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Differentiation of *Mycoplasma mycoides* subsp. *mycoides* from certain closely related caprine mycoplasmas by mycoplasmaemia and cross-protection tests in mice

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SUMMARY

In recent years, mycoplasma taxonomists have found that numerous mycoplasma strains from goats are serologically indistinguishable from *Mycoplasma mycoides* subsp. *mycoides*, the causative agent of contagious bovine pleuropneumonia (CBPP), by routinely used tests, e.g. the metabolism- and growth-inhibition tests. As a result, such organisms are now openly referred to as *M. mycoides* subsp. *mycoides*.

Seven of these so-called *M. mycoides* subsp. *mycoides* strains from goats were compared with two strains of *M. mycoides* subsp. *mycoides* from CBPP, and with one strain of *M. mycoides* subsp. *capri*, by means of two in-vivo tests, namely, (1) a test of the ability of each strain, injected intraperitoneally into mice, to produce mycoplasmaemia, and (2) a cross-protection test in mice. Of the seven strains, only one ('O goat') was indistinguishable from genuine *M. mycoides* subsp. *mycoides;* it also had small colonies resembling those of genuine *M. mycoides* subsp. *mycoides*. The other six were easily distinguished from genuine *M. mycoides* subsp. *mycoides*, and they produced large colonies. These six strains and others like them should no longer be given a name that fails to distinguish them from the causative agent of CBPP.

Cross-protection tests showed that the seven goat strains referred to above differed from *M. mycoides* subsp. *capri*.

INTRODUCTION

Serological cross-reactions between *Mycoplasma mycoides* subsp. *mycoides*, the aetiological agent of contagious bovine pleuropneumonia (CBPP) and *Mycoplasma mycoides* subsp. *capri*, the aetiological agent of contagious caprine pleuropneumonia (CCPP), have been demonstrated by many workers (Cottew & Leach, 1969), but there is abundant evidence that the organisms represent distinct subspecies (Freundt, 1974).

However, in recent years there has been much confusion concerning certain caprine and ovine mycoplasmas that are indistinguishable from *M. mycoides*

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subsp. *mycoides* by the serological methods routinely used for the identification of mycoplasmas.

In a review of mycoplasma diseases of goats, Hudson, Cottew & Adler (1967) noted that several goat strains were serologically similar to *M. mycoides* subsp. *mycoides*. In a comparison of 47 bovine, caprine and ovine strains by biochemical and serological methods, Al-Aubaidi, Dardiri & Fabricant (1972) concluded that many strains had previously been misidentified, and that more than 20 of their strains (designated 'group 8') from goats and sheep should be considered to be *M. mycoides* subsp. *mycoides*. Stone & Yedloutschnig (1973) referred to the difficulties involved in assessing the pathogenicity of these strains for cattle by experimental means. An article (Anon., 1974) describing the work of the FAO/WHO Programme on Comparative Mycoplasmology referred frankly to *M. mycoides* subsp. *mycoides* from goats and sheep. The occurrence of *M. mycoides* subsp. *mycoides* has also been reported in goats by El Nasri (1967), Perreau (1971), Ojo (1973, 1977), MacOwan (1976) and Littlejohns & Cottew (1977), and in 'maned sheep' (*Ammotragus lervia*) by Ernø *et al.* (1972).

CBPP is still a disease of exceptional importance (Chalmers, 1975) and it is a matter for concern that organisms stated to be *M. mycoides* subsp. *mycoides* have recently been isolated from goats and sheep in parts of the world that include not only Europe, but also the U.S.A., from which CBPP was eradicated more than 80 years ago, and Australia (Gee, 1977) which was finally freed from the disease only in 1973 after enormous effort and expense.

Work at this laboratory (see Smith, 1971) showed that the intraperitoneal inoculation of mice with freshly isolated *M. mycoides* subsp. *mycoides* from CBPP resulted in symptomless infection accompanied by protracted mycoplasmaemia, and that the development of mycoplasmaemia could be prevented by active and passive immunization. Dyson & Smith (1975) showed that mouse-protective antibody was produced in cattle as a result of CBPP infection or vaccination, and that it was distinct from complement-fixing and precipitating antibody. Smith (1968) and Dyson & Smith (1976) showed that the degree of mycoplasmaemia produced by attenuated CBPP-vaccine strains was strikingly lower than that produced by freshly isolated strains.

This report describes an investigation into the relation between *M. mycoides* subsp. *mycoides* from CBPP, so-called *M. mycoides* subsp. *mycoides* from goats, and *M. mycoides* subsp. *capri*. The study was based on (1) the ability of strains to produce mycoplasmaemia in mice, and (2) cross-protection tests in mice.

MATERIALS AND METHODS

Mycoplasma strains

The 10 strains used (Table 1) consisted of two well known strains of *M. mycoides* subsp. *mycoides* from CBPP, seven strains of so-called *M. mycoides* subsp. *mycoides* from various pathological conditions in goats, and one strain of *M. mycoides* subsp. *capri* from CCPP. Table 1 also gives the information available on the history of laboratory subculture of each strain.

Table 1. *Mycoplasma strains*

Strain	Reference	Number of subcultures since isolation	Further information
Blenheim*	Smith (1968)	Few	Virulent CBPP strain, Australia
KH_3J*	Smith (1968)	*ca.* 90	Highly attenuated CBPP-vaccine strain, Sudan
O goat†‡	Hudson *et al.* (1967)	Probably few§	From a goat with polyarthritis and lymphadenitis, New Guinea
Y goat†‡	Laws (1956)	Probably few§	From a goat with fibrinous peritonitis, Australia
Ojo I†	Ojo (1973)	Probably few§	From a goat with pneumonia, Nigeria
Ojo II†	Ojo (1973)	Probably few§	From a goat with pneumonia, Nigeria
Cov 2†	Cottew *et al.* (1969)	Probably few§	From the milk of a goat with oedema disease, Turkey
74/2488†	Littlejohns & Cottew (1977)	Probably few§	From the kidney of a goat with arthritis, serositis and pneumonia, Australia
143-A66 Conn‡‖	Jonas & Barber (1969)	Probably few§	From a goat with periorbital oedema and conjunctivitis, U.S.A.
Smith (1423)¶	Smith (1967, 1969*b*), Cottew *et al.* (1969), Al-Aubaidi *et al.* (1972)	Few	Virulent CCPP strain, Turkey

* Genuine *M. mycoides* subsp. *mycoides* of bovine origin.
† Identified and supplied by Dr G. S. Cottew as *M. mycoides* subsp. *mycoides* of caprine origin.
‡ Identified by Al-Aubaidi *et al.* (1972) as *M. mycoides* subsp. *mycoides* of caprine origin ('group 8' strain).
‖ Supplied by Dr R. H. Leach.
§ 'Probably few' means almost certainly less than 20, and possibly less than 10.
¶ *M. mycoides* subsp. *capri.*

Viable counts

The numbers of colony-forming units (c.f.u.) in liquid cultures were assessed by the method of Miles, Misra & Irwin (1938) on blood agar (BA; Oxoid blood agar base No. 2 containing defibrinated horse blood 15%, v/v), the means of duplicate counts being taken as the true values.

Mycoplasmaemia tests in mice

The method was essentially that described by Smith (1971). Groups of female white mice (Tuck No. 1 strain) weighing 18–20 g were inoculated intraperitoneally with appropriate dilutions (0·5 ml per mouse) in BVF-OS medium (Turner, Campbell & Dick, 1935) of 3-day mycoplasma cultures in BVF-OS. Mycoplasmaemia was assessed 24 h after inoculation, by a selective blood-culture technique (see below).

Cross-protection tests in mice

The method was essentially that described by Smith (1969*a*). Groups of females (Tuck No. 1 strain) weighing 16–18 g were immunized intravenously with undiluted mycoplasma culture (0·25 ml per mouse) grown from a large inoculum for 3 days in BVF-OS and killed by heating at 56 °C for 30 min in a water bath. Control mice received 0·25 ml of sterile BVF-OS intravenously. The mice were challenged 3 weeks later by the intraperitoneal injection (0·5 ml per mouse) of an appropriate dilution in BVF-OS of a 3-day BVF-OS culture of strain Blenheim or strain O goat. Protection was assessed by the presence or absence of mycoplasmaemia 24 h after challenge as judged by a selective blood-culture technique (see below).

Selective blood-culture technique for mice

The method was essentially that described by Smith (1971). Two of three different media, all containing penicillin (100 units/ml) and thallium acetate (0·05%, w/v), were seeded with blood (one drop) from the tail-tip of each mouse. The three media consisted of (1) 5 ml volumes of BVF-OS, (2) 5 ml volumes of Oxoid nutrient broth No. 2 containing Wellcome Calf Serum No. 1, 20% v/v ('ONB-OS'), and (3) BA. BVF-OS and ONB-OS blood cultures were subcultured, after 7 days' incubation at 37 °C, on BA containing penicillin and thallium acetate. All BA cultures were examined for the presence or absence of mycoplasma growth after 7 days' incubation at 37 °C in a humid aerobic atmosphere. BVF-OS and BA media were used for blood cultures in the earlier experiments of the series, and ONB-OS and BA in the later experiments. The variation between the results given by each of the three media was very slight. It is worthy of note that ONB-OS, which, unlike BVF-OS, can be prepared easily and rapidly, is as efficient as BVF-OS for re-isolating the mycoplasmas listed in Table 1 from mice (J. M. Hooker & R. A. Milligan, unpublished observation).

Mycoplasmaemia (M) produced in groups of eight mice by different doses (D)*, 24 h after inoculation in experiment no.

	1†		2		3		4		5		6	
Strain	D	M	D	M	D	M	D	M	D	M	D	M
Blenheim	350	6	1·5	7	12	8	2	8	11	8	150	8
	35	6	0·15	8	1·2	8	0·2	7	1·1	8	1·5	8
	3·5	6	0·015	4	0·12	8	0·02	1	0·11	4	0·15	7
KH_3J	23	5	—	—	—	—	5	6	28	1	230	6
	2·3	1	—	—	—	—	0·5	0	5·6	4	47	5
	0·23	0	—	—	—	—	0·05	0	0·56	0	4·7	5
O goat	390	5	—	—	—	—	2	8	11	8	7	7
	39	5	—	—	—	—	0·2	4	1·1	8	0·7	7
	3·9	5	—	—	—	—	0·02	1	0·11	2	0·07	8
Y goat	500	4	—	—	—	—	350	7	—	—	—	—
	50	1	—	—	—	—	35	1	—	—	—	—
							3·5	0	—	—	—	—
Ojo I	220	3	—	—	—	—	230	6	—	—	—	—
	22	1	—	—	—	—	23	0	—	—	—	—
							2·3	1	—	—	—	—
Ojo II	250	4	—	—	—	—	490	4	—	—	—	—
	25	0	—	—	—	—	49	1	—	—	—	—
							4·9	0	—	—	—	—
Cov 2	400	4	170	3	280	6	—	—	—	—	—	—
	40	1	17	2	28	0	—	—	—	—	—	—
			1·7	0	2·8	0	—	—	—	—	—	—
74/2488	180	5	140	6	16	3	—	—	280	7	24	6
	18	2	14	3	1·6	3	—	—	28	5	2·4	2
			1·4	2	0·16	0	—	—	2·8	1	0·24	1
143-A66 Conn	300	6	340	8	180	6	—	—	—	—	—	—
	30	1	34	5	18	2	—	—	—	—	—	—
			3·4	0	1·8	1	—	—	—	—	—	—
Smith (1423)	240	2	—	—	—	—	400	4	—	—	—	—
	24	0	—	—	—	—	40	2	—	—	—	—
							4	0	—	—	—	—

* Dose per mouse given in c.f.u. (10^6).

† Six mice only per group in experiment 1.

— = Not done.

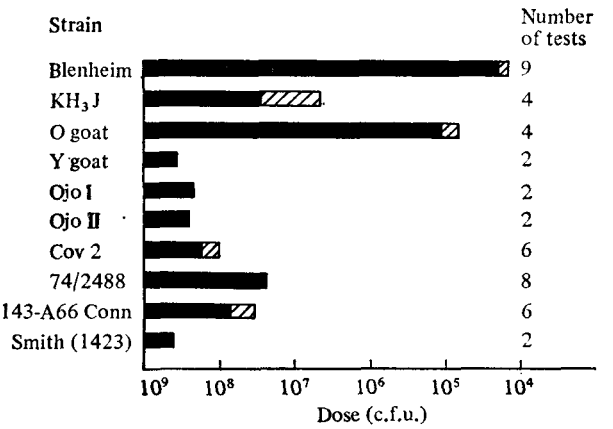

Fig. 1. Doses of 10 mycoplasma strains necessary to produce mycoplasmaemia in mice. For the purposes of Fig. 1, 'mycoplasmaemia' means positive blood cultures from at least 50 % of a group of mice 24 h after inoculation. ■, Mycoplasmaemia occurred in all tests; ▨ mycoplasmaemia occurred in some but not all tests.

RESULTS

Growth in culture

The *M. mycoides* subsp. *mycoides* strains from CBPP (Blenheim and KH_3J) produced pin-point colonies on blood agar after growth for 2–3 days and colonies about 0·5 mm in diameter after growth for 7 days. In BFV-OS medium seeded with a small piece of blood agar bearing 4-day-old colonies, growth first became visible after 2 days' incubation and maximum turbidity – attained after 3 days' incubation – was light. The caprine strain, O goat, exactly resembled Blenheim and KH_3J in these respects.

The other seven strains listed in Table 1 were strikingly different from Blenheim KH_3J and O goat. On blood agar, all produced visible colonies after overnight incubation, and after 7 days' incubation all produced colonies up to 2 mm in diameter. In BVF-OS medium seeded as described above, growth first became visible after incubation for 1 day and the maximum turbidity attained after 2 days' incubation was greater than that produced by Blenheim, KH_3J and O goat.

Mycoplasmaemia tests in mice

The 10 strains listed in Table 1 were examined in the course of six experiments, the details and results of which are shown in Table 2. Fig. 1, based on these results and on those in Table 3 (see below), shows the dose of each strain required to produce mycoplasmaemia in $\geqslant 50\,\%$ of mice.

The Blenheim strain, like other fresh isolates of *M. mycoides* subsp. *mycoides* from CBPP (Smith 1968), produced mycoplasmaemia very readily. Doses at least as small as 10^5 c.f.u. gave rise to positive blood cultures in $\geqslant 50\,\%$ of mice 24 h after inoculation; doses approximately 10 times greater gave rise to positive blood cultures in virtually all mice. Strain O goat was similar to Blenheim, though slightly less active.

Table 3. *The ability of Blenheim, 143-A66 Conn, Cov 2 and 74/2488 cultures of different ages to produce mycoplasmaemia in mice*

Mycoplasma strain	Age of culture (h)	Dose of mycoplasmas per mouse (c.f.u., 10^6)	Mice with mycoplasmaemia in groups of 6 at the stated time (h) after inoculation	
			24	48
Blenheim	24	200	6	6
	24	20	6	5
	24	2	6	4
	48	540	6	5
	48	54	6	5
	48	5·4	6	6
	72	730	6	6
	72	73	6	6
	72	7·3	6	6
143-A66 Conn	24	500	5	6
	24	50	4	4
	48	700	6	4
	48	70	2	1
	72	750	3	4
	72	75	3	1
Cov 2	24	100	3	1
	48	600	4	1
	72	890	4	1
74/2488	24	480	4	5
	48	900	5	5
	72	740	3	4

The other eight strains produced mycoplasmaemia much less readily. To produce effects comparable with that produced by a relatively small dose of the Blenheim strain, doses greater by hundreds or more often thousands of times were required. One of these eight strains was KH_3J – the well known CBPP-vaccine strain, highly attenuated by about 90 repeated subcultures; mycoplasmaemia was produced more readily by KH_3J than by any of the other seven strains except 74/2488, which was similar to KH_3J.

Because 3-day BVF-OS cultures were used to provide all the mouse inocula, cultures of the rapidly growing strains were at a stage of growth different from that of cultures of the slowly growing strains (Blenheim, KH_3J and O goat) at the time of injection. It seemed possible that a culture's stage of growth might influence its ability to produce mycoplasmaemia. Single cultures of each of four strains were therefore injected after 1, 2 and 3 days' growth into groups of mice. The rapidly growing strains were represented by 143-A66 Conn, Cov 2 and 74/2488, chosen at random, and the slowly growing strains by Blenheim. The results (Table 3) indicated that the mycoplasmaemia was not influenced by the stage of growth of the organisms in the inoculum.

Cross-protection tests in mice

In each of five experiments made on different occasions, the 10 strains listed in Table 1 were used to immunize groups of mice. Three weeks later, each group was subdivided to allow for challenge with Blenheim and O goat – the only two strains readily capable of producing mycoplasmaemia. Table 4 shows the results of blood cultures made 24 h after challenge in each of the five experiments; it also shows the aggregated results.

It is immediately apparent that Blenheim, KH_3J and O goat formed a group that differed from all other strains. Blenheim and O goat immunized completely against themselves and against each other, and KH_3J immunized completely against both challenge strains.

The aggregated results for the five experiments show that the remaining seven mycoplasma strains differed strikingly from Blenheim, KH_3J and O goat, in that vaccination with each of them failed to prevent mycoplasmaemia in 44–94% of mice subsequently challenged with Blenheim or O goat. Nevertheless, it is clear from the aggregated results that Y goat, Ojo I, Ojo II, Cov 2 and 74/2488 all gave partial protection ($P < 0{\cdot}001$) against challenge with Blenheim and O goat; strain 143-A66 Conn also gave partial protection, but to a lesser degree, against challenge with Blenheim ($P < 0{\cdot}01$) and O goat ($P < 0{\cdot}05$).

Strain Smith (1423) – the only representative of the species *M. mycoides* subsp. *capri* – gave no protection against either challenge strain.

Of the six strains that gave partial protection, 143-A66 Conn may have been exceptional. Not only did it give an unusually low degree of partial protection but, as a result, was difficult to distinguish from *M. mycoides* subsp. *capri* (strain Smith 1423) on the basis of cross-protection: the aggregated results show that it could be distinguished from strain Smith (1423) by challenge with Blenheim ($P < 0{\cdot}022$) but not O goat ($P > 0{\cdot}05$), whereas Y goat, Ojo I, Ojo II, Cov 2 and 74/2488 could be distinguished from Smith (1423) readily by the use of either challenge strain ($P < 0{\cdot}005$).

DISCUSSION

Great reliance is placed by mycoplasma taxonomists on in-vitro serological methods, notably on the metabolism-inhibition and growth-inhibition tests. As already stated, such methods have failed to distinguish O goat, Y goat, Ojo I, Ojo II, Cov 2, 74/2488 and 143-A66 Conn from CBPP strains of *M. mycoides* subsp. *mycoides*, even though all except O goat produce colonies that are much larger than those of CBPP strains. The in-vivo methods used in the present study proved to be more successful.

It was possible to pick out, on the basis of complete cross-protection, the small-colony goat strain (O goat) as being the only goat strain that was indistinguishable from genuine *M. mycoides* subsp. *mycoides*. On the basis of partial cross-protection, Y goat, Ojo I, Ojo II, Cov 2, 74/2488 and 143-A66 Conn could be distinguished from genuine *M. mycoides* subsp. *mycoides* and from *M. mycoides* subsp. *capri*.

Table 4. *Cross-protection tests between CBPP strains of* M. mycoides *subsp.* mycoides, *caprine strains of so-called* M. mycoides *subsp.* mycoides, *and* M. mycoides *subsp.* capri

Mice vaccinated with strain	Mycoplasmaemia in vaccinated mice 24 h after challenge* with Blenheim (B) and O goat (O) in experiment no.															
	1		2		3		4		5		1–5 (aggregated results)					
	B	O	B	O	B	O	B	O	B	O	B	O				
Blenheim	0/6	0/6	0/6	0/6	0/8	0/8	0/6	0/6	0/12	0/12	0/38	0/38				
KH_3J	0/6	0/6	0/6	0/6	0/8	0/8	0/6	0/6	0/12	0/12	0/38	0/38				
O goat	0/6	0/6	0/6	0/6	0/8	0/8	0/6	0/6	0/12	0/12	0/38	0/38				
Y goat	4/6	3/6	4/6	5/6	6/8	5/8	4/6	3/6	2/8	3/8	20/34	19/34				
Ojo I	6/6	4/6	6/6	6/6	2/8	1/8	2/6	2/6	0/7	2/8	16/33	15/34				
Ojo II	5/6	3/6	6/6	4/6	3/8	3/8	3/6	4/6	4/8	3/8	21/34	17/34				
Cov 2	3/6	2/6	6/6	4/6	3/8	4/8	4/6	3/6	5/8	4/8	21/34	17/34				
74/2488	5/6	5/6	6/6	4/6	4/8	6/8	1/6	3/5	3/8	4/8	19/34	22/33				
143-A66 Conn	4/5	5/6	5/6	6/6	4/8	8/8	5/6	3/6	7/8	5/8	25/34	27/34				
Smith (1423)	6/6	5/6	6/6	6/6	6/8	7/8	6/6	6/6	8/8	8/8	32/34	32/34				
Controls	13/16	15/20	10/10	10/10	17/20	18/20	20/20	20/20	19/20	20/20	79/86	83/90				

Numerator = mice with mycoplasmaemia; denominator = mice challenged.

* Challenge doses (c.f.u., 10^6) in experiments 1, 2, 3, 4 and 5 were as follows: (Blenheim) 57, 14, 42, 16 and 470 respectively; (O goat) 46, 140 40, 27 and 400 respectively.

The mycoplasmaemia tests gave good support for these findings. The fresh field strain (Blenheim) of *M. mycoides* subsp. *mycoides* from CBPP, and strain O goat, produced mycoplasmaemia very readily. The highly attenuated CBPP-vaccine strain, KH_3J, although much less capable than Blenheim and O goat of producing mycoplasmaemia, was at least as capable as the remaining seven goat mycoplasma strains, and in most instances much more so. The information available (Table 1) suggests that the goat mycoplasma strains had not been subjected to a large number of subcultures since isolation. Strain KH_3J, on the other hand, had been subjected to about 90 subcultures in the course of attenuation.

The optical densities of the various heat-killed vaccines used in the cross-protection tests were not all identical, but the following evidence shows that this did not lead to spurious results. The three vaccines (Blenheim, KH_3J and O goat) that gave complete cross-protection were slightly less dense than the remaining seven vaccines, none of which gave more than partial cross-protection. In case it should be argued that the strains that gave partial instead of complete cross-protection did so because the doses of vaccine were excessive – an unlikely possibility – it should be mentioned that similar vaccines prepared from these strains gave no more than the usual partial protection when they were used in 10-fold to 20-fold dilutions (unpublished experiment).

The complete inability of vaccine prepared from strain Smith (1423) of *M. mycoides* subsp. *capri* to protect against challenge with strains Blenheim and O goat supports the earlier findings of Smith (1969*b*); the earlier findings showed however that immunization with Blenheim gave partial protection against challenge with *M. mycoides* subsp. *capri*, strain Smith (1423), suspended in mucin.

Most of the caprine strains of so-called *M. mycoides* subsp. *mycoides* that have been tested by appropriate methods are thought to be non-pathogenic for cattle (Cottew, 1976; Gee, 1977) but strain O goat injected into calves, produced clinical, serological and pathological reactions identical with those produced by CBPP strains of moderate virulence (Hudson *et al.* 1967). Strain O goat was isolated in 1954 from a goat in New Guinea – a country free from CBPP. It is interesting that, of seven so-called *M. mycoides* subsp. *mycoides* strains from goats, O goat was the only one that we could not distinguish from the causative organism of CBPP. The other six strains were easily distinguished from it by our methods, and to continue to use the name *M. mycoides* subsp. *mycoides* for such strains would be not only unjustifiable, but also misleading, both to microbiologists and to animal health authorities.

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