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Experimental infection of castrated lambs with *Mycoplasma agalactiae*

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SUMMARY

The course of experimental infection in groups of 6-month-old castrated lambs with field isolates of *Mycoplasma agalactiae* from France was followed culturally and serologically for 7 months. Infection with an ovine field isolate following inoculation by different routes and contact exposure was compared with that caused similarly by a caprine field isolate. The prolonged infections produced were symptomless apart from limited arthritis in one animal inoculated with the isolate from sheep and increased lachrymation in another associated with the goat isolate. The ovine isolate was more virulent in that ante- and post-mortem recoveries of the organism were more consistent and the serological responses more pronounced. Serological responses varied between animals and between strain infections, and the results of the film inhibition test were more consistent than those of the complement fixation test. The limitations of both these tests for detecting carrier infections are discussed.

INTRODUCTION

Since *Mycoplasma agalactiae* was recognized as the cause of contagious agalactia of sheep and goats (Bridre & Donatien, 1923) the agent has been isolated in many countries (Cottew, 1979). In dairy animals infection has generally been associated with typical clinical signs of mastitis, conjunctivitis and arthritis. Zavagli (1951) found the highest incidence of clinical disease occurred during late pregnancy and early lactation, although frequently a long period elapsed between the observation of ocular or joint lesions in summer and the onset of the syndrome in lactating animals. This author also established that in flocks of males or sterile females infection was often clinically symptomless. Nevertheless these animals were carriers and when healthy lactating ewes were introduced they became infected. In experimental studies adults, lactating females and lambs injected with *M. agalactiae*, and lambs infected orally developed clinical signs (Shabanov *et al.* 1973; Toshkov *et al.* 1974, 1975, 1976; Zavagli, 1951). Transient infection following injection of attenuated culture vaccine has also been recorded (Foggie *et al.* 1970).

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Although *M. agalactiae* has not been reported from sheep or goats in Britain, it was decided for disease control purposes to assess procedures for detection of infection. Castrated lambs were inoculated and challenged by contact with two imported strains of *M. agalactiae*, and the course of infection followed culturally and serologically. The complement fixation test (CFT) is widely used for the diagnosis of contagious agalactia (Bridre & Donatien, 1925; Etheridge *et al.* 1969; Morozzi, Dominici & Cardaras, 1973; Perreau, Le Goff & Giauffret, 1976; Schaeren & Nicolet, 1982). However, it is regarded generally as a herd or flock test because not all infected animals develop detectable complement fixing antibodies to *M. agalactiae*. For this reason the more recently developed film inhibition test (FIT) was also investigated (Howard, Collins and Gourlay, 1976; Ruhnke, Thawley & Nelson, 1976; Thorns & Boughton, 1978).

MATERIALS AND METHODS

Inocula

Two strains of *M. agalactiae* isolated in France were supplied as pure cultures at low passage levels by Dr P. Perreau, Maisons-Alfort, Paris. Strain V8 was isolated from an ovine case of contagious agalactia in the Basque region, and the second strain (Annecy) was from a caprine case near Annecy.

The inocula of strains V8 and Annecy were broth cultures in the log phase of growth containing respectively $10^{8 \cdot 8}$ and $10^{8 \cdot 4}$ colony forming units per ml. Both inocula were confirmed free of bacterial contaminants.

In the growth-inhibition test, colonies of both strains were inhibited by antiserum prepared in rabbits to the type strain of *M. agalactiae* (PG_2). Young colonies of strains V8 and Annecy also fluoresced strongly with the same antiserum in the indirect epi-immunofluorescent test. After incubation at 37 °C for 2–4 days colonies of both strains caused film and spot formation on the surface of solid medium.

Experimental design

Nine 6-month-old castrated Blackface sheep were selected from the closed laboratory flock. Prior to the experiment all the animals in the flock were bled and no CF antibodies to *M. agalactiae* were detected. Of the nine lambs selected, three were retained as unexposed controls and housed separately. The remaining six were kept in two separate groups of three animals in accommodation ventilated by filtered air. One group was infected with strain V8 and the other group with strain Annecy. Within each infected group one sheep was inoculated with 2 ml intravenously and 6 ml intratracheally, a second received 4 ml subcutaneously over the shoulder and 4 ml intranasally, while the third was exposed by contact. All the animals were examined clinically each day and rectal temperatures recorded. Every week they were bled for cultural, serological and haematological examinations. During the first week post-inoculation (p.i.) and in animals with bacteraemia, blood samples for culture were collected every 1–2 days. Each week nasal, ocular and rectal swabs from all animals were cultured.

The infected sheep remained under experiment for 207–216 days. At post-mortem examination the following samples were cultured for mycoplasma: nasal, ocular,

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rectal, carpal joint, stifle joint and hock joint swabs, retropharyngeal, mediastinal and mesenteric lymph nodes, lung, liver, spleen, kidney, small and large intestine. From animals inoculated subcutaneously the prescapular lymph node on the inoculated side was also cultured.

Cultural procedures

The procedures for isolation of mycoplasma from experimental sheep were similar to those described previously (MacOwan *et al.* 1982). For culture of mycoplasma from the blood 9·5 ml of broth were inoculated with 0·5 ml of fresh whole blood. To reduce bacterial contamination of primary cultures from rectal swabs penicillin was replaced by ampicillin at a final concentration of 1 mg/ml. For demonstration of film and spot formation the pig serum in the medium was replaced with 20 % inactivated horse serum.

Serological procedures

Preparation of antigen for immunization and CFT, and the methods for growth inhibition and immunofluorescent tests have been described previously (MacOwan *et al.* 1982).

In the CFT, working strength of PG_2 antigen was determined by titration and a test procedure similar to that described by Morgan *et al.* (1978) was followed. Three units of complement were used and fixation was carried out overnight at 4 °C instead of 37 °C for 30 min. Sera were tested in parallel after inactivation at 57 °C for 60 min and after inactivation at 60 °C for the same period. All samples were screened at dilutions from 1/20 to 1/160. Fixation of complement was graded from 1 to 4 on a regression of haemolysis basis (1 being 75 % haemolysis).

For the film inhibition test, agar medium was poured into plates 120 mm^2 to a depth of 4 mm and flooded with log phase broth culture of strain V8 containing more than 10^8 c.f.u./ml, drained, the surface dried at 37 °C and nine wells 5 mm in diameter cut approximately 4 cm apart. Except where stated otherwise, sera for examination were inactivated at 57 °C for 60 min in a water bath. The wells were filled with heat-inactivated serum and the plates incubated for 2 days at 30 °C followed by 2 days at 37 °C. The test was read against a dark background and the area of film inhibition calculated.

Haematological examinations

Weekly blood samples were collected into ethylene diamine tetra-acetic acid (EDTA), for red and white cell estimation using a Coulter Counter (Model ZF) according to the manufacturers' instructions.

Histological examination

Tissue samples of lung, liver, kidney, spleen, heart, trachea, mesenteric and mediastinal lymph nodes were collected at post-mortem examination from each animal. Carpal tissues were also collected from the animal which became lame after infection with strain V8. After primary fixation in 10 % buffered formalin, the tissues were routinely processed through alcohols, embedded in paraffin wax, and prepared 5 μm sections were stained with haematoxylin and eosin.

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Table 1. *Cultural recoveries of* M. agalactiae *during the experiment*

Isolated from	Days after challenge					
	V8 infected			Annecy infected		
	IT/IV	SC/IN	Contact	IT/TV	SC/IN	Contact
Blood	3–8	1–28	22–32	—	—	—
Rectum	72	—	35	—	—	—
Nose	—	—	—	—	—	—
Eye	77	—	—	—	—	60

Key: IT, intratracheal; IV, intravenous; SC, subcutaneous; IN, intranasal.

RESULTS

Clinical findings

Throughout, rectal temperatures of all experimental animals remained within the normal range. Few clinical signs were recorded. The animal inoculated intravenously and intratracheally with strain V8 developed mild and transient lameness associated with pain in the right carpal joint, between days 13 and 32 p.i. In the sheep exposed to strain Annecy by contact, slightly increased lachrymation of the right eye was observed from 17 days post exposure. Total red and white blood cell counts from all the animals remained within the normal range.

Mycoplasma isolates during life

Mycoplasma isolates which were sensitive to digitonin, caused film and spot formation and fluoresced strongly with antiserum to PG2 were recognized as recoveries of *M. agalactiae*. During the experiment the organism was recovered from the blood, rectum and eye (Table I).

Post-mortem examination

At post-mortem examination all the animals were in good bodily condition. On the anterior aspect of the right carpal joint of the sheep which had shown transient lameness early in the experiment, a pad of white tissue (3 × 1 cm) was adherent to the fascia overlying the joint capsule. Histologically this lesion consisted of dense fibro-granulation tissue with aggregates of macrophages around developing capillaries. Within the joint inflammatory changes had developed. Neutrophils and macrophages had invaded the synovial connective tissue spaces and disrupted the lining epithelium. Strands of adhering fibrinocellular debris were attached to the disrupted synovial lining. No significant gross or histological lesions were observed in any of the other animals, although there was a slight excess of peritoneal fluid in the sheep exposed by contact to infection with strain Annecy.

Mycoplasma isolation after slaughter

M. agalactiae was recovered culturally from samples taken at several sites during post-mortem examination (Table 2). More recoveries were obtained from the sheep infected with strain V8. The mesenteric lymph nodes of the inoculated sheep in both groups yielded the organism and it was also recovered from the small

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Table 2. *Cultural recoveries of* M. agalactiae *from samples taken at post-mortem examination*

Isolated from	V8 infected			Annecy infected		
	IT/IV	SC/IN	Contact	IT/IV	SC/IN	Contact
Nose	−	−	−	+	−	−
Eye	−	+	−	−	−	−
Retropharyngeal LN	−	+	−	−	−	−
Mediastinal LN	+	−	−	−	+	−
Mesenteric LN	+	+	−	+	+	−
Small intestine	+	+	+	−	−	−
Kidney	−	−	+	−	−	−
Peritoneal fluid	−	−	−	−	−	+
Carpal joint	−	+	+	−	−	−
Carpal lesion		+				

Key: LN, lymph node.

intestinal mucosa of all sheep infected with strain V8. There were no recoveries of *M. agalactiae* from the control animals during the experiment or from samples taken at post-mortem examination.

Complement-fixation reactions

When serum samples inactivated at 60 °C were examined by CFT, antibodies to *M. agalactiae* were detected in all the sheep infected with strain V8 and in one animal inoculated with strain Annecy. No antibodies were detected in any of the control sheep sera at a dilution of 1/20.

Following inoculation of strain V8 titres of 3/20 to 1/40 developed by 4 weeks while the in-contact sheep developed a titre of 2/40 by seven weeks (Table 3). The titre of one inoculated animal rose to 4/160 or more by 13 weeks and remained at this level. In contrast, the titre of the second inoculated sheep fell below 1/40 after 4 weeks, remained below this level for a further 4 weeks, and then rose to remain over 1/160 from 20 weeks. For the contact animal the titre fell below 2/20 by week 8 and remained below this level for 4 weeks before rising to 4/160 by week 20.

Of the three sheep in the Annecy infected group only one inoculated animal developed a titre. In this animal the titre was first detected 15 weeks p.i. and rose steadily over the subsequent weeks to reach a level of 1/160 on week 20 before falling on week 26 to reach 1/20 by week 30 (Table 3).

When sera were inactivated at 57 °C prior to examination by CFT the titres were generally higher than following inactivation at 60 °C but they varied widely from week to week and at irregular intervals low titre reactions occurred in the sera of the control animals. A low proportion of the sera were also anticomplementary.

Film-inhibition reactions

The sera of animals inoculated with strain V8 inhibited film formation by 2 weeks, and by 3 weeks the serum of the in-contact animal was also inhibitory. The area of film inhibition varied from 240 to 800 mm², and the sera collected between

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Table 3. *Complement fixation test titres of sera inactivated at 60 °C*

Week post exposure	V8 infected			Annecy infected		
	IT/IV	SC/IN	Contact	IT/IV	SC/IN	Contact
1	—	—	—	—	—	—
4	1/40	3/20	—	—	—	—
7	2/20	2/80	2/40	—	—	—
8	—	2/40	—	—	—	—
12	—	4/20	—	—	—	—
13	1/40	4/160	3/80	—	—	—
14	2/40	4/160	3/40	—	—	—
15	3/80	4/160	2/160	1/20	—	—
16	1/80	4/160	3/160	2/40	—	—
17	3/80	4/160	1/160	1/40	—	—
18	3/40	4/160	2/80	—	—	—
19	3/40	4/160	2/80	1/40	—	—
20	3/160	4/160	4/160	1/160	—	—
23	4/160	4/160	4/160	1/160	—	—
25	1/160	4/160	4/160	1/160	—	—
26	1/160	4/160	4/160	1/80	—	—
30	1/160	4/160	4/160	1/20	—	—

7 and 19 weeks after infection gave the strongest inhibition (490–800 mm²). Inhibitory effects remained until the end of the experiment.

The two sheep inoculated with strain Annecy also developed film-inhibiting antibodies by 2 weeks although zones of inhibition were smaller, varying from 165 to 686 mm². Weekly serum samples from one animal remained inhibitory until the end of the experiment while the other gave only partial inhibition of film production after 21 weeks. The serum of the sheep exposed by contact to infection with strain Annecy did not inhibit film production, neither did sera from the control sheep. Occasional samples from these latter sheep caused partial inhibition but affected areas did not exceed 80 mm² and they were considered negative. Serum samples from ten weekly bleedings of all the sheep were tested after inactivation at 57 °C for 60 min and at 60 °C for the same period. Both comparative sets of sera gave similar inhibitory results.

DISCUSSION

The cultural recovery of *M. agalactiae* in samples taken during post-mortem examination from all the inoculated and in-contact animals indicated that under experimental conditions the organism was infectious and contagious to castrated lambs. Signficantly only one animal, the sheep inoculated intravenously and intratracheally with strain V8, exhibited clinical disease, which was manifested as transient lameness. As the cultures inoculated were at a low passage level, it seems likely that the absence of clinical disease was a reflection of the type of animal used, namely castrated lambs. This view is consistent with the field observations of Zavagli (1951) on infected barren ewes and males. Such animals developed no clinical signs but transmitted infection and disease to healthy lactating ewes which

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were introduced to the flock. In our study there was contagious spread from largely symptomless carrier animals infected by inoculation to in-contact animals.

In natural disease it is generally accepted that spread of infection occurs by ingestion of the organism (Zavagli, 1951). Demonstration of *M. agalactiae* in rectal swab samples during the experiment, and in the intestinal mucosa and mesenteric lymph nodes at post-mortem examination supported this view. In addition, the persistence of the organism for over 6 months in animals exposed to the infection is consistent with the observation by Zavagli (1951) that minimal infection may remain, incubating for several months until particular conditions such as fatigue, parturition or lactation stimulate increased multiplication with possible clinical disease.

Bacteraemia has been reported in naturally occurring clinical cases and in experimental animals following inoculation of *M. agalactiae* (Shabanov *et al.* 1973; Toshkov *et al.* 1974, 1975, 1976; Zavagli, 1951). Both sheep inoculated with strain V8 became bacteraemic within 3 days. In the sheep exposed by contact, bacteraemia developed after 22 days and persisted for 10 days. To our knowledge this is the first report of bacteraemia in an animal exposed experimentally by contact to *M. agalactiae* infection. From our observations on animals infected with the V8 strain, the development of bacteraemia is independent of the route of challenge and represents a possible phase in the establishment of infection and localization of the organism in selected sites, including joints. Bacteraemia was not detected in the animals exposed to strain Annecy either by inoculation or by contact. However, recovery of *M. agalactiae* from these animals in samples taken at post-mortem confirmed that infection had been established and suggested that bacteraemia had occurred at a level too low for the sensitivity of our cultural procedures. These variations between the two groups of animals may indicate differences in virulence of the two strains employed, perhaps reflecting the species of origin.

Serologically, evidence of challenge was detected by both CFT and FIT. The results of the CFT were satisfactory only if sera were inactivated for 60 minutes at 60 °C. In a proportion of sera inactivated at 57 °C heat labile components capable of fixing complement remained. These sera were not anticomplementary and further investigations of the heat labile reaction are indicated. In contrast, the results of the FIT following inactivation of sera at either temperature were similar.

Complement fixing antibodies to *M. agalactiae* were detected in four of the six infected sheep. The test failed to detect antibodies in one animal inoculated with strain Annecy and in the animal infected by contact with this strain. Of the four animals which developed complement fixing antibodies the titre of two fell below 1/20 within 4 weeks remaining below this level for at least 4 weeks before becoming consistently higher for the rest of the experiment. Owing to the persistence of infection it seems unlikely that lateral spread of infection accounted for this variation. Other explanations could include the influence of intermittent low level bacteraemia which would not be detected by our cultural procedures. In three of the four animals which developed CFT titres a reliable indication of previous infection was evident only by 13–15 weeks post-challenge.

Although the CFT has been widely used as an aid to the diagnosis of contagious agalactia caused by *M. agalactiae*, most workers have studied the serological response in clinically affected animals. Our findings indicate the CFT is less reliable

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for the detection of symptomless carrier animals and this is consistent with the observations of Perreau, Le Goff & Giauffret (1976) on naturally occurring chronic carriers. However, further studies would be justified to ascertain whether antigen prepared by other methods or from the homologous infecting strain would detect antibodies earlier.

The results of the FIT were more consistent than those of the CFT. Evidence of challenge was detected in all inoculated sheep by 2 weeks and by 3 weeks in the sheep infected by contact with strain V8. These reactions persisted throughout the experiment except in one of the sheep inoculated with strain Annecy that became negative after 21 weeks. This test failed to provide serological evidence of infection only in the animal infected by contact with strain Annecy. This is the first report of the application of the FIT for detection of antibodies to *M. agalactiae*. Although the results are encouraging, to substantiate the value of this test for diagnosis further studies are required both in experimental and field infections.

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REFERENCES

Bridre, J. & Donatien, A. (1923). Le microbe de l'agalaxie contagieuse et sa culture in vitro. *Comptes rendus hebdomadaire des séances de l'Académie des Sciences, Paris* **177**, 841–843.

Bridre, J. & Donatien, A. (1925). Le microbe de l'agalaxie contagieuse du mouton et de la chevre. *Annales de l'Institut Pasteur* **39**, 925–951.

Cottew, G. S. (1979). Caprine–ovine mycoplasmas. In *The Mycoplasmas*. Volume 2. *Human and Animal Mycoplasmas.* (ed. J. G. Tully and R. F. Whitcomb), chapter 3, pp. 107–109. New York: Academic Press.

Etheridge, J. R., Foggie, A., Arisoy, F. & Erdag, O. (1969). Contagious agalactia of sheep and goats. A diagnostic complement fixation test. *Pendik Veteriner Kontrol Ve Arastirma Enstitusu Dergisi* **2**, 101–119.

Foggie, A., Etheridge, J. R., Erdag, O. & Arisoy, F. (1970). Contagious agalactia of sheep and goats. The serial passage in goats of an attenuated strain of *Mycoplasma agalactiae* AIK40. *Research in Veterinary Science* **11**, 477–479.

Howard, C. J., Collins, J. & Gourlay, R. N. (1976). A radial growth precipitation test and its comparison with certain other serological tests for the detection of antibody in bovine sera to *Mycoplasma agalactiae* subsp. *bovis*. *Veterinary Microbiology*, **1**, 23–30.

MacOwan, K. J., Randall, C. J., Jones, H. G. R. & Brand, T. F. (1982). Association of *Mycoplasma synoviae* with respiratory disease of broilers. *Avian Pathology* **11**, 235–244.

Morgan, W. J. B., MacKinnon, D. J., Gill, K. P. W., Gower, S. G. M. & Noris, P. I. W. (1978). The complement fixation test. (CFT) In *Brucellosis diagnosis*, *Standard Laboratory Techniques*, 2nd ed, pp. 17–37. Ministry of Agriculture, Fisheries and Food (publications), Tolcarne Drive, Pinner, Middlesex HA5 2DT.

Morozzi, A., Diminici, S. & Cardaras, P. (1973). A serological survey on contagious agalactia in sheep: the development of effective diagnostic methods. *Atti della Società italiana delle scienze veterinaire* **27**, 628–633.

Perreau, P., Le Goff, C. & Giauffret, A. (1976). Le diagnostic serologique de l'agalaxie contagieuse des petits ruminants: un test de fixation du complement. *Bulletin d'Académie Vétérinaire de France* **49**, 185–192.

Ruhnke, H. L., Thawley, D. & Nelson, F. C. (1976). Bovine mastitis in Ontario due to *Mycoplasma agalactiae* subsp *bovis*. *Canadian Journal of Comparative Medicine* **40**, 142–148.

Schaeren, W. & Nicolet, J. (1982). Application of a micro-Elisa to the serology of infectious agalactia of goats. *Schweizer Archiv für Tierheilkunde*, **124**, 163–177.

https://doi.org/10.1017/S0022172400065050 Published online by Cambridge University Press

Shabanov, M., Toshkov, A., Mikhailova, L. & Shirova, L. (1973). Experimental mastitis in ewes due to *Mycoplasma agalactiae*. *Veterinarno Meditsinski Nauki Sofia* **10**, 81–84.

Thorns, C. J. & Boughton, E. (1978). Studies on film production and its specific inhibition with special reference to *Mycoplasma bovis* (*M. agalactiae* var. *bovis*). *Zentralblatt für Veterinarmedizen* B **25**, 657–667.

Toshkov, A., Mikhailova, L., Shirova, L. & Shabanov, M. (1974). Investigations of the infection and immune process in mycoplasmoses. 1. On some features accompanying experimental agalactia in sheep and goats. *Izvestiya na Mikrobiologicheskiya Institut na Bulgarskata Akademiya na Naukite* **25**, 39–45.

Toshkov, A., Shirova, L., Mikhailova, L. & Shabanov, M. (1975). Investigations of the infection and immune process in mycoplasmoses. V. Experimental infection with *Mycoplasma agalactiae* in lambs. *Acta Microbiologica Virologica et Immunologica* **2**, 42–47.

Toshkov, A., Shirova, L., Mikhailova, L. & Shabanov, M. (1976). Study of the infectious and immunisation processes in mycoplasmoses. VIII. Experimental chronic infection of sheep with *Mycoplasma agalactiae* during pregnancy, lambing and lactation. *Acta microbiologica virologica et immunologica* **4**, 52–57.

Zavagli, V. (1951). L'agalaxie contagieuse des brebis et des chevres. *Bulletin de l'office International des Epizooties* **35–36**, 336–362.

https://doi.org/10.1017/S0022172400065050 Published online by Cambridge University Press