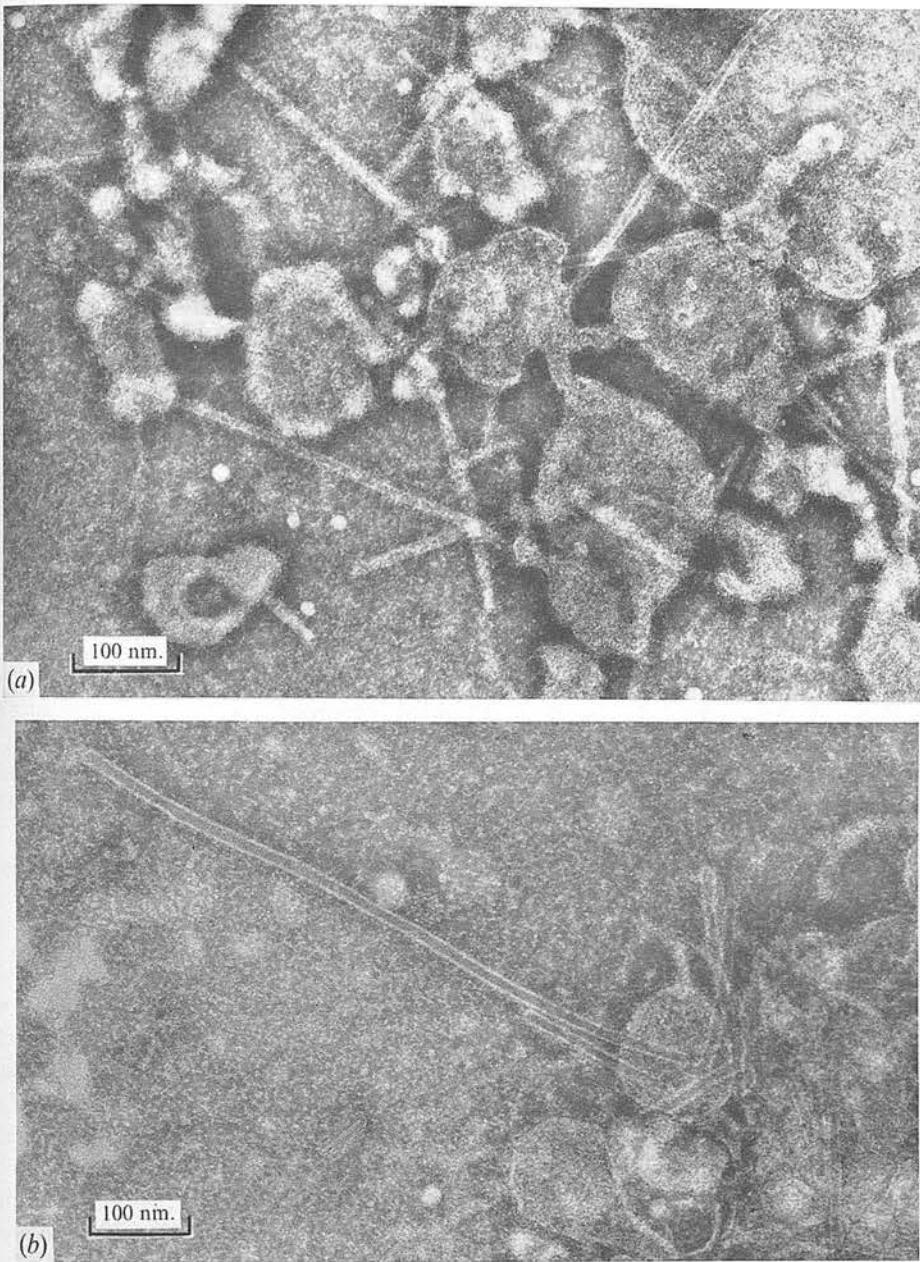


Fig. 5. (a) Cells of *Acholeplasma laidlawii* from broth culture with associated long forms. Negatively stained with 2 % potassium phosphotungstate. (b) A hollow form of greater diameter, that is 30 nm.

particles; it is presumed that they are nucleoproteins since their buoyant density was the same as that for particles in infective preparations.

Similar long rods were observed attached to mycoplasmal cells in centrifuged deposits of infected broth cultures. Numerous particles of length between 100 and 500 nm. were attached by one end to cellular membranes (Fig. 4b) and one of these rods is penetrated by

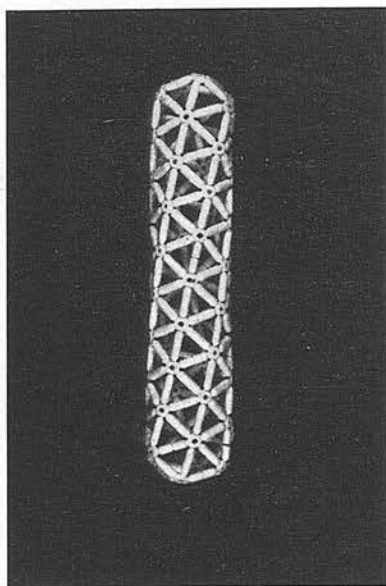


Fig. 6. Proposed model of virus structure showing two-start helix of hexagonally arranged subunits.

stain. Other samples of centrifuged deposits yielded numerous apparently hollow long rods (Fig. 5*a*) of diameter similar to that of normal rods. These long rods appeared most frequently in cultures of cells inoculated with virus late in log phase and in those incubated for over 4 hr after infection. Less frequently we found rods with greater diameter (about 30 nm.) and wall thickness about 7.5 nm. (Fig. 5*b*).

Deposits from cultures of *A. laidlawii* which had not been inoculated with virus showed no rods of the types described.

DISCUSSION

Our results suggest that MV-L1 is an unenveloped and helically symmetrical DNA virus which is probably the first of this type to be described. The normally occurring particle in infective preparations is a slightly sinuous rod of 16 nm. diameter and 90 nm. length. Optical diffraction studies indicate that the tubular portion of the particle consists of subunits arranged in hexagons to form a helix. Under normal staining conditions the lattice spacing from optical diffraction patterns of hexagonally arranged protein subunits has been shown to be half the centre-to-centre spacing of the hexagons (Bancroft, Hills & Markham, 1967; Hitchborn & Hills, 1968); on this basis the centre-to-centre spacing of the protein hexagons of MV-L1 particles is approximately 9.6 nm. Hull, Hills & Markham (1969) demonstrated that it was possible to form the hemispherical ends of bacilliform particles by using half icosahedra and that the structure of the tubular part depended upon the axis across which the icosahedron was cut. The structure deduced from the optical diffraction patterns of MV-L1 is consistent with one based upon a 12 morphological subunit icosahedron ($T = 1$) cut across its two-fold axis. This gives a particle of 16 nm. diameter and a tubular portion consisting of morphological subunits arranged in a two-start helix with 5.6 subunits per turn on a pitch angle of about 20° (Fig. 6).

The two preferred lengths of virus particles (Fig. 2) correspond to particles with either

both ends rounded (90 nm. long) or with one end rounded (80 nm.) and the other showing disintegration. The difference in preferred lengths is consistent with the loss of a half icosahedron (8 nm.) from one end. Virus particles appeared to attach by one end only to host membranes. No particles were observed to be attached at both ends. As the unattached end was always rounded it is likely that degradation occurs at the end normally involved in attachment.

The long rods described may not be infective although some are nucleoproteins. Failure in the mechanism which normally closes the protein tube into a particle of standard length may be due to the inclusion of either a multiple length of virus nucleic acid or possibly of host DNA. The long rods of different morphology were seen only in broth cultures and may be polymers of virus or host protein lacking nucleic acid.

The authors thank Professor R. W. Horne for kindly supplying the photomicrograph (taken with a JEOL JEM 100B microscope) shown in Fig. 1*b*.

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Some Biological Characteristics of Mycoplasmatales *Virus-laidlawii* 1

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SUMMARY

Mycoplasmatales virus-laidlawii 1 (MV-L1) produced plaques on lawns of 11 out of 17 strains of *Acholeplasma laidlawii* but not on 12 other mycoplasma species examined. Clones of *A. laidlawii* resistant to lysis by the virus could readily be obtained from survivors of the susceptible BNI strain. These resistant clones carry virus apparently serologically similar to MV-L1 as does the susceptible BNI strain itself.

The virus was sensitive to the action of u.v. light and chloroform but relatively insensitive to ether. Nonidet-P 40, heat and pH levels of between 8 and 10. MV-L1 passed readily through Millipore VS filters of 25 nm. pore diameter with loss of titre of 0.5 log p.f.u./ml. or less.

In one-step growth experiments the latent period was between 30 and 60 min. and the burst size varied from 4 to 213. Maximum yield was obtained when virus was added early in the logarithmic stage of *acholeplasma* growth. MV-L1 appeared to cause gradual lysis of the *acholeplasma* culture.

INTRODUCTION

A recent report (Gourlay, 1970) described the isolation and some properties of a virus which infects a member of the Order Mycoplasmatales, namely *Acholeplasma laidlawii*. In a subsequent article (Gourlay, Bruce & Garwes, 1971) this virus, designated Mycoplasmatales virus-laidlawii 1 (MV-L1), was shown to be bacilliform with a mean diameter of 14.6 nm. and a mean length of 89.8 nm. Its nucleic acid appeared to be DNA. In this paper we present the results of work on the virus sensitivity, replication and host range. Some of the results of the virus sensitivity were reported briefly in the earlier communications.

METHODS

***Acholeplasma* growth.** The medium used for the culture of *Acholeplasma* was glucose-serum (GS) broth or agar (Gourlay & Leach, 1970). Later the DNA was omitted, 10% foetal calf serum (FCS) was used instead of 20%, and in solid medium, 0.5% agarose was used instead of 0.65%. This solid medium was poured on a base composed of PPLO broth (Difco), agarose, penicillin, thallium acetate and phenol red. *Acholeplasma* cultures were stored at -70°.

***Acholeplasma* assay.** The titre of *acholeplasma* cultures was estimated on GS agar plates by the method of Miles & Misra (1938).

Virus production. Virus was propagated by inoculating a small amount of stock suspension of the virus into either broth cultures of the BNI strain of *A. laidlawii* (Gourlay, 1970) or on to solid medium plates in which *acholeplasmas* were incorporated in the top layer.

Frequently virus was also incorporated in the top layer together with the acholeplasmas. After incubation at 37° for 18 to 24 hr, virus was harvested as broth in the case of broth cultures or by flooding solid medium plates with phosphate buffered saline (PBS) pH 7.3 for 3 to 18 hr at room temperature (22°) after which the PBS containing the virus was removed. Stock virus suspensions were usually heated at 60° for 30 min. to inactivate viable acholeplasmas and were stored at -70°.

Virus assay. The titre of virus suspension was estimated either by a pour-plate method or by a modification of the Miles & Misra (1938) method. In the former, serial ten-fold dilutions of virus were prepared in PBS containing 5% FCS; equal volumes, 0.1 ml., of the virus dilutions and an acholeplasma culture (titre 1 to 6×10^8 c.f.u./ml.) were mixed with 2 ml. melted GS agar at 45° to 50° and rapidly poured on to a PPLO base. In the Miles & Misra method, dilutions of the virus were applied by means of a standard 0.02 ml. dropper to a GS agar plate prepared and seeded with acholeplasmas as above. Plaques were counted after incubation at 37° for 24 to 48 hr. All titrations were performed in duplicate.

Virus yield in relation to acholeplasma growth. One hundred ml. of GS broth were inoculated with 1.65×10^8 c.f.u. of the BNI strain of *A. laidlawii* removed from storage at -70°. The broth was incubated at 37° and at various times after inoculation 1 ml. and 3 ml. samples were removed. The 1 ml. samples were used for titration of the acholeplasmas and for recording extinction at 660 nm, while the 3 ml. samples were inoculated with MV-Li (m.o.i. about 0.1) and reincubated at 37°. After 24 hr the 3 ml. sample was titrated for virus activity. This experiment was repeated once using slightly different times.

Preparation of antiserum. Antiserum was prepared in rabbits, which received an initial inoculation with 4.0×10^{10} p.f.u. purified virus (Gourlay *et al.* 1970) with sodium alginate and calcium gluconate (Medical Alginates Ltd., Perivale, Greenford, Middlesex) as adjuvant, followed 2 weeks later by four intravenous injections of equivalent amounts of virus, without adjuvant, at weekly intervals. The rabbits were bled for serum 4 days after the last injection.

Virus filtration. Virus harvested from lawns of the host acholeplasma was diluted 1/10 with PBS + 5% FCS and then serially filtered through Millipore membrane filters of decreasing pore size under 5 to 10 lb. positive pressure. The initial virus dilution and the filtrates were titrated.

Host range of virus. The host range of the virus was determined by placing a drop of high titre virus suspension (about 2×10^9 p.f.u./ml.) on to a lawn prepared from each of the mycoplasmas being tested. The lawn was examined for plaque formation after incubation at 37° for 24 to 48 hr. Each mycoplasma was tested on at least three separate occasions.

Chloroform sensitivity. Stock virus suspension was diluted 1/10 in PBS + 10% FCS. Chloroform (Analar) was added to half the virus dilution to give a final concentration of 5% while an equal volume of PBS was added to the other half. The virus dilutions were then shaken for 10 min. at 4° or at room temperature and then the chloroform was removed by centrifugation at 850 g for 10 min. or by evaporation at 37°. The chloroform-treated and control virus suspensions were then titrated.

Ether sensitivity. In two experiments stock virus suspension was diluted 1/10 in PBS containing 10% FCS, and 20% diethyl ether (Analar) was added to half of it and 20% PBS was added to the other half as control. The mixtures were left at 4° for 18 hr after which the ether was allowed to evaporate at 37°. The ether-treated and control virus suspensions were titrated. Two further experiments were performed in a similar manner except that the virus was diluted in PBS alone.

Sensitivity to Nonidet-P 40. Stock virus suspension diluted 1/10 in PBS was mixed with an equal volume of 0.8% Nonidet-P 40 (B.D.H. Ltd.) for 15 min. at 37°. A similar virus dilution

mixed with an equal volume of PBS was used as a control. The Nonidet-treated and control materials were then titrated.

Sensitivity to u.v. light. Five ml. samples of the virus suspension, diluted 1/10 in PBS, were exposed in 5 cm diameter plastic Petri dishes (Nunc, Sterilin Ltd., Richmond, Surrey) at a distance of 12 cm. from an u.v. light source (Universal UV lamp, 254 nm. Gelman-Camag. Model 54102) and titrated after exposure for various lengths of time. The titrations were performed under ordinary light conditions and the plates were then incubated at 37° in the dark.

Thermal inactivation of virus. Universal containers with 10 ml. PBS were immersed in water baths at various temperatures. Ten min. were allowed for temperature equilibration, after which 1 ml. of virus suspension was added to each tube to give 5×10^9 p.f.u./ml. At intervals 0.5 ml. samples were transferred to small bottles chilled in ice water. All samples were titrated at the end of the experiment.

pH sensitivity. Samples of normal saline were adjusted to pH values varying from 1.2 to 12.2 by the addition of 1 M-HCl or 1 M-NaOH and 4.5 ml. amounts were added to universal containers held at 37°. After 15 min., 0.5 ml. of virus suspension diluted 1/10 in normal saline to give about 2×10^9 p.f.u./ml. was added to each and the container shaken vigorously. After a further 24 hr 0.2 ml. samples were titrated from each container.

One-step growth experiment. This was performed by the one-step growth method of Adams (1959).

Acholeplasma lysis by MV-L1. Ten ml. broth cultures of *A. laidlawii* BNI were incubated overnight at 37° and treated with an M.S.E. ultrasonic disintegrator for 45 sec. at maximum output and returned to 37° for a further 1½ hr at which time their viable titres were 3.8×10^7 to 3.8×10^8 c.f.u. of acholeplasmas/ml. Sufficient virus was then added to achieve a m.o.i. of about 1. PBS or virus inactivated with 5% chloroform was added to a control culture. The cultures were then held at 37° and the mycoplasma concentration estimated at intervals by optical absorbance at 660 nm. and by titration after treatment with MV-L1 antiserum for 10 min. at 37°. Growth curves were constructed.

Effect of ribonuclease on plaque formation. The activity of MV-L1 on the host acholeplasmas grown on solid medium plates containing 1 mg./ml. of ribonuclease-A (Sigma, type 1-A) in the top layer of the plate, was tested (Bradley, 1966).

RESULTS

Host range of MV-L1

MV-L1 was first detected on a lawn prepared from a broth culture of a mycoplasma which was subsequently cloned, identified as *Acholeplasma laidlawii* and designated the BNI strain (Gourlay, 1970). MV-L1 was examined for its ability to lyse 16 other strains of *A. laidlawii* and 12 other species of mycoplasma details of which are given in Table 1. Plaques were produced on 10 cloned strains of *A. laidlawii*, namely M1304/68, M1305/68, M1307/68, M1301/68, M1302/68, 03, TGE(C), 151, 152 and 179. The plaques varied, from a few faint discrete plaques to distinct confluent zones covering the whole area of the drop. On some lawns plaques were produced only after the acholeplasma constituting the lawn had undergone one or two subcultures in GS broth. No plaques were observed on the lawns of any of the other species of mycoplasma examined.

Plaque morphology

The plaques produced by MV-L1 on solid medium plates were larger than those described in the earlier work (Gourlay, 1970). At high dilutions single isolated plaques up to 6 mm. in

diameter were observed, they were turbid and frequently possessed an indistinct halo around the periphery (Fig. 1). Plaques could be produced on lawns incubated either at 37° or at room temperature. On one occasion, following three successive passes of virus in which the inoculating suspension was not heated to 60°, large plaques up to 12 mm. in diameter were produced.

Table 1. *Species and strains of mycoplasma tested for susceptibility of MV-L1*

Species or strain of mycoplasma	Source	Received from
<i>A. laidlawii</i> (PG8)	Sewage	N.C.T.C. Colindale, London
<i>A. laidlawii</i> M1304/68*	Cattle	Dr R. H. Leach
<i>A. laidlawii</i> M1305/68*	Cattle	Dr R. H. Leach
<i>A. laidlawii</i> M1306/68	Cattle	Dr R. H. Leach
<i>A. laidlawii</i> M1307/68*	Cattle	Dr R. H. Leach
<i>A. laidlawii</i> O-324	Sheep	Dr R. H. Leach
<i>A. laidlawii</i> M1301/68*	Swine	Dr R. H. Leach
<i>A. laidlawii</i> M1302/68*	Swine	Dr R. H. Leach
<i>A. laidlawii</i> M1308/68	Swine	Dr R. H. Leach
<i>A. laidlawii</i> O3*	Cattle	I.R.A.D. Compton
<i>A. laidlawii</i> O11	Cattle	I.R.A.D. Compton
<i>A. laidlawii</i> O12	Cattle	I.R.A.D. Compton
<i>A. laidlawii</i> TGE(C)*	Tissue culture	I.R.A.D. Compton
<i>A. laidlawii</i> 151*	Cattle	Dr D. A. Haig
<i>A. laidlawii</i> 152*	Cattle	Dr D. A. Haig
<i>A. laidlawii</i> 179*	Cattle	Dr D. A. Haig
<i>A. granularum</i>	Swine	Dr R. T. Hodges
<i>A. granularum</i> M217/69	Swine	Dr R. H. Leach
<i>Acholeplasma</i> sp. M221/69	Swine	Dr R. H. Leach
<i>M. hyorhinis</i>	Swine	Dr R. T. Hodges
<i>M. bovirhinis</i> (PG43)	Cattle	N.C.T.C. Colindale, London
<i>M. dispar</i> (462/2)	Cattle	I.R.A.D. Compton
Serological group 6 (Squire)	Cattle	Dr R. H. Leach
Serological group 7 (N29)	Cattle	Dr R. H. Leach
<i>M. mycoides</i> var. <i>mycoides</i> (v5)	Cattle	I.R.A.D. Compton
<i>M. mycoides</i> var. <i>mycoides</i> (Gladysdale)	Cattle	I.R.A.D. Compton
<i>M. mycoides</i> var. <i>mycoides</i> (Irii)	Cattle	I.R.A.D. Compton
<i>M. mycoides</i> var. <i>mycoides</i> (S1)	Cattle	I.R.A.D. Compton
<i>M. mycoides</i> var. <i>mycoides</i> (T2/33)	Cattle	I.R.A.D. Compton
<i>M. mycoides</i> var. <i>mycoides</i> (T3)	Cattle	I.R.A.D. Compton
<i>M. mycoides</i> var. <i>mycoides</i> (KH31)	Cattle	I.R.A.D. Compton
<i>M. pneumoniae</i> (FH)	Human	N.C.T.C. Colindale, London
<i>M. salivarium</i> (PG20)(H11)	Human	N.C.T.C. Colindale, London
<i>M. fermentans</i> (PG18)	Human	N.C.T.C. Colindale, London
<i>M. hominis</i> (PG21)(H50)	Human	N.C.T.C. Colindale, London
<i>M. orale</i> type 1 (CH19299)	Human	N.C.T.C. Colindale, London

* Strains susceptible to MV-L1.



Fig. 1. Plaques formed by MV-L1 on a plate of *Acholeplasma laidlawii* strain BNI. Stained neutral red. $\times 17$.

Selection of clones of *A. laidlawii* BNI resistant to MV-L1

After 24 hr growth of MV-L1 in a broth culture of the BNI strain of *A. laidlawii*, a sample of the culture was inoculated on to a GS agar plate. From the acholeplasma colonies that grew, four were selected and cloned by picking single colonies on three successive occasions. The four clones were propagated in GS broth and lawns were prepared subsequently from each. Drops of MV-L1 suspension (titre 1×10^8 p.f.u./ml.) were placed on each lawn and on a control lawn of the parent BNI strain. After incubation the lawns were examined for plaques.

Distinct plaques were visible on the control lawn and on one of the other lawns; but none were visible on the three remaining lawns, which were apparently resistant to infection with MV-L1.

Examination of *A. laidlawii* strain BNI and clones of BNI resistant to MV-L1 for the presence of carrier virus.

A. laidlawii BNI, and the 3 BNI clones resistant to MV-L1 together with a non-susceptible *Acholeplasma* sp. M221/69 were each seeded onto GS agar plates. When the lawns were well grown the plates were washed with PBS for 3 to 5 hr at room temperature (22°). Samples from each washing were then dropped on to fresh lawns of each organism. MV-L1 suspension was used as a control.

The results (Table 2) show that plaques were produced on a lawn of the BNI strain by washings from the 3 clones of BNI resistant to MV-L1 and also by the washing from the parent BNI strain but not by the washing from the M221/69 strain. No plaques were produced on any of the other lawns.

Table 2. Examination of washings from *A. laidlawii* strain BNI, clones of *A. laidlawii* BNI resistant to MV-L1 and *Acholeplasma* sp. M221/69 for the presence of plaque-forming agents on lawns prepared from each of them

Washings from	The presence of plaques produced on lawns prepared from				
	M221/69	Resistant A	Resistant C	Resistant D	BNI
M221/69	—	—	—	—	—
Resistant/A	—	—	—	—	+
Resistant/C	—	—	—	—	+
Resistant/D	—	—	—	—	+
BNI	—	—	—	—	+
MV-L1 control	—	—	—	—	+

The agents in the various washings responsible for plaque formation were then examined for sensitivity to inhibition by MV-L1 antiserum. Lawns of BNI were inoculated with 2 drops of a 1/10 dilution of each of the washings. When the drops were dry a loopful of MV-L1 antiserum was placed in the centre of one drop and normal rabbit serum in the centre of the other drop. After incubation at 37° for 24 hr the plaques were examined for inhibition indicated by a circle of acholeplasma growth within the plaque.

The plaques produced by the washing from BNI and by washings from the MV-L1 resistant clones of BNI were inhibited by antiserum prepared against MV-L1, indicating that the agents carried by these strains are serologically similar to MV-L1.

The titre of agent in the washing from one of the MV-L1 resistant clones was about 1×10^8 p.f.u./ml. and in the BNI washing was about 1×10^6 p.f.u./ml. These agents produced isolated plaques at high dilution and the addition of high dilution of the BNI agent to a culture of *A. laidlawii* BNI resulted in a 4 log increase in titre.

Clones of BNI, resistant to the virus in the BNI washing, were then obtained. Lawns prepared from four of these clones were then examined for resistance to MV-L1 stock suspension and also to the virus in both the washing from one of the MV-L1 resistant BNI clones and to the virus in the BNI washing. No plaques were produced on any of the lawns by any of the viruses, indicating that clones of BNI resistant to the virus which was obtained from BNI itself were also resistant to MV-L1 and to the virus from the MV-L1-resistant clones.

Further attempts to obtain a virus from washing lawns of *A. laidlawii* BNI did not always succeed, but similar virus was isolated, on at least seven separate occasions.

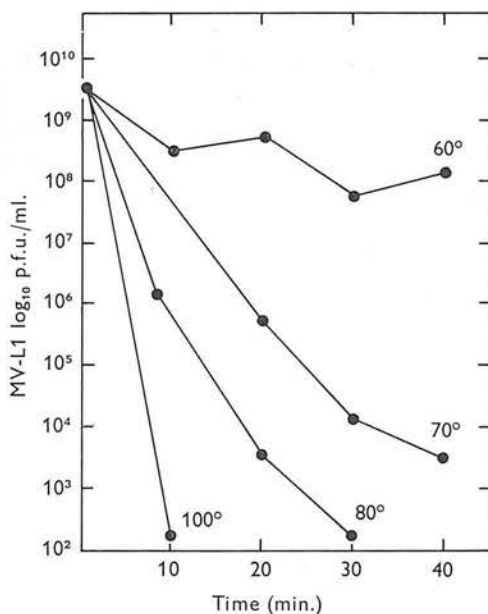


Fig. 2. Inactivation of MV-L1 at various temperatures.

Sensitivity to physical and chemical treatments

When held at 60° for 30 min. activity of MV-L1 suspension decreased by about 1.0 log p.f.u./ml. At higher temperatures activity decreased further until no activity could be detected in a sample held at 80° for 30 min. (Fig. 2). MV-L1 was stable between pH 8 and pH 10 when held at 37° for 24 hr. At higher and lower pH levels, viral activity was progressively lost. In two experiments, u.v. light inactivated 90% of the virus in 19 and 23 sec. respectively. Three separate filtration experiments were performed and the results of titrations of the virus before and after filtration are given in Table 3. From these results it can be seen that MV-L1 passes through Millipore filters of 25 nm. pore diameter with a loss of titre of 0.5 log p.f.u./ml. or less. In five separate experiments chloroform caused a drop in titre of between 6.5 and 7.5 log p.f.u./ml. Ether caused a drop in titre of 1.4 and 1.97 log p.f.u./ml. when PBS was used as diluent, whereas when PBS containing FCS was used as diluent the drop in titre was nil and 1.25 log p.f.u./ml. In five experiments in which the virus was treated with Nonidet-P40 the titre either remained unchanged (in two experiments) or was reduced by a maximum of 1.0 log p.f.u./ml.

Yield of MV-L1 in relation to mycoplasma age

The results of the two experiments are shown in Fig. 3. Maximum yield of virus was obtained when the virus was added early in the logarithmic stage of acholeplasma growth, i.e. 6 to 12 hr after incubation commenced. If virus was added late in the log phase or in the retardation or stationary phase of growth, the yield of virus was reduced drastically and considerably less virus was obtained than was added. After 18 hr, an equilibrium was reached

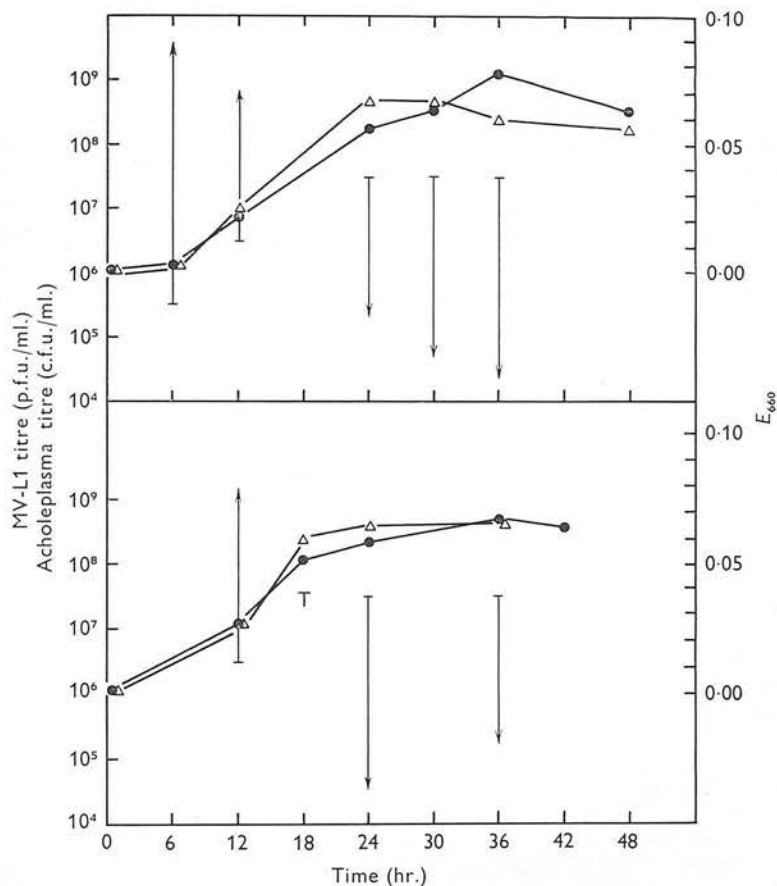


Fig. 3. Yield of MV-L1 in relation to the age of *Acholeplasma laidlawii* strain BNI at the time of MV-L1 inoculation. ●—●, Acholeplasma titre of culture, △—△, optical extinction of acholeplasma culture; ↑, titre of MV-L1 inoculated into sample of culture; ↓, titre of MV-L1 harvested 24 hr later.

Table 3. Effect of filtration on titres of MV-L1 diluted in PBS + 10 per cent FCS

Titre of MV-L1 before filtration p.f.u. ml.	Titre of MV-L1 after filtration through membranes of pore diameter			
	220 nm.	100 nm.	50 nm.	25 nm.
1.5×10^{10}	2.5×10^{10}	1.75×10^{10}	1.75×10^{10}	1.25×10^{10}
5.0×10^7	NT	2.0×10^8	2.25×10^8	7.5×10^7
1.0×10^{10}	NT	NT	NT	5.0×10^9

NT = not titrated.

when almost the same number of virus particles were yielded as were added. The mean generation time of *A. laidlawii* in the GS broth during these experiments was calculated from the growth curves as about 130 min.

Yield of virus

The titre of virus obtained from growth on solid medium plates was usually higher than that obtained from growth in broth culture. In the former the titre was frequently over 1×10^{10} p.f.u./ml. and occasionally over 1×10^{11} p.f.u./ml.

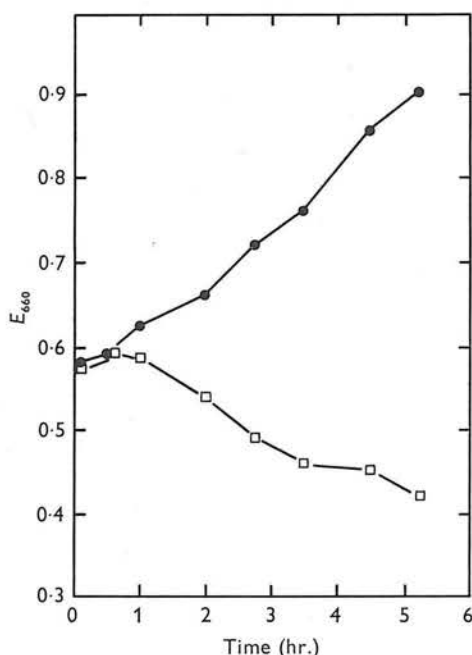


Fig. 4. Rate of growth of *Acholeplasma laidlawii* strain BNI in broth cultures inoculated with viable or inactivated MV-L1. Growth measured by optical extinction. ●—●, Culture infected with inactivated MV-L1; □—□, culture infected with viable MV-L1.

One-step growth experiment

A number of separate experiments were performed but the results were very variable. It was evident, however, that the latent period was between 30 and 60 min. The rise period was between 1 and 2 hr and the burst size varied from 4 to 213.

Effect of ribonuclease on plaque formation

In two experiments, the addition of ribonuclease to the medium had no effect on the formation of plaques, as the titre of the virus grown on ribonuclease-containing plates and normal plates was similar.

Acholeplasma lysis by MV-L1

A typical growth curve chosen from a number of experiments is shown in Fig. 4. The optical extinction and viable mycoplasma titre of the inactivated virus control culture rose steadily over a period of hours, while the extinction of the culture inoculated with live virus rose in a similar manner for the first 30–60 min. after which the extinction fell steadily until it was below that at which it started. The viable *acholeplasma* titre followed a similar pattern.

DISCUSSION

The sensitivity of *rv* strains of *A. laidlawii* to lysis by MV-L1 varied from complete insensitivity to almost full sensitivity. Amongst the sensitive strains it was observed that there was a gradation of sensitivity, as shown by the number of plaques produced on the relevant lawns by a standard number of virus particles. It was also noticed that the sensitivity of a number of these *A. laidlawii* strains varied from time to time. The reasons for this are not clear but they appear to be associated with growth of the acholeplasmas.

The production of turbid plaques and the evidence that resistant clones of the acholeplasma can readily be obtained, indicate that lysogeny occurs. Washings of lawns of the resistant clones and the susceptible parent BNI strain of *A. laidlawii* contain agents which produce plaques on lawns of the BNI strain. These agents are serologically similar to MV-L1 and at least two of them appear to be viral in nature as they produce single isolated plaques at high dilution and, in addition, the BNI agent multiplies in a broth culture of the acholeplasma. Clones of the BNI strain of *A. laidlawii* resistant to the agent in the BNI washings were shown to be resistant also to MV-L1 and to the agent in the washings of the MV-L1 resistant clones of *A. laidlawii*. This is a further example of the similarity of these agents and MV-L1, and also suggests that MV-L1 does not contain more agents than are contained in the washings from BNI or the MV-L1 resistant clones. The lysogenic condition appears complex and the implications interesting but further work is required to clarify the situation.

The results of the experiments on the yield of virus in relation to the age of the acholeplasma culture are straightforward and show that maximum yield of virus is obtained when the virus is added early in the log phase of acholeplasma growth. The variable results of the burst size in the one-step growth experiments indicate that the method of acholeplasma enumeration employed is probably not entirely suitable for this type of work, since colony-forming units may consist of many clumped acholeplasma cells. The use of ultrasonic disintegration in an attempt to disperse these clumps was used in the experiments on acholeplasma lysis. These lysis experiments showed that the addition of viable virus to a growing culture of *A. laidlawii* led to a drop in the optical extinction of the culture compared with its starting extinction and compared to a control culture inoculated with inactivated virus. This suggests that the virus lyses the acholeplasmas. This lysis apparently occurs gradually over a period of time rather than as a sudden event.

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Mycoplasmatales Virus-laidlawii 2, a New Virus Isolated from *Acholeplasma laidlawii*

(Accepted 15 April 1971)

Recent reports (Gourlay, 1970; Gourlay, Bruce & Garwes, 1971) described the first isolation and gave some characteristics of a virus which infects a member of the order Mycoplasmatales, namely *Acholeplasma laidlawii* (formerly *Mycoplasma laidlawii*). This virus, designated Mycoplasmatales virus-laidlawii 1 (MV-L1), was shown to be bacilliform with a mean width of 14.6 nm. and a mean length of 89.8 nm. It was insensitive to ether and detergent and withstood a temperature of 60° for 30 min.; its nucleic acid appeared to be DNA. This report describes the isolation of another virus which infects *A. laidlawii* and which differs from MV-L1.

Nineteen strains of *A. laidlawii* were examined for virus activity by the filtrate method described earlier (Gourlay, 1970), and also by a method in which each strain was grown on a GS agar plate (Gourlay & Leach, 1970) until good colony growth was visible. Each plate was then washed with 3 ml. phosphate-buffered saline, pH 7.3 for 18 hr at room temperature and the washing was harvested. The filtrates and washings were then examined for the presence of virus by placing a drop of each on to GS agar plates seeded with cultures of each of the acholeplasma strains. The plates were examined after 24 to 48 hr incubation at 37°. Most showed a confluent lawn of acholeplasma colonies but there were a number on which plaques were visible. These plaques were present at the sites of application of various washings. No plaques were visible at the site of application of any of the filtrates.

The washing of *A. laidlawii* strain 0-324 produced plaques on lawns of six different strains of *A. laidlawii*, namely BNI, M221/70, TGE(C), M1305/68, M1307/68 and M1308/68. The plaques varied; those on M1307/68, M1308/68 and TGE(C) were few in number and about 1 mm. in diameter, those on BNI and M221/70 were similar in size but more numerous, and those on M1305/68 consisted of one large confluent zone covering the whole area of the drop. MV-L1 produced plaques on lawns of all these strains except 0-324 and M1308/68.

The 0-324 washing was titrated by preparing serial tenfold dilutions in buffered saline containing 5% foetal calf serum. Drops of each dilution were placed on a lawn prepared from a culture of M1305/68 and the lawn incubated at 37° for 48 hr. The titre of the agent was 5×10^5 p.f.u./ml. The plaque-forming agent was cloned by picking a single plaque on three successive occasions. A stock of agent was then prepared by growing it on lawns of the host acholeplasma (M1305/68) and harvesting the washings which were stored at -70°. At high dilutions the stock material produced single discrete plaques, about 1 to 2 mm. in diameter. (Fig. 1). Plaques were formed during incubation at 37° but not during incubation at 22°.

When the agent, diluted in buffered saline, was exposed for 2½ min. at a distance of 12 cm. from an ultraviolet light source (Universal u.v. lamp, 254 nm., Gelman-Camag, model 54102) or exposed to a temperature of 56° for 10 min. the titre was reduced from 6.1 log p.f.u./ml. to 3.9 log p.f.u./ml. and from 5.1 log p.f.u./ml. to less than 2.25 log p.f.u./ml., respectively.

The agent, diluted in buffered saline, was treated with the detergent Nonidet-P40 (British Drug Houses Ltd, Poole, Dorset), 0.4% (v/v) for 15 min. at 37° and the titre was reduced to

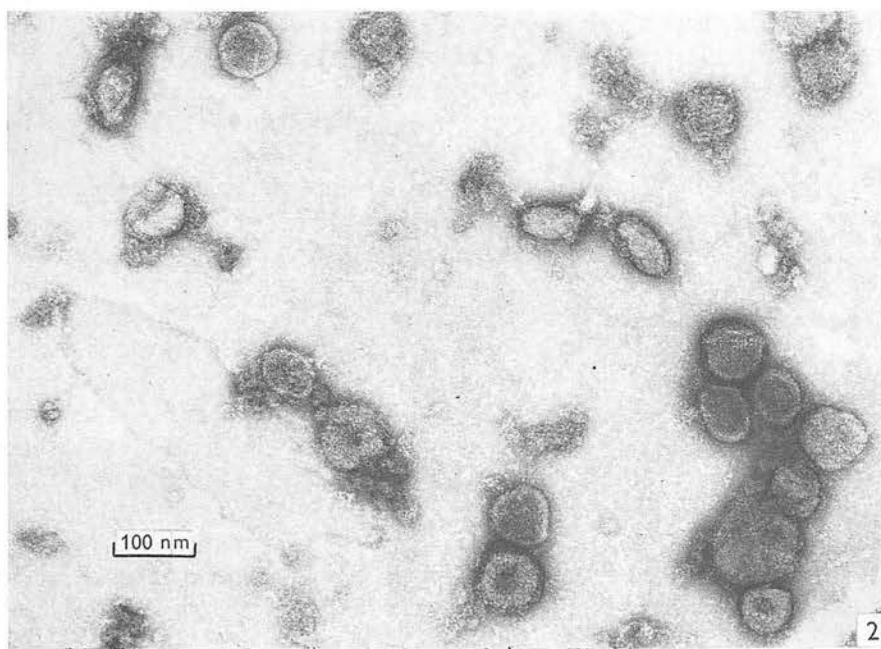
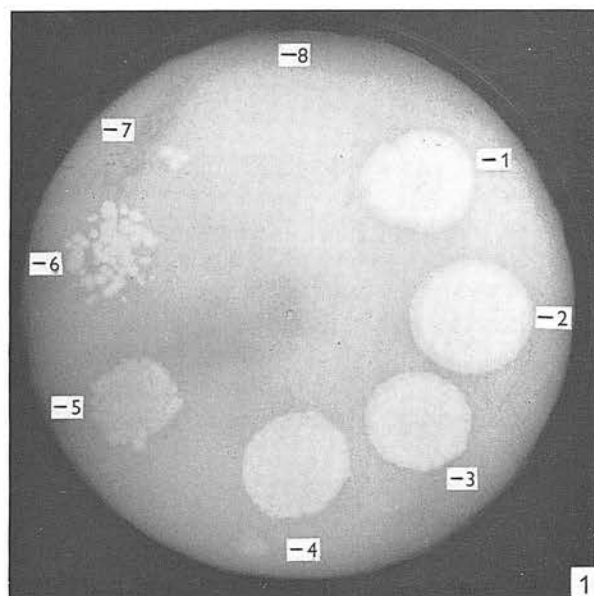


Fig. 1. Plaques formed by serial tenfold dilutions of virus on a plate of *Acholeplasma laidlawii* strain M' 305/68. Stained neutral red.

Fig. 2. Electron micrograph of centrifuged deposit of filtered stock virus. Negatively stained with uranyl acetate.

less than 2.25 log p.f.u./ml. This compares with a titre of 7.95 log p.f.u./ml. for a buffered saline control. Similarly, when the agent, diluted as above, was treated with 20% diethyl ether at 4° for 18 hr or 5% chloroform for 10 min. at 22° the titres were reduced to 1.25 log p.f.u./ml. and less than 1.25 log p.f.u./ml. compared with buffered saline control titres of 6.1 and 5.9 log p.f.u./ml., respectively.

The stock material diluted in serum saline was serially filtered under a positive pressure of 5 to 10 lb./in.² through Millipore membrane filters of decreasing pore size. In four separate attempts the agent (titre 5×10^4 to 5×10^7 p.f.u./ml.) passed readily through filters of 200 nm. pore size without loss of titre. There was, however, a loss in titre of 0.4 to 0.75 log p.f.u. when passed through a 100 nm. filter and 1.25 to 3.1 log p.f.u. through a 50 nm. filter. No plaque forming activity was detected in the 25 nm. filtrates.

Antiserum prepared in rabbits to purified MV-L1 inhibited the formation of plaques by MV-L1 on lawns of BN1 and M1305/68 but failed to inhibit the formation of plaques by the 0-324 agent on the same lawns.

On three occasions the addition of 3.2 to 4.2 log p.f.u./ml. agent to GS broth cultures of growing acholeplasmas resulted in a 3 to 4 log increase in titre of the agent after 24 hr incubation at 37°. There was no evidence of replication in the absence of actively growing cells of the acholeplasma.

Stock agent was passed through a 220 nm. Millipore filter and the filtrate was centrifuged at 150,000 g for 2 hr and the pellet resuspended in serum saline, fixed with 3% glutaraldehyde in 0.1 M-sodium cacodylate and stained with 1.5% uranyl acetate and examined in an AEI EM 6 electron microscope. Numerous roughly spherical, apparently enveloped bodies whose diameters ranged from 50 to 120 nm. were seen (Fig. 2).

The characteristics of this agent, detailed above, are consistent with those of a virus. There are some obvious differences between this virus and MV-L1. For example, it differs in morphology, is larger in size, is serologically dissimilar, is more sensitive to heat and is sensitive to ether and Nonidet-P40. Individual plaques on M1305/68 and BN1 also differ in that they are smaller than those produced by MV-L1 on the same lawns and, unlike MV-L1, are not formed at 22°. In view of these differences, it is clearly a new and fundamentally different virus and represents a new group of mycoplasma viruses, for which the designation Mycoplasmatales virus-laidlawii 2 (MV-L2) is proposed.

I wish to thank Dr R. H. Leach, Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9, for supplying the *A. laidlawii* strains 0-324, M1305/68, M1307/68, M1308/68 and M221/70 and Miss Judy Bruce and Mr I. Jebbett for the electron-micrograph and photograph respectively, and Miss Sara Wyld for excellent technical assistance.

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Isolation and characterization of mycoplasma viruses

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It is now about two years since a virus which infects a member of the order Mycoplasmatales, namely *Acholeplasma laidlawii*, and designated Mycoplasmatales Virus-Laidlawii 1 (MV-L1) was first isolated (Gourlay 1970; Gourlay *et al.* 1971). Since then a number of other viruses have been isolated at Compton from *A. laidlawii*, one of which was different from MV-L1 and for which the designation MV-L2 was proposed (Gourlay 1971). The other viruses were serologically similar to either MV-L1 or MV-L2 (Gourlay 1972). Liss & Maniloff (1971) have also isolated a number of viruses from *A. laidlawii*, which they designated MV-L52 to MV-L60, and also a virus (MV-G51) from a mycoplasma species other than *A. laidlawii*, namely *Mycoplasma* species strain 14 (goat). The relationships between the viruses isolated at Compton and those isolated by Liss & Maniloff in America are not clear.

ISOLATION OF MYCOPLASMA VIRUSES

A standard procedure for the isolation of bacteriophages involves adding filtrates from bacterial cultures or raw sewage to test host organisms. Clearing of broth cultures or the appearance of zones of lysis on seeded solid medium plates indicate phage activity.

The initial isolation of MV-L1 (Gourlay 1970) was made by adding filtrates of broth cultures of mycoplasmas to solid medium plates seeded with other mycoplasmas. Broth cultures of eight different isolates of mycoplasmas obtained from the bovine nose were prepared. When the cultures had grown they were each divided into two aliquots. One aliquot of each was filtered through a Millipore 220 nm membrane filter and the other aliquot was used to prepare a 'lawn' by seeding it on a GS solid medium plate (Gourlay & Leach

1970). After the plates had dried, each filtrate was tested for viral activity by placing a drop on each of the seeded plates. When these drops were dry the plates were incubated at 37°C for 24 to 48 hours. With one exception all showed confluent lawns of mycoplasma colonies. In the exception a distinct clear zone or plaque was seen at the site of application of one of the drops on one of the lawns. The plaque-forming agent present in the filtrate was subsequently shown to be a virus and was designated MV-L1.

The culture which produced the lawn on which the plaque was seen was inoculated onto a solid medium plate and the mycoplasmas that grew were purified by picking single colonies on three successive occasions. The cloned culture (strain BN1) continued to present plaques when the virus was added, and was shown to be *A. laidlawii*.

The above method of virus isolation—the filtrate method—was modified in the light of work with clones of *A. laidlawii* BN1 resistant to MV-L1 infection (see later). The modification—the washing method—entailed growing each strain of mycoplasma on a solid medium plate as the top layer of a pour plate (Gourlay & Wyld 1972). When good growth of the colony was visible each plate was washed for 5 to 18 hours at room temperature with enough phosphate-buffered saline (PBS), pH 7.3, to cover the surface. The PBS washings were then removed and examined for the presence of viruses by placing a drop of each onto solid medium plates seeded with cultures of each of the mycoplasma strains as before. Usually the strains were seeded as top layers of pour plates (layer plates). Nineteen strains of *A. laidlawii* were examined by the filtrate and washing methods and, whereas no plaques were obtained from the filtrates, 30 plaques were produced by the washings, indicating the superiority of the latter method. These plaques were produced on plates prepared from 11 different *A. laidlawii* strains.

Twenty-three of these plaques were produced by agents serologically similar to MV-L1. Seventeen were from washings of the BN1 and M221/70 strains (the latter being a subculture of the BN1 strain that was sent to Dr R. H. Leach, Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale, London, and subsequently returned), whereas the remaining six plaques were produced by washings from four different strains, namely M1301/68, M1302/68, M1304/68 and M1308/68. Seven plaques were produced by agents serologically dissimilar to MV-L1. Six of the latter were obtained from one of the washings on different acholeplasma lawns. The agent in this washing (0-324) proved to be a virus which was fundamentally different from MV-L1 and this was designated MV-L2 (Gourlay 1971). The remaining plaque was produced by the washing from M1307/68.

The washing method of isolation was used by Liss & Maniloff (1971) to

isolate nine viruses from *A. laidlawii* and one from *Mycoplasma* species strain 14 (goat).

Recently the washing method has been used again at Compton to isolate more viruses from acholeplasma species. Washings from layer plates were prepared from 20 strains of *A. laidlawii* and two strains of *A. granularum*, including many of the strains that were used in the earlier isolation attempts. Fifty-two plaques were produced, many of them on layer plates of the BN1 and M1305/68 strains of *A. laidlawii*. In addition, a few plaques were also produced on layer plates of seven other strains of *A. laidlawii* (5N, 03, M1301/68, M1302/68, M1307/68, M1308/68 and 151). Plaques were produced by washings from 14 different acholeplasma strains, one of them being 0-324 from which MV-L2 was isolated. The 14 washings that produced plaques were subinoculated onto M1305/68 (1305) layer plates where plaques were produced on this strain, or onto the appropriate lawn where they were not. In all cases these latter washes subsequently produced plaques on the 1305 strain and this strain was used for future passages.

After the identification and purification of clear and turbid plaque-forming strains of MV-L1 (see later) it was decided to distinguish between these two types of plaque-forming agents on the isolation plates. The clear and turbid plaques were therefore cut out separately from the layer plates and washed in PBS. These plaque-forming agents were then purified by picking single plaques on 3 successive occasions; the resulting cloned agents were grown on 1305 layer plates and the subsequent washings stored at -70°C .

SELECTION OF *A. LAIDLAWII* BN1 AND 1305 CLONES RESISTANT TO MV-L1 AND MV-L2

The plaques produced by MV-L1 and MV-L2 were turbid, suggesting that some colonies were resistant to the effects of the viruses. It has already been reported that clones of BN1 resistant to MV-L1 can be obtained by selecting survivors within the plaques on solid medium or in broth cultures of the acholeplasma inoculated with the virus (Gourlay & Wyld 1972). These resistant clones were shown to carry virus when layer plates prepared from them were washed with PBS and the washings tested for viral activity. The carried virus was serologically similar to MV-L1. When the BN1 strain itself was washed, the washing was also shown to contain a similar virus (Gourlay & Wyld 1972).

Since the above report we have shown that MV-L2 behaves in a similar manner, as clones of 1305 resistant to this virus can readily be obtained by

similar methods. The resistant clones also carry a virus serologically similar to MV-L2. So far we have not isolated any viruses from the 1305 strain of *A. laidlawii*, which is the one we use to propagate MV-L2 and now also MV-L1. Resistant clones of this strain can, however, readily be obtained to each virus.

When MV-L1 was first propagated on the 1305 strain of *A. laidlawii* it was noticed that two different plaque types were visible: a turbid plaque type, which appeared predominant, with a titre of about 3.5×10^9 /ml, and a clear plaque type with a titre of about 1×10^7 /ml. These two plaques were purified separately by picking single plaques on three successive occasions and were shown to be stable. When these two types, named turbid (tur) and clear (clr), were put on the layer plates of the BN1 strain these differences were barely apparent.

The virus obtained from the BN1 strain itself appeared to be a pure culture of the turbid type of MV-L1 when put onto 1305 layer plates.

From the various isolation attempts described earlier a total of 25 isolates have been obtained from 15 acholeplasma strains. This does not include MV-L1, about whose origin there is some doubt (see p. 154). All were strains of *A. laidlawii* except one, M217/69, which was a strain of *A. granularum*. Details of these agents are given in Table 1. Where more than one isolate was made from a single acholeplasma strain, the turbidity or clarity of the plaques produced was used for differentiation. However, in the case of 0-324 two large plaques (lrg) were observed among the very large number of small ones (MV-L2), and one of these was picked and cloned.

CHARACTERIZATION OF VIRUS ISOLATES

These 25 isolates were examined by a number of different methods which had proved of value in examining and differentiating between MV-L1 and MV-L2, to see whether they were different from these two viruses.

Susceptible host

MV-L1 was tested for its ability to form plaques on a number of different strains of *A. laidlawii*, other acholeplasma strains and other mycoplasma species. It formed plaques on 11 out of 17 strains of *A. laidlawii* but not on 12 other mycoplasma and acholeplasma species examined (Gourlay & Wyld 1972). The species tested were *Mycoplasma mycoides* var. *mycoides* (7 strains), *M. pneumoniae*, *M. salivarium*, *M. fermentans*, *M. hominis*, *M. orale* type 1, *M. hyorhinis*, *M. bovirhinis*, *M. dispar*, bovine serological groups 6 and 7

TABLE 1

Species and strains of *Acholeplasma* from which viruses were isolated

Strain of <i>Acholeplasma</i>	Source	Virus isolate
<i>A. laidlawii</i> (uncloned) ^a	Bovine nose	MV-L1
<i>A. laidlawii</i> 10 ^a	Bovine nose	10 tur
<i>A. laidlawii</i> D143 ^a	Bovine nose	143 clr
		143 tur
<i>A. laidlawii</i> D172 ^a	Bovine nose	172 clr
		172 tur
<i>A. laidlawii</i> D182 ^a	Bovine nose	182 clr
		182 tur
<i>A. laidlawii</i> BN1 ^a	Bovine nose	BN1/1
<i>A. laidlawii</i> 03 ^a	Bovine eye	03 clr
		03 tur
<i>A. laidlawii</i> 011 ^a	Bovine eye	011 clr
		011 tur
<i>A. laidlawii</i> 152 ^a	Bovine oviduct	152 clr
		152 tur
<i>A. laidlawii</i> 179 ^a	Bovine oviduct	179 clr
<i>A. laidlawii</i> M1304/68 ^b	Cow	1304 clr
		1304 tur
<i>A. laidlawii</i> M1307/68 ^b	Cow	1307
		1307 tur
<i>A. laidlawii</i> M1308/68 ^b	Pig	1308 tur
<i>A. laidlawii</i> 0-324 ^b	Sheep	MV-L2
		0-324 lrg
<i>A. granularum</i> M217/69 ^b	Pig	217 tur
<i>Acholeplasma</i> sp. M221/69 ^b	Pig	221 clr
		221 tur

^a Isolated at the Institute for Research on Animal Diseases, Compton, Newbury, Berkshire.^b Supplied by Dr. R. H. Leach, Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London N.W. 9.

(Leach 1967) and *A. granularum* (2 strains). In addition Liss & Maniloff (1971) showed that MV-L1 did not form plaques on *Mycoplasma* species strain 14 (goat), *M. gallisepticum*, *M. hominis* and *M. avian* serotype 1 strain. Two of Liss & Maniloff's other virus isolates, MV-G51 and MV-L52, also did not produce plaques on these same strains of mycoplasma.

MV-L2 was also tested for its ability to form plaques on a number of *A. laidlawii* and other mycoplasma species. It produced plaques on six out of 17 strains of *A. laidlawii* but not on seven other species tested (*A. granularum*, *M. hominis*, *M. salivarium*, *M. orale* type 1 and bovine serological groups 6 and 7).

Viruses have now been isolated from many different strains of *A. laidlawii*,

and from *A. granularum* and *Mycoplasma* species strain 14 (goat). All these viruses, however, produce plaques on *A. laidlawii* and not on any of the limited number of other mycoplasma or acholeplasma species so far tested. This would indicate that the virus source is not a good means of virus differentiation, but the infective host, on the other hand, may be of value. In this respect all the viruses so far isolated are *A. laidlawii* infective viruses and as such are apparently specific in their host choice, perhaps even differentiating within the Acholeplasmataceae.

As mentioned above MV-L1 and MV-L2 vary to some extent in their host range, MV-L1 infecting more strains of *A. laidlawii* than MV-L2, although MV-L2 produced a few plaques on the M1308/68 strain of *A. laidlawii* whereas MV-L1 did not (Gourlay 1971). Liss & Maniloff (1971) showed that the host range of MV-L52 differed from that of MV-L1 and MV-G51, which were the same, on the six lawns used. This evidence, incidentally, suggests that the goat isolate was similar to the *A. laidlawii* isolate and further supports the contention that the virus source is of limited value for virus differentiation. Within the *A. laidlawii* strains themselves consistency of plaque formation by a particular virus on a susceptible host strain was not always attained, particularly with recently isolated *A. laidlawii* strains, and there appeared to be a gradation of sensitivity from relatively insensitive to fully sensitive (Gourlay & Wyld 1972). This makes fine differentiation between strains difficult to establish with certainty.

Serological test

Antisera to MV-L1 (Gourlay & Wyld 1972) and MV-L2 were prepared in rabbits. Purification of the virus used for rabbit inoculation was essential as antibodies against the acholeplasma constituents inhibited growth of the acholeplasmas. The serological test was performed by inoculating layer plates of the *A. laidlawii* with two drops of virus suspension. When the drops were dry, a loopful of antiserum was placed in the centre of one drop and normal rabbit serum in the centre of the other drop. After incubation at 37°C for 24 hours the plaques were examined for inhibition, indicated by a circle of acholeplasma growth within the plaque.

By means of this test MV-L1 and MV-L2 could be readily differentiated; MV-L1 and MV-L2 being inhibited by the homologous antiserum and not by the heterologous. The results of serological tests with the other virus isolates showed that all except 1307 were inhibited by antiserum to MV-L1 and not by MV-L2 antiserum, whereas 1307 was inhibited only by MV-L2 antiserum.

Detergent and ether sensitivity

On the basis of their sensitivity to 0.4% (v/v) detergent (Nonidet P-40) for 15 minutes at 37°C and 20% diethyl ether at 4°C for 18 hours MV-L1 and MV-L2 can readily be differentiated (Gourlay 1971). MV-L1 is not sensitive whereas MV-L2 is. The various isolates were treated with Nonidet and all except 1307 were insensitive, their titres being reduced by 1.0 log plaque-forming unit (p.f.u.)/ml or less. The titre of 1307 was reduced by at least 4.5 log p.f.u./ml. The isolates were treated with ether and all proved relatively sensitive. The clear plaque-forming isolates were more sensitive than those that produced turbid plaques, their titres being reduced by an average of 6.0 log p.f.u./ml. The turbid plaque-formers lost an average of 3.3 log p.f.u./ml. The titre of 1307 was reduced by 2.0 log p.f.u./ml.

Heat sensitivity

MV-L1 and MV-L2 can be distinguished by their sensitivity to 60°C for 30 minutes, MV-L1 being relatively resistant whereas MV-L2 is inactivated. When the various isolates were held at 60°C for 30 minutes the titres of all except 1307 were reduced by 1.0 to 2.0 log p.f.u./ml. The titre of 1307 was reduced by 6.0 log p.f.u./ml.

Virus morphology

Preparations obtained by centrifugation of MV-L1 and MV-L2 showed distinctly different particles when examined in the electron microscope. MV-L1 was seen to be a rod-shaped virus (Fig. 1) of about 90×15 nm (Gourlay *et al.* 1971), whereas MV-L2 was spherical (Fig. 2), about 50–100 nm in diameter and apparently enveloped (Gourlay 1971). Liss & Maniloff (1971) showed that MV-L52 and MV-G51 were morphologically similar to MV-L1, as were their isolates MV-L59 and MV-L60. We examined eight of our virus isolates, namely 03clr, 10tur, 011clr, 179tur, 143tur, 182tur, 1304clr and 1307, in the electron microscope and it appeared that all but 1307 were rod-shaped particles similar in size to MV-L1. Isolate 1307 was morphologically similar to MV-L2.

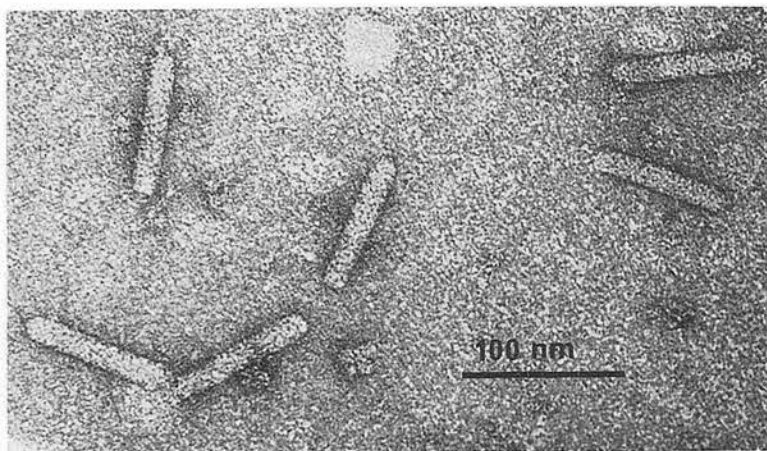


FIG. 1. Purified MV-L1 preparation negatively stained with uranyl acetate.

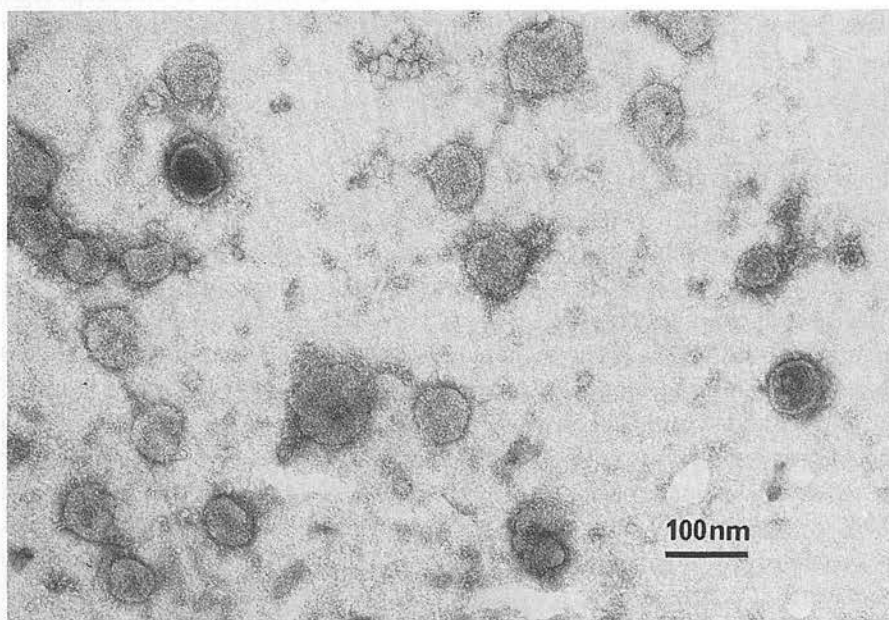


FIG. 2. Concentrated MV-L2 preparation negatively stained with uranyl acetate.

Plaque morphology and temperature of development

Although the plaques produced by MV-L2 are smaller than those produced by MV-L1 (Gourlay 1971), this property is of limited value for differentiation, unless it is well controlled. Even then it may not be constant enough for differentiation when tests are performed at different laboratories. Ideally viruses should be tested not only on the same host, but at the same time. When the isolates were examined under these conditions for plaque size all except 1307 gave large plaques. In addition to plaque size the turbidity or clarity of plaques is of value in differentiating between different isolates.

A better criterion, perhaps, than plaque size for differentiation is the temperature at which plaques form. It has already been shown that MV-L2 differs from MV-L1 in that it does not produce plaques on either BN1 or 1305 layer plates incubated at 22°C whereas MV-L1 does (Gourlay 1971). We have examined this phenomenon more closely and have shown that MV-L1 will form plaques on 1305 layer plates at any temperature between 22°C and 37°C (the range of temperatures examined), whereas MV-L2 will form plaques at 35°C and 37°C but not at 30°C or lower, even though the acholeplasmas grow well at these lower temperatures.

When we examined all the virus isolates on 1305 layer plates at 22°C and 37°C it was apparent that they all produced plaques at both temperatures, except 1307 which produced plaques only at 37°C.

Susceptibility of resistant clones

Layer plates were prepared from three clones of the 1305 strain of *A. laidlawii* resistant to MV-L1 and three clones of 1305 resistant to MV-L2. Control plates were also prepared from the fully susceptible 1305 strain. MV-L1 and MV-L2 virus suspensions were then titrated, and drops of the virus dilutions were placed on each plate. After incubation at 37°C for 24 hours, MV-L1 produced plaques on the 1305 control plate and on the plates prepared from the clones resistant to MV-L2 but not on the clone resistant to MV-L1. Conversely, MV-L2 produced plaques on the control plate and on the plates resistant to MV-L1 but not on the plates resistant to MV-L2. Detailed results are given in Table 2.

This method of differentiation, with the 1305 resistant clones, was used to examine the various virus isolates, and it was seen that all but 1307 produced plaques on the MV-L2 resistant layer plates but not on the MV-L1 resistant plates, whereas 1307 showed the converse reactions.

TABLE 2

Titration of MV-L1 and MV-L2 on layer plates of *A. laidlawii* 1305 clones susceptible and resistant to MV-L1 and MV-L2

Host strain	Titre of virus p.f.u./ml	
	MV-L1	MV-L2
MV-L1 resistant 1	$<2.5 \times 10^2$	6.5×10^8
MV-L1 resistant 2	$<2.5 \times 10^2$	4.5×10^8
MV-L1 resistant 3	$<2.5 \times 10^2$	9.5×10^8
MV-L2 resistant 1	2.5×10^9	$<2.5 \times 10^2$
MV-L2 resistant 2	5.0×10^9	$<2.5 \times 10^2$
MV-L2 resistant 3	4.0×10^9	$<2.5 \times 10^2$
1305 control	3.5×10^9	7.0×10^8

DISCUSSION

There is some doubt as to the origin of the first MV-L1 isolate. It was obviously present in the mycoplasma culture examined. It may have come either from a mycoplasma or been free in the culture, as the culture had undergone very few subcultures since isolation from the bovine nose. In order to try and trace its origin, the culture was inoculated onto a solid medium plate, and from the mycoplasma colonies that grew eight were selected and cloned. Each clone was then grown as a lawn on solid medium plates and washed as described previously. Each washing was then tested for viral activity on lawns of BN1. A plaque was observed at the site of application of one of the drops, and the clone from which this washing was obtained was shown to be *A. laidlawii*. It would appear, therefore, that MV-L1 probably originated from an acholeplasma present in the original culture. The high titre of virus in the original filtrate (2.5×10^9 p.f.u./ml) and the common occurrence of viruses in *A. laidlawii* would support this contention.

MV-L1 and MV-L2 were classified as viruses for the following reasons: (1) they were filtrable, passing through Millipore membrane filters of 25 and 50 nm pore size respectively; (2) they were inactivated by ultraviolet light (254 nm), indicating that they contained nucleic acid; (3) single discrete plaques were produced at high dilutions on lawns of the appropriate acholeplasma, indicating that the agents were particulate; (4) addition of high dilutions of the agents to broth cultures of growing acholeplasmas resulted in a 3–5 log increase in titre of the agents, showing that they replicate; and (5) there was no evidence of replication in the absence of actively growing cells of the acholeplasma.

Not all these criteria have been applied to each of the new isolates. However, each isolate produced single discrete plaques at high dilution and resembled

either MV-L1 or MV-L2 serologically and by most of the other methods used for differentiation purposes, including electron micrographs of a number of them, which showed particles morphologically resembling either MV-L1 or MV-L2.

Examination of the various isolates suggests that all except 1307 resemble MV-L1, while 1307 is similar to MV-L2. These isolates can therefore probably be considered different strains of MV-L1 and MV-L2.

The demonstration of both clear and turbid plaque forms of these viruses may indicate the ease of mutation of these viruses. A large plaque mutant of MV-L1 has already been described (Gourlay & Wyld 1972) and it was noticeable that the clear plaque mutant produced larger plaques than did the turbid one and was also more sensitive to ether. It was interesting that the strain of *A. laidlawii* used as host was important in demonstrating the two types of plaques, as it was only when MV-L1 was placed on the 1305 strain that they were observed.

Ether sensitivity proved of no value for differentiation purposes. The sensitivity to ether of the isolates (which apparently resemble MV-L1 by all other criteria) is difficult to explain, but may be due to the failure to heat them between each subculture as was the custom with MV-L1. This was not done as the heat sensitivity of each isolate was unknown at the time.

It is apparent from our observations and those of Liss & Maniloff (1971) that many strains of *A. laidlawii* carry a virus and some even carry two viruses: for example M1307/68 and 0-324 carry both MV-L2 and MV-L1. The two recorded instances of viruses being isolated from a mycoplasma other than *A. laidlawii* are the isolations from *Mycoplasma* strain 14 (goat) (Liss & Maniloff 1971) and from *A. granularum* M217/69. Although these viruses have been isolated from species other than *A. laidlawii* they infect and therefore presumably belong to the MV-L group.

SUMMARY

Twenty-six isolates, including Mycoplasmatales Virus-Laidlawii 1 (MV-L1) and MV-L2, were obtained from 14 different strains of *Acholeplasma laidlawii* and one strain of *Acholeplasma granularum*. All but MV-L1 were obtained by the 'washing' method of virus isolation.

The 26 isolates were examined in various ways including those that had previously proved useful in distinguishing between MV-L1 and MV-L2; these were determination of the infective host, serology, sensitivity to detergent, sensitivity to heat, plaque morphology, temperature of plaque development and ability to infect clones of *A. laidlawii* resistant to MV-L1 and MV-L2. In

addition, eight isolates were examined morphologically in the electron microscope.

By these criteria all but one of the agents appeared to be similar to MV-L1. The remaining one (1307) resembled MV-L2.

The two strains of *A. laidlawii* from which MV-L2 and the 1307 isolate were obtained also released a virus similar to MV-L1. The MV-L1 type isolate acquired from *A. granularum* produced plaques on *A. laidlawii*.

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Discussion

Pirie: No doubt you are tired of having Jonathan Swift's rhyme quoted at you:

*So, naturalists observe, a flea
Hath smaller fleas that on him prey;
And these have smaller fleas to bite 'em,
And so proceed ad infinitum.*

Is a resistant strain resistant to lysis or to multiplication?

Gourlay: It is resistant in that it will not form plaques when the virus is put on. We do not know whether the acholeplasma is resistant to attachment or is immune. Many acholeplasma strains apparently carry the MV-L1 virus,

since we can isolate virus from them. However, we can apparently still use these strains as hosts to grow serologically similar virus.

Pirie: Are you sure that the viruses that are stuck to the mycoplasma by one end are coming to it and not being extruded from it?

Gourlay: It is possible that they are coming out or going in and are not simply attached.

Harrison: How big is MV-L2?

Gourlay: From filtration studies and rough measurements in an electron microscope it seems to vary from about 50 nm to about 120 nm in diameter.

Harrison: Have you tried using phenol to prepare infective nucleic acid? If this is possible, the type of nucleic acid could be studied by enzyme inactivation experiments. In morphology MV-L2 superficially resembles some RNA-containing viruses of plants and vertebrates.

Gourlay: No.

Chen: In those plates where you do not find any plaques do you think the virus will be freely and gradually released from the cells? Do mycoplasmas release the viruses only after they die?

Gourlay: I do not know. Virus release is rather difficult to understand; one can try many times to release virus from a particular acholeplasma lawn and only succeed once or twice. We have tried various means of releasing the virus from acholeplasma lawns; by washing with different fluids, by lysing cells with detergent and by treatment with ultraviolet light followed by washing, but have found no way of doing it reliably (Gourlay, unpublished findings, 1971). On the other hand, virus can readily be obtained from lawns prepared from 'resistant' acholeplasmas.

Taylor-Robinson: Milne, Thompson and I (1972) have looked at MV-L1 in the electron microscope, both by negative staining and by sectioning infected cells. In negatively stained MV-L1 preparations we see bacilliform bodies 86 nm long and about 13 nm wide, which are similar to your measurements, Dr Gourlay. We have also seen a great variety of other different forms, particularly tubular structures which were between 50 nm and 1 μ m long and about 13–18 nm wide. They were quite clearly emerging or being extruded from the mycoplasma membrane. We never saw these structures in uninfected cultures and we do not know what they are, but perhaps they could be the result of aberrant virus synthesis. In sections of infected cells we saw virus particles attached to the outside of the cells and radiating from them; they were about 75 nm long and about 7.5 nm wide, so there is a discrepancy between the size of the particles here and the size in the negatively stained preparations. We think that perhaps the nucleic acid is stained but not the protein and that this

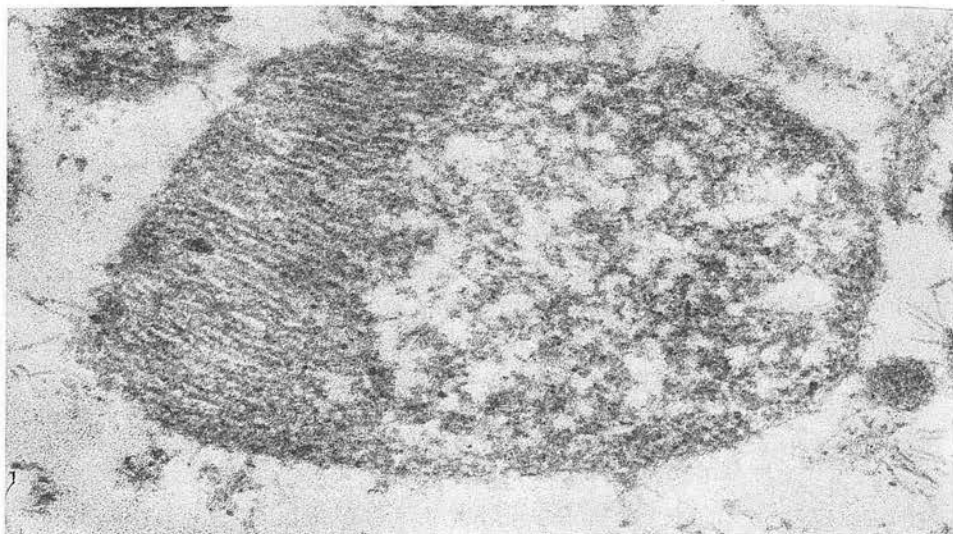


FIG. 1 (*Taylor-Robinson*). Section of a cell of *A. laidlawii* infected with MV-L1 showing virus particles inside the cell. $\times 77\,000$.

accounts for the discrepancy in size. Milne (1967) has observed the same thing in electron microscopy studies of other viruses. Of particular interest is that about one in four of the achleoplasma cells contain striated masses (Fig. 1) which we believe to be virus particles; again they are about 7.5 nm wide, but it is difficult to tell how long they are. We believe that this observation supports the idea of Liss & Maniloff (1971) that the virus particles emerge from the cell by its rupture. It seems inconceivable that each of the particles is extruded through the cell membrane, and I suggest that those which are seen projecting radially from the cell have been adsorbed back onto the membrane rather than extruded singly through it.

Also, in about one in 20 of the cells we saw rather spiky things which were about 12 nm across and at least 116 nm long before they apparently disappeared into the plane of the section. We do not know what they are, but perhaps they are helical and if so we suggest that they may be the nucleocapsid components of MV-L2.

Pirie: Many years ago it was observed that influenza virus seemed to be extruded from the cell and then broken off in pieces. How does that work stand up now?

Horne: This was first observed by Hoyle (1954), and it was from subsequent electron microscope studies that both complete and incomplete forms of myxoviruses could be seen being released by this mechanism. It appears that

the haemagglutinin component and ribonucleoprotein part of the virus is packaged at the cell surface in an envelope which forms part of the host cell plasmalemma. This can be seen to a greater extent in cells infected with the larger myxoviruses such as mumps and Newcastle disease (see Wolstenholme & Knight 1964).

Maramorosch: Vago & Giannotti (1971) have shown in Stolbur-infected plants mycoplasma-like bodies that had similar structures, and they interpreted them as viruses.

Maniloff: Dr Taylor-Robinson, how old was the infection shown in Fig. 1?

Taylor-Robinson: It was about 48–72 hours after virus inoculation.

Maniloff: Do you know what the multiplicity of infection (MOI) was?

Taylor-Robinson: Not really. We believe we infected a very high proportion of the cells and so increased our chance of seeing things within them.

Maniloff: We have looked at one-step growth curves of the MV-L1 type viruses, and clearly different eluate isolates have different biological properties (Liss & Maniloff 1971). The curves resemble animal virus replication or filamentous bacteriophage replication. The burst size is characteristic of the virus; for MV-L1 it is about 5, for some of the others it is 80–100. We measured the colony forming units during virus infection and found no loss of viability, so we do not believe that there is cell lysis in the bacteriophage sense. We think the cells release viruses and that if one plates them on agar one, in essence, rescues them, and they can form colonies.

We think we get a plaque on lawns because the cells grow past their optimal growth phase and are either growth-inhibited or lysed. We tried to look at the early events of infection. In general we try to work at an MOI of one or below. After infection with an MOI of a half, since the average number of viruses per cell is a half, if we see a cell with more than one virus on it the viruses must have been made by the cell. We see lots of membrane floating about and patches of virus. The viruses do not seem to be randomly distributed on the membrane, there seem to be patches of membrane where they come out. And, as in Dr Taylor-Robinson's figure (Fig. 1), there is an unresolvable globule inside that membrane.

We modified Dr Gourlay's isolation method and used the following method. In a fixed-angle tube we layered in 1 ml of 30% sucrose and 10 ml of culture, 10^8 colony forming units per ml, and spun hard enough to pellet the virus; the cell pellet ends up plastered against the sides of the tube. If one then takes out 1 ml from the bottom with a needle and plates it, plaques form. But plaques form every time we try it. We have used this method to isolate viruses from mycoplasmas that we had never been able to isolate them from by the washing method. This procedure appears to strip viruses off cells; it is not

merely physically separating extracellular viruses from cells. By this technique we have isolated virus from *M. pneumoniae*, *M. gallisepticum*, *M. arthritidis* H39 and Connecticut goat. We have completed the acholeplasma sequence; Dr Gourlay has found viruses in *A. granularum* and *A. laidlawii* and we have one from *A. axanthum*. But as Dr Gourlay said, we can only grow viruses on *A. laidlawii*.

Neimark: Do you isolate phage from liquid cultures of these other species?

Maniloff: We grow a tube of *M. gallisepticum*, layer it over sucrose and spin.

Razin: Is it not strange to isolate from different mycoplasmas viruses which will infect only *A. laidlawii*? Is it possible that you are working with cultures which contain *M. laidlawii*?

Maniloff: We have cloned and serologically typed all our cultures. The *A. axanthum* came from Dr Tully, and we just plated it without passing it first in our own laboratory.

Razin: It is not so strange for Dr Gourlay to isolate the virus from *A. granularum* because *A. granularum* is so similar to *A. laidlawii*. But *M. pneumoniae* and *A. laidlawii* are very different.

Taylor-Robinson: It is very odd that many people are trying to isolate mycoplasma viruses and failing, whereas you can isolate them from everything!

Neimark: Some of the problems you and others are having arise, I believe, because MV-L1 is lysogenic. After some effort we were able to isolate an indicator strain from *A. laidlawii* BN1 which is free of this phage. On this basis we have developed a satisfactory system (H. Neimark & Z. Abzug, unpublished findings, 1971); plaques on this indicator strain are uniform and clear. One can demonstrate that *A. laidlawii* BN1 is lysogenic by adding the indicator strain in an agar layer over lawns of *A. laidlawii* BN1 spotted on agar plates; plaques form in the indicator layer only above the areas of *A. laidlawii* BN1 growth.

Taylor-Robinson: Dr Maniloff, could your lawn of *A. laidlawii* contain virus which you stimulate to grow by adding the various inocula to it?

Maniloff: We had no success until we switched to the sucrose technique. We have plated many other things, such as uninoculated cultures and buffer solutions. We are clearly not working with the same viruses because there are at least two different electrophoretic patterns to the virus proteins and their biological properties are clearly different.

Tully: What criteria could one use to be sure that a mycoplasma was free of virus? Perhaps a sedimentation profile of the DNA of the BN1 strain of *A. laidlawii* in a sucrose gradient would reveal whether two DNA populations were present. As long as you have evidence that there may be latent phage in the

test lawn strain (BN1), I do not see how you can accurately determine whether there are viruses in other mycoplasmas.

Gourlay: This is crucial; but to get a mycoplasma free from virus is extremely difficult.

Tully: Have you tried to see if any of these strains have two DNA populations as demonstrated by Dugle & Dugle (1971) for another *A. laidlawii* strain?

Gourlay: No.

Maniloff: Strains of *Escherichia coli* are lysogenic, and many of my colleagues say that everything they work with is lysogenic. Our problem is to get to the stage where we are satisfied that we understand the system and know when it might be back-firing on us.

Hayflick: Does the material you remove from the centrifuged cultures of *M. pneumoniae*, *M. gallisepticum* and so on, and then put on lawns of *A. laidlawii* result in replication of a particle that, in your judgement, is an *A. laidlawii* virus or a virus indigenous to other mycoplasma species?

Maniloff: Before we switched experimental methods I thought we were isolating viruses from different mycoplasmas. Now I do not know.

Hayflick: Have you mixed *M. pneumoniae*, for example, with *A. laidlawii*?

Maniloff: No.

Maramorosch: Did you find viruses in extracts from plants?

Maniloff: Dr Whitcomb and I (unpublished findings, 1971) isolated an *A. laidlawii* strain from both leaf hoppers and corn stunt which had a disc electrophoretic membrane pattern unlike anything that has been described. And it does put out viruses.

Tully: I was under the impression that you were not able to repeat these isolations on other corn stunt and leaf hopper material supplied by Dr Whitcomb.

Chen: Dr Maniloff, what kind of culture do you get from the corn stunt material?

Maniloff: I have no idea where it came from because at that time I did not know about such things as surface sterilization. We just ground up the leaf hoppers, filtered them and plated the filtrate.

Razin: Have you any indication of how the phage infects the mycoplasma? Does it inject its nucleic acid or is it incorporated, as it is, completely by the mycoplasma?

Gourlay: Unlike many bacteriophages, MV-L1 does not appear to have an injection mechanism. As the acholeplasma cell has no cell wall it may not need it. I do not know how the nucleic acid enters the cell. It may enter through holes in the membrane or it may be actively engulfed by the host.

Horne: From your sucrose experiment, Dr Maniloff, is it possible that the

particles as separate entities are damaged particles? Are you saying that this would account for the asymmetric shape of the particle—with one end broken off?

Maniloff: I do not believe that at all. I believe that Dr Gourlay's picture (fig. 1, p. 152) is right: MV-L1 seems to be bullet-shaped, round at one end and flat at the other.

Neimark: We see the same morphology.

Gourlay: MV-L1 appears to have an attachment end. At least one end is different from the other, and when we examine virus particles attached to acholeplasma cells or debris we do not observe virus particles attached by both ends (Bruce *et al.* 1972). But we have not seen any obvious attachment areas on the surface of MV-L2.

Harrison: Have you tried the effects on the replication of MV-L2 of the inhibitors that are normally used to distinguish RNA from DNA viruses?

Gourlay: No. We have been trying to see what the inside of MV-L2 is like. We have sectioned the virus, but the material inside appears as a small round mass. From the way it aggregates, it may have a rather loose type of internal structure.

Tully: Is there any evidence that virus infections in mycoplasma might alter the antigenic characteristics of these organisms? We have 12–15 acholeplasmas that are not *A. laidlawii*, *A. granularum*, or *A. axanthum*, and they have biological characteristics that are different from these three established species. We have not been very successful in demonstrating mycoplasma viruses in them. However, from a taxonomic standpoint we are going to have to be sure that no viruses are present in these strains before naming a new species since, in my opinion, transduction of antigens from *A. laidlawii* is a very real possibility.

Gourlay: One can certainly find differences in the colonial morphology of infected and non-infected *A. laidlawii*. The infected colonies are smaller and rougher (Gourlay, unpublished findings, 1971) and I see no reason why the conversion phenomenon, in which bacterial antigens can be altered by phage infection, should not apply to acholeplasmas infected with virus.

Tully: For this reason, I believe we need to have some methods to exclude virus infections from candidates for new species of *Mycoplasma* or *Acholeplasma*.

Ehrman: In 1968 we looked at the seminal fluid of the sterile hybrid male *Drosophila paulistorum* by phase contrast microscopy and saw some very large particles that resembled RNA viruses (Kernaghan & Ehrman 1968; Tandler *et al.* 1968). These sterile hybrids never produce any motile sperm; they produce sperm tails but the heads are defective. The semen came from the posterior ejaculatory duct. At that time I did not know how the mycoplasma-

like organism I was studying was inherited. I thought perhaps it was injected into the body of the female by the male during copulation. We located these RNA entities in the paragonia of *D. paulistorum* males where they are to be found in intracellular cytoplasmic vacuoles. I could never trace them into the body of the female.

Taylor-Robinson: This is a conference on pathogenic mycoplasmas. Is it possible to twist things and ask whether the virus could have an effect on the pathogenicity of the mycoplasmas?

Gourlay: Yes, perhaps it could. It is possible that a situation may occur with mycoplasmas similar to that with *Corynebacterium diphtheriae* in which non-toxicogenic varieties can be made toxigenic by infection with bacteriophage (Groman 1959).

Bové: Have you investigated the G + C content of the virus DNA?

Gourlay: No, the problem is getting enough virus DNA.

Bové: Is the protein firmly bound to the rest of the DNA or can you detach it with detergent?

Gourlay: I do not yet know.

Razin: There is a question about the nomenclature, Dr Maniloff, as your MV-G51 and MV-L52 may be similar or identical to Dr Gourlay's MV-L1.

Maniloff: We should not have numbered our isolates. However, by our biological criteria they are biologically different from MV-L1 (Liss & Maniloff 1971).

Taylor-Robinson: But virologists do not work like that. They rely more on antigenic differences based on serology. Have you typed your viruses serologically?

Maniloff: One of our three isolates has a different disc gel pattern (A. Liss & J. Maniloff, unpublished findings, 1971), but they are serologically similar to MV-L1, as determined by Dr Gourlay.

Harrison: Dr Gourlay, it appears that the vast majority of your isolates are serologically related to each other, and there is every reason to think they are morphologically similar; they are what I would call the same virus. I would like to see a name put forward which would include all your isolates that are related to one another together with any others that might be found to be related to your type strain.

Gourlay: I think we have only two viruses: MV-L1 and MV-L2. All the other isolates are related to one of these. However, Dr Maniloff has now called his isolates MV-G51 and MV-L52 etc. and this has caused confusion. We have named these viruses Mycoplasmatales viruses and I think it is premature to change this now.

Taylor-Robinson: If there was to be a change I would like to see MV-L1

changed to ML-VI, because there is no *A. laidlawii* 1, it is the virus that is 1.

Gourlay: There is already an MLV, the Melilotis latent virus, and, furthermore, it would have to be called ALV (*Acholeplasma laidlawii* virus).

Whittlestone: It might be useful to concoct a name that related to its DNA content and its rod shape.

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Further Studies on the Morphology and Composition of Mycoplasmatales Virus-laidlawii 2

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SUMMARY

Mycoplasmatales virus-laidlawii 2 (MV-L2) was purified by ammonium sulphate precipitation and by sucrose density gradient fractionation. Electron micrographs of virus showed predominantly spherical enveloped particles with a mean diameter of about 80 nm (range 52 to 125 nm). The envelope had a 'unit membrane' structure and was probably serologically dissimilar to the 'unit membrane' of the host *Acholeplasma laidlawii*. No obvious isometric or helical capsid was observed within the envelope. The nucleic acid appeared to be DNA.

INTRODUCTION

Mycoplasmatales virus-laidlawii 2 (MV-L2) was isolated from a strain of *Acholeplasma laidlawii*, a member of the Mycoplasmatales (Gourlay, 1971). This virus differed morphologically, serologically and biologically from Mycoplasmatales virus-laidlawii 1 (MV-L1), the first virus isolated from *A. laidlawii* (Gourlay, 1970), which is a small rod probably containing DNA (Gourlay, Bruce & Garwes, 1971). Electron micrographs of negatively stained MV-L2 showed roughly spherical, apparently enveloped particles, about 50 to 120 nm in diameter (Gourlay, 1971).

This paper describes the method used for purification of MV-L2, further electron microscope and serological studies and studies designed to examine its nucleic acid type.

METHODS

Virus production and assay. *A. laidlawii* strains 1302/68 and 1305/68 (Gourlay & Wyld, 1972) were used as host for this virus. The acholeplasma strains were grown and the virus produced and assayed (by the modified Miles and Misra method), as previously described for MV-L1 (Gourlay & Wyld, 1972), except that incubation at 37 °C was carried out for 36 to 48 h instead of 18 to 24 h.

Preparation of antiserum. For the preparation of MV-L2 antisera, rabbits received an initial intramuscular injection containing 5×10^{10} p.f.u. of purified virus combined with sodium alginate (Medical Alginates Ltd, Perivale, Greenford, Middlesex) as adjuvant, followed 2 weeks later by three weekly intravenous injections of equivalent amounts of virus. The rabbits were bled for serum 4 and 7 days after the last injection. *A. laidlawii* antiserum was prepared by a similar regimen, using antigen prepared from the 152 strain of *A. laidlawii*.

Examination of the serological relationship between MV-L2 and A. laidlawii. This was examined indirectly by growth inhibition and neutralization of the achleoplasma and virus, respectively.

In the first experiment, a lawn was prepared with the 1305/68 strain of *A. laidlawii*. When the plate was dry, 0.02 ml of *A. laidlawii* antiserum was dropped on to the lawn, 0.02 ml of MV-L2 antiserum was dropped on to another area of the lawn and finally a third area of lawn was inoculated with 0.04 ml of MV-L2 suspension at a concentration which produced confluent plaques. When the virus suspension had dried, 0.02 ml of MV-L2 antiserum was dropped within the area of the MV-L2 suspension drop. The plate was then incubated at 37 °C. In the second experiment, 0.2 ml of *A. laidlawii* antiserum was added to 0.2 ml of MV-L2 suspension, diluted in phosphate buffered saline (PBS), pH 7.3; the mixture was incubated for 2 h at 37 °C, after which it was assayed for MV-L2 activity. Controls with normal rabbit and MV-L2 antisera were prepared.

Purification of MV-L2. MV-L2 virus suspension, produced on solid medium plates (Gourlay & Wyld, 1972) using the 1305/68 strain of *A. laidlawii* as host, was made up to 55 % saturation with ammonium sulphate in an ice bath. The mixture was centrifuged at 10000g for 45 min at 4 °C. The resulting supernatant fluid was discarded and the precipitate was dissolved in a small volume of 0.15 M-sodium chloride buffered with 10 mM-tris, pH 7.4 (TS), and dialysed against 500 vol. of TS overnight at 4 °C. The dialysed precipitate was then layered over a two-step sucrose gradient consisting of 13 ml of 20 % (w/w) sucrose in TS layered over 4.5 ml of 60 % (w/w) sucrose in TS, in a 20 ml polycarbonate tube. This was centrifuged in a 3 × 23 ml swing-out rotor at 30000 rev/min (94000g av.) for 3.5 h at 4 °C. The gradient was fractionated into 0.7 ml samples by pumping from the bottom of the tube through a capillary tube. MV-L2 was located by infectivity assay and by extinction at 260 and 280 nm. Fractions containing most virus were pooled, dialysed against TS, then layered over a 12.5 ml linear gradient from 20 to 60 % (w/w) sucrose in TS. The gradient was centrifuged in a 6 × 15 ml swing-out rotor at 25000 rev/min (59000g av.) for 17 h at 4 °C and then fractionated into 0.4 ml samples by the method described for the previous gradient. Virus was located as described above.

Incorporation of [³H]-labelled nucleic acid precursors. *A. laidlawii* 1305/68 was inoculated in two aliquots of GS broth, without added DNA, one containing 0.03 mM-[³H]-methyl-thymidine and the other 0.03 mM-[³H]-uridine-5' (both at a specific activity of 1000 mCi/m-mol). The broth cultures were incubated overnight at 37 °C and then used to produce layer plates on bases containing the same nucleotides at a concentration of 0.01 mM and a specific activity of 1000 mCi/m-mol. The layer plates were infected with MV-L2 and the virus produced was purified as described above, samples being removed at each step in the purification procedure for total and acid-insoluble radioactivity. *A. laidlawii* 1305/68 was inoculated into two samples of GS broth, without added DNA, one containing 0.01 mM-[³H]-thymidine and the other 0.01 mM-[³H]-uridine (both at a specific activity of 1000 mCi/m-mol). The cultures were incubated at 37 °C and sampled at 6 h intervals for acid-insoluble radioactivity. Radioactivity was measured with a Packard model 2425 liquid scintillation counter.

Colorimetric determination of virus nucleic acid. Aliquots of the purified virus suspension were analysed using the diphenylamine reaction for DNA (Burton, 1956) and the orcinol test for RNA (Mejbaum, 1939). Solutions of calf thymus DNA (BDH Ltd) and yeast soluble RNA (Sigma Chemical Co.) were used to construct standard curves in both tests and the relative sensitivity of the orcinol test for RNA and DNA was calculated.

The effect of ribonuclease on plaque formation. The activity of MV-L2 on the 1305/68 strain

of *A. laidlawii* grown on solid medium plates containing 1 mg/ml of ribonuclease-A (Sigma, type 1-A) in the top layer of the plate was tested (Bradley, 1966).

Electron microscopy. For direct examination specimens were applied to carbon-collodion copper grids and negatively stained for about 30 s with 2 % uranyl acetate. Specimens for sectioning were fixed in 3 % glutaraldehyde in 0.1 M-phosphate buffer for 3 h, washed and postfixed in 2 % osmium buffered solution (Millonig's fixative) for 1 h. Pellets were embedded in Araldite and cut on a Reichart OMU 2 ultramicrotome. Sections were stained with 3 % uranyl acetate in 50 % methanol for 15 min followed by 2 % lead acetate for 5 min before examination. All specimens were examined with an AEI EM6 electron microscope. The dimensions of 100 negatively stained virus particles were measured from photographic enlargements of accurate magnification. Each particle was measured twice, along the maximum and minimum diameter, and the average diameter calculated.

RESULTS

Purification of virus

The MV-L2 suspension was concentrated from 2360 ml to 2 ml during the purification process. The initial virus titre was 1×10^9 p.f.u./ml and the final titre after purification was 1×10^{12} p.f.u./ml. Electron micrographs of purified material showed numerous virus particles and very little contaminating material.

Virus, subsequently located by infectivity assay, was observed as a narrow band in the linear sucrose density gradient at a concentration corresponding to a buoyant density of 1.19 g/ml.

A significant degree of purification was indicated by the fact that antisera prepared in rabbits by inoculation of purified virus possessed no demonstrable growth inhibitory antibodies to *A. laidlawii*.

Investigation of the nucleic acid type

In two experiments, the addition of ribonuclease to the medium had no effect on the formation of plaques as the titres of virus grown on ribonuclease-containing plates and normal plates were similar.

The experiments involving incorporation of [3 H]-labelled nucleic acid precursors showed that radioactivity was found only in association with virus grown in the presence of labelled thymidine and not uridine (Fig. 1). As a control, the incorporation of [3 H]-thymidine and [3 H]-uridine into uninfected *A. laidlawii* cells was examined and it was shown that both nucleic acid precursors were incorporated into the acid-insoluble material with high efficiency.

The positive reaction of purified virus in the diphenylamine test supported the presence of DNA in the virus particle. The result of the orcinol test, when adjusted for the DNA present, indicated that the particle contained no RNA.

Electron microscopy

Examination of images of negatively stained concentrated or purified virus suspension showed numerous roughly spherical particles 52 to 125 nm in diameter with a mean diameter of about 80 nm (Fig. 2). They generally excluded stain (Fig. 3a, b, c). Frequently, however, stain penetrated into the interior of the particles leaving an unstained peripheral band about 10 nm wide (Fig. 3d, e, f). The interior was usually densely stained and no obvious structure could be visualized but, occasionally, when the stain was less dense there was an indication of a fine coiled structure within the envelope (Fig. 3d, e, f).

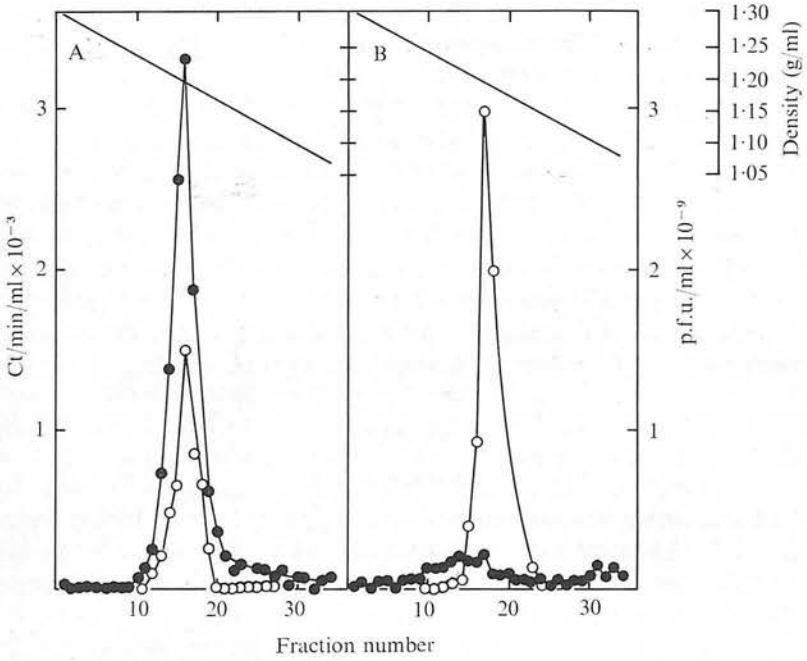


Fig. 1. The distribution of virus grown in medium containing: A, $[^3\text{H}]$ -thymidine; B, $[^3\text{H}]$ -uridine, after centrifuging to equilibrium in linear sucrose gradients. $[^3\text{H}]$ ct/min (●—●); p.f.u./ml (○—○); the continuous line represents buoyant density.

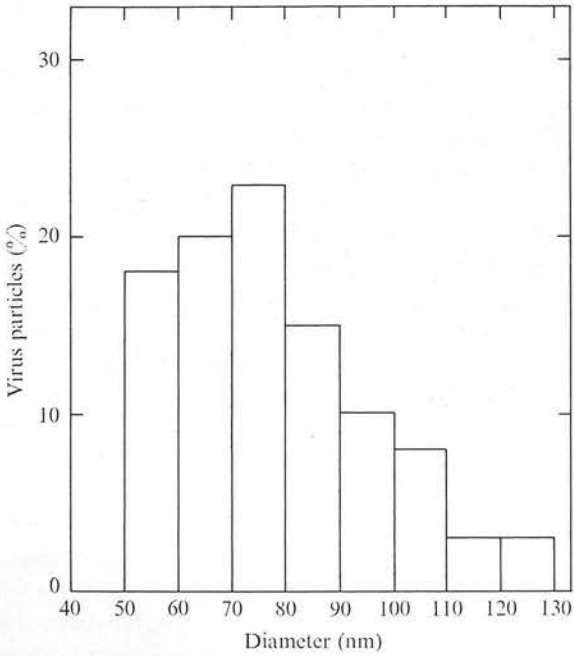


Fig. 2. Histogram to show average diameter of virus particles.

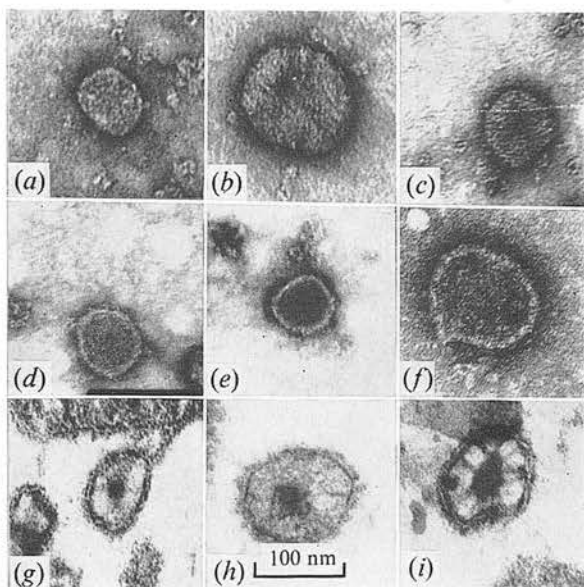


Fig. 3. Electron micrographs of MV-L2. (a) to (f) negatively stained with uranyl acetate; (a) to (c) stain excluded; (d) to (f) showing stain penetration; (g) to (i) sectioned and stained with uranyl acetate and lead citrate.

In thin sections, positively stained particles had a distinct triple-layered periphery indicated by two electron-dense layers separated by an electron transparent layer (Fig. 3g, h, i). Dense irregular depositions of stain appeared in the centre of the particles (Fig. 3g, h, i), occasionally possessing stained strands radiating from the dense centre to the periphery (Fig. 3i).

Examination of thin sections of *A. laidlawii* 1302/68 that had been infected with MV-L2 for 3.5 h showed many virus particles attached to the acholeplasma cell membrane (Fig. 4a, b, c). After infection for 7 h numerous free virus particles could be seen, frequently congregated together as though just released from the same source (Fig. 5). Sometimes the virus particles were observed adjacent to (Fig. 6) or almost encircling (Fig. 7) apparently disintegrating acholeplasma cells with no discernible membrane.

Examination of the serological relationship between MV-L2 and A. laidlawii

In the first experiment, on solid medium, *A. laidlawii* antiserum produced an area of inhibition of acholeplasma growth at the site of application of the drop, with a narrow band of enhanced growth at the periphery of the inhibition area. MV-L2 antiserum produced a distinct area of inhibition of MV-L2 plaque formation, indicated by a circle of acholeplasma growth within the confluent virus plaques. MV-L2 antiserum produced a slight enhancement of growth where it had been dropped on to the acholeplasma lawn.

In the second experiment, MV-L2 treated with normal rabbit serum and *A. laidlawii* antiserum had a titre of 2×10^6 p.f.u./ml, whereas MV-L2 treated with MV-L2 antiserum had a titre of $< 2.5 \times 10^1$ p.f.u./ml.

These results show that MV-L2 antiserum does not inhibit the growth of *A. laidlawii* and that *A. laidlawii* antiserum does not inhibit the multiplication of MV-L2.

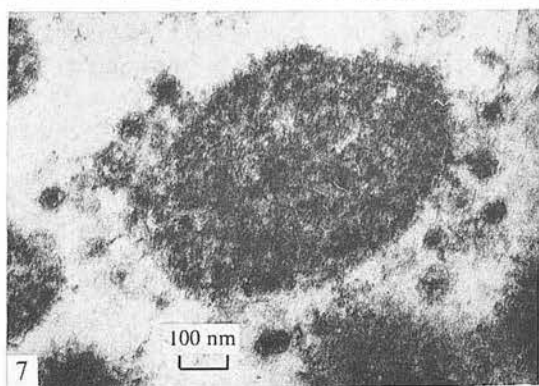
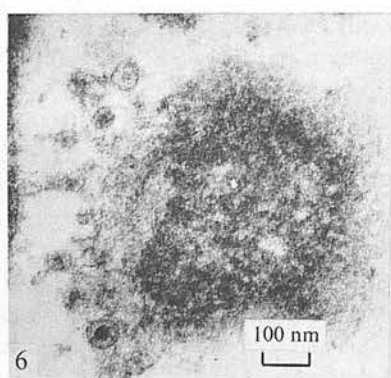
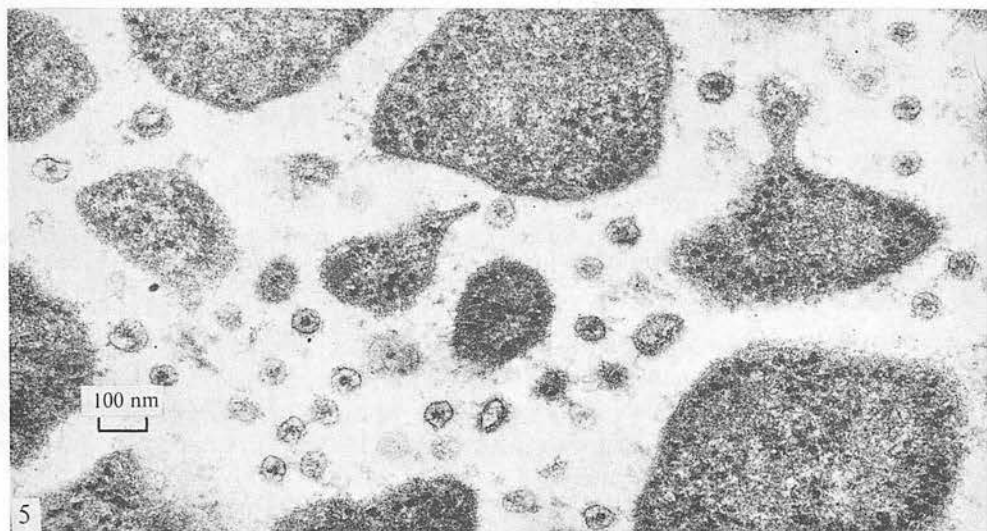
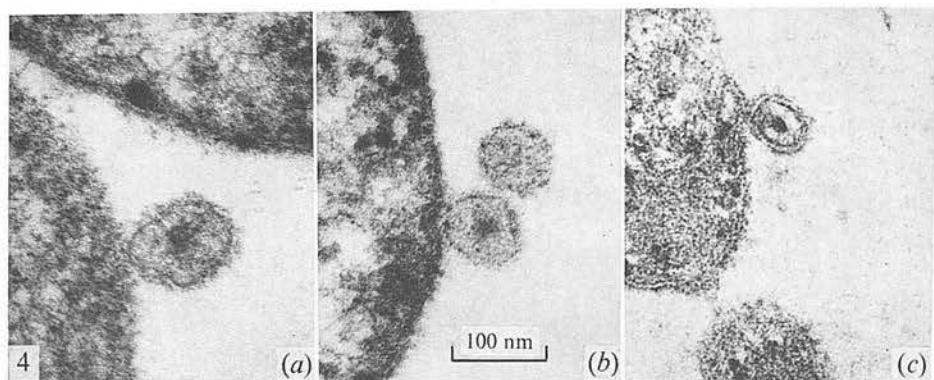


Fig. 4. Electron micrographs of thin sections of *Achleplasma laidlawii* 3.5 h after infection with MV-L2, showing MV-L2 attached to *A. laidlawii* cell membrane.

Fig. 5. Electron micrograph of thin section of *A. laidlawii* 7 h after infection, with MV-L2, showing numerous free virus particles.

Fig. 6. Electron micrograph of thin section of *A. laidlawii* 7 h after infection with MV-L2, showing virus particles adjacent to an apparently disintegrating achleoplasma cell.

Fig. 7. Electron micrograph of thin section of *A. laidlawii* 7 h after infection with MV-L2, showing virus particles almost encircling an apparently disintegrating achleoplasma cell.

DISCUSSION

Electron micrographs of negatively stained preparations of MV-L2 showed no obvious polyhedral structures; the particles appeared somewhat pleomorphic but predominantly spherical. Stain penetration of some virus particles indicated that the structure usually observed in these negatively stained preparations is an envelope, without visible projections, about 10 nm in thickness. Within the envelope no obvious isometric or helical capsid is visible. In the occasional virus, however, there is an indication of a fine coiled structure within the envelope. Sections also support the evidence of the loose nature of the internal nucleic acid material which apparently contracts into a small core on fixation.

Examination of sections of virus confirmed the presence of an envelope, which apparently has a 'unit membrane' structure.

The association of radioactivity with virus grown in the presence of labelled thymidine and the results of the colorimetric determination provide strong evidence that the virus genome is composed of DNA. This is further supported by the failure of ribonuclease to inhibit plaque formation by the virus.

The buoyant density of MV-L2 in sucrose solution of 1.19 g/ml is in agreement with the evidence that the virus is bounded by a lipoprotein membrane, suggested by electron microscopy and the sensitivity of virus infectivity to lipid solvents (Gourlay, 1971).

Thin sections of infected acholeplasma cells show no evidence of virus budding, although the virus particles observed attached to the cells may be in the late stages of budding from the cell surface. The variation in size of the virus particles suggests that they bud and this variation reflects the imprecision of the process of envelope formation at the cell surface. A number of acholeplasma cells surrounded by virus particles appear disintegrated having no distinct cell membrane; this would suggest that lysis can occur whether budding occurs or not.

Neither mature virus particles nor provirus have been observed with certainty within the acholeplasma cell. However, particles resembling mature virus has been observed within cells that are apparently liberating virus and these particles are usually seen within less electron-dense areas which may really be extracellular invaginations.

Failure to inhibit growth of MV-L2 by *A. laidlawii* antiserum and *A. laidlawii* by MV-L2 antiserum, perhaps indicates that no serological relationship exists between the envelope of MV-L2 and the membrane of *A. laidlawii*.

We wish to thank Mrs Brenda Pike for skilled technical assistance.

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Isolation of Mycoplasmatales Virus-laidlawii 3, a New Virus Infecting *Acholeplasma laidlawii*

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Two fundamentally different viruses that infect *Acholeplasma laidlawii* have so far been isolated. They have been designated Mycoplasmatales virus-laidlawii 1 (MV-L1) and Mycoplasmatales virus-laidlawii 2 (MV-L2). MV-L1 is an unenveloped helical DNA virus, 16 nm in diameter and 90 nm in length (Bruce *et al.* 1972); while MV-L2 is a predominantly spherical enveloped virus with a mean diameter of 80 nm (range 52 to 125 nm). Its nucleic acid appears to be DNA (Gourlay *et al.* 1973).

This report describes the isolation of another virus which infects *A. laidlawii* and which differs from both MV-L1 and MV-L2.

Four serial tenfold dilutions of two clones of the M1305/68 strain of *Acholeplasma laidlawii* resistant to MV-L2 (Gourlay, 1972) were prepared in phosphate-buffered saline, pH 7.3, containing 5% foetal calf serum. Drops (0.02 ml) of each dilution were placed on glucose serum (GS) agar plates (Gourlay & Wyld, 1972) and the plates were examined after incubation at 37 °C for 48 h. The plate inoculated with acholeplasmas of clone 1 revealed confluent growth, within the areas of the drops, at all dilutions; the dilutions being distinguished one from the other only by variations in density of growth. The acholeplasmas of clone 2 had grown similarly but at the 10^{-1} dilution, 20 to 30 very small plaques were visible within the area of the drop, and at the 10^{-2} dilution, three slightly larger plaques were observed.

No plaques were visible at the neat and 10^{-3} dilutions. These plaques had apparently formed spontaneously and could be reproduced by the same method with clone 2.

The plaque-forming agent was propagated on a lawn of the M1305/68 strain of *Acholeplasma laidlawii*. Tenfold dilutions of clone 2 were prepared in buffered saline and drops were placed on the lawn. After incubation for 48 h confluent plaques were visible at the site of the neat, 10^{-1} and 10^{-2} dilution drops and an almost confluent plaque at the 10^{-3} dilution drop. All these plaques were cut out of the agar plate and placed in buffered saline for 5 h, and this fluid constituted the starting material for plaque-purification and characterization of the agent. The titre of the agent in this fluid was $6.25 \log$ p.f.u./ml. The agent was cloned by picking a single plaque into buffered saline and subinoculating dilutions of the fluid onto a lawn of the M1305/68 strain of *A. laidlawii* on four successive occasions. A stock of the cloned material was then prepared (titre $10.2 \log$ p.f.u./ml) and stored at -70 °C. Three separate clones were obtained in this way and all behaved similarly in subsequent characterization studies.

At high dilutions stock material produced single discrete plaques, about 0.5 to 2 mm in diameter (Fig. 1). Plaques were formed during incubation at 22 and 37 °C.

When the agent, diluted 1/1000 in buffered saline, was exposed on two separate occasions for 3 min at a distance of 12 cm from a u.v. light source (Universal U.V. lamp, 254 nm, Gelman-Camag, model 54102) the titre was reduced by an average of $5.0 \log$ p.f.u./ml. Exposure of the agent diluted 1/10 to a temperature of 60 °C for 30 min on three occasions reduced the titre by an average of $0.48 \log$ p.f.u./ml. The agent, diluted 1/10 in buffered saline, was treated on two occasions with the detergent Nonidet-P40 (British Drug Houses,

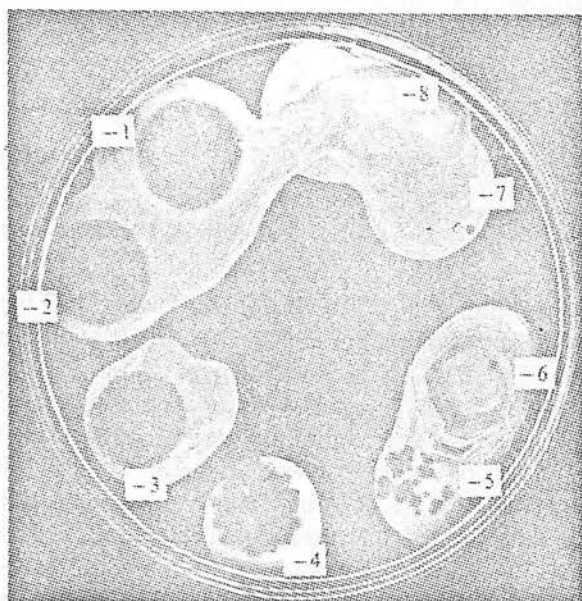


Fig. 1. Plaques formed by serial tenfold dilutions of virus on a lawn of *Acholeplasma laidlawii* strain M1305/68.

Poole, Dorset), 0.4 % (v/v) for 15 min at 37 °C and in both instances the titre was increased very slightly by an average of 0.09 log p.f.u./ml, compared with a buffered saline control. When one part of diethyl ether was added to 4 parts of the agent diluted 1/10 in buffered saline and placed at 4 °C for 18 h no reduction in activity was observed.

A sample of stock material diluted in serum saline was serially filtered under a positive pressure of 5 to 10 lb/in² through Millipore filters of decreasing pore size. In three separate attempts the agent (titre 8.2 to 9.2 log p.f.u./ml.) passed readily through a filter of 220 nm pore size without loss of titre. There was, however, an average loss in titre of 0.8 log p.f.u./ml when passed through a 100 nm filter, and a further average loss in titre of 1.6 log p.f.u./ml when passed through a 50 nm filter. No plaque-forming activity was detected in the 25 nm filtrates.

Antisera prepared in rabbits to MV-L1 and MV-L2 inhibited the formation of plaques by MV-L1 and MV-L2 respectively on lawns of the M1305/68 strain of *Acholeplasma laidlawii*, but both antisera failed to inhibit the formation of plaques by the new agent on lawns of the same acholeplasma.

In a serum neutralization test, 0.2 ml of antisera to MV-L1 and MV-L2, diluted from 1/4 to 1/64 in buffered saline, were added respectively to 0.2 ml saline suspensions of the agent (titre 5.1 log p.f.u./ml); the mixtures were incubated for 2 h at 37 °C and were then assayed for plaque-forming activity. Normal rabbit serum diluted 1/4 was used as control. Neither MV-L1 nor MV-L2 antisera, even at the 1/4 dilution, caused any reduction in titre of the agent. When MV-L1 and MV-L2 antisera, diluted 1/64, were tested against the homologous viruses, the titres were reduced; MV-L1 from 5.37 to < 1.25 log p.u./ml and MV-L2 from 5.2 to < 1.25 log p.f.u./ml.

The addition of 1 mg/ml of ribonuclease-A (Sigma, type 1-A) to the solid medium plate had no effect on the formation of plaques, as the titre of the agent grown in ribonuclease-containing plates and normal plates was similar.

Table 1. Titration of MV-L1, MV-L2 and the new agent on lawns prepared from clones of *Acholeplasma laidlawii* strain M1305/68 susceptible and resistant to each organism

Host strain	Titre of organisms log p.f.u./ml		
	MV-L1	MV-L2	new agent
Susceptible M1305/68	9.1	7.6	10.1
M1305/68 resistant to MV-L1	< 2.2	7.7	9.6
M1305/68 resistant to MV-L2	9.1	2.2	9.8
M1305/68 resistant to the new agent	9.2	8.1	< 2.2

Lawns were prepared from three clones of M1305/68, one clone resistant to MV-L1, one resistant to MV-L2 (not clone 2) and one resistant to the new agent. Control lawns were also prepared from fully susceptible M1305/68. MV-L1, MV-L2 and the new agent were each titrated and drops of each dilution placed on each lawn. After incubation at 37 °C for 48 h, MV-L1, MV-L2 and the new agent were all observed to produce plaques on the control M1305/68 lawns; MV-L1 and the new agent produced plaques on the MV-L2 resistant lawns, MV-L2 and the new agent produced plaques on the MV-L1 resistant lawns and both MV-L1 and MV-L2 produced plaques on lawns resistant to the new agent. Detailed results are given in Table 1.

In three out of three occasions the addition of 4.2 to 5.4 log p.f.u./ml of the agent to GS broth cultures of growing *acholeplasmas* resulted in a 3.8 to 5.2 log increase in titre of the agent after 22 h incubation at 37 °C. There was no evidence of replication in the absence of actively growing cells of the *acholeplasma*.

Stock material diluted 1/10 to 1/100 in serum saline was passed through a 220 nm Millipore filter and the filtrate was centrifuged at 150000 *g* for 1.5 h. The pellet was resuspended in a minimal amount of supernatant fluid, applied to carbon-collodion coated grids and negatively stained for a few seconds with 2 % potassium phosphotungstate at pH 7.2. Specimens were examined with a Philips EM300 electron microscope. Numerous polyhedral particles were observed (Fig. 2*a-c*). They appeared uniform in size (about 54 nm in diameter) and some particles were hexagonal in profile. No tails were visible, although the particles did sometimes appear to be attached to cellular material by a short process (Fig. 2*c*).

The characteristics of the new plaque-forming agent, described above, are consistent with those of a virus. This virus, however, differs in a number of important respects from both MV-L1 and MV-L2. First, electron micrographs reveal that the virus is morphologically unlike either MV-L1 or MV-L2. Secondly it forms plaques on clones of M1305/68 immune to both MV-L1 and MV-L2 and conversely clones of M1305/68 immune to the virus are not immune to either MV-L1 or MV-L2. Finally, the virus is serologically dissimilar to both MV-L1 and MV-L2. In view of these differences it is obvious that this virus is new and intrinsically dissimilar to both MV-L1 and MV-L2 and represents a new group of mycoplasma viruses, for which the designation *Mycoplasma* *laidlawii* 3 (MV-L3) is proposed.

The observations that the spontaneously produced plaques were seen only on lawns prepared from diluted *acholeplasma* cultures indicates that the density of lawn may be important for the recognition of small plaque-forming mycoplasma viruses.

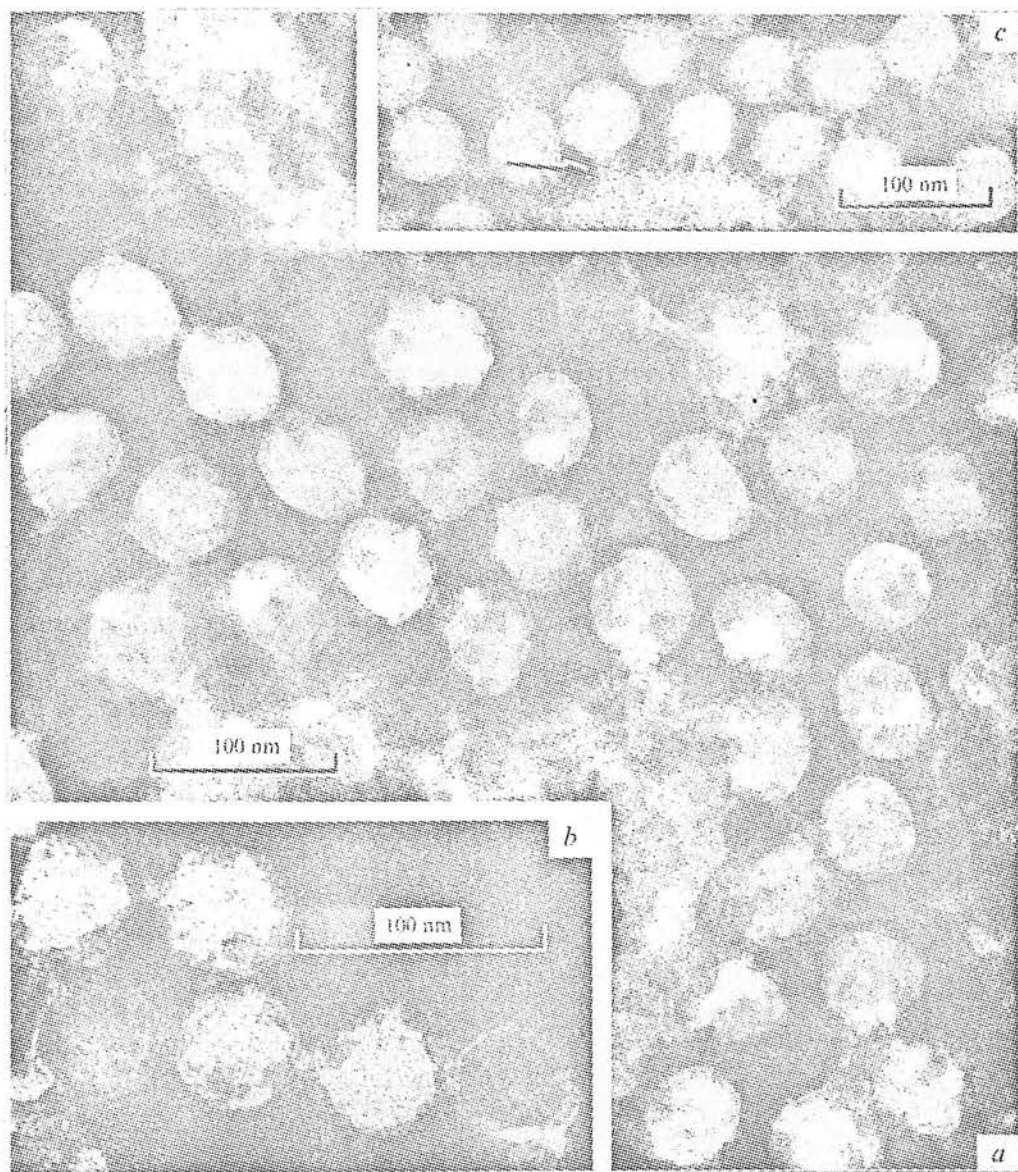


Fig. 2. Electron micrograph of centrifuged deposit of filtered stock virus. Negatively stained with potassium phosphotungstate. Numerous polyhedral particles, presenting various views, can be observed in (a), (b) and (c). Empty particles are visible in (a) and (b). A short process apparently attaching a virus particle to cellular material is indicated by an arrow in (c).

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