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The relationship of two equine mycoplasmas to *Mycoplasma mycoides*

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SUMMARY

Two unidentified mycoplasmas, N3 and N11, isolated from the respiratory tract of horses, were found to cross-react with strains of *M. mycoides* subsp. *mycoides* in indirect immunofluorescence tests, growth-inhibition tests carried out by the running drop/agar-well method, and in complement-fixation and double immunodiffusion tests. Serologically, the equine mycoplasmas were not completely identical with any of the reference strains of *M. mycoides* with which they were compared. Their cultural characteristics, ability to digest coagulated serum and casein, and survival at 45 °C, however, suggested that they were more closely related to strains of *M. mycoides* subsp. *mycoides*, such as Y-goat, which are found in goats, than to strains of that subspecies which are pathogenic for cattle.

INTRODUCTION

The isolation from the equine respiratory tract of two unidentified mycoplasmas, strains N3 and N11, belonging to the genus *Mycoplasma*, has already been reported (Allam & Lemcke, 1975). It was originally thought that these strains, which were characterized by their rapid growth and large colony size, were unrelated to other recognized species. Neither strain was inhibited by antiserum to *M. mycoides* subsp. *mycoides*, strain Gladysdale, in disc growth-inhibition or in metabolism-inhibition tests. Similarly, strain Gladysdale was not inhibited by antiserum against N3. However, more detailed investigations at the FAO/WHO Collaborating Centre for Animal Mycoplasmas and at the Lister Institute, using other serological techniques as well as a different method for the growth-inhibition test, have revealed cross-reactions with strains of *Mycoplasma mycoides* subsp. *mycoides* and subsp. *capri*. The results of these investigations are presented here.

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Table 1. *Reference strains of Mycoplasma*

Species	Strain	Reference
M. mycoides subsp. *mycoides*	PG1, Y-goat, Gladysdale, G1/61	
M. mycoides subsp. *capri*	PG3	
Bovine Group 7 or Serotype L	PG50, B144P	Leach (1967, 1973); Al-Aubaidi and Fabricant (1971); Askaa, Ernø and Ojo (1978)
M. capricolum	California kid	Tully *et al.* (1974)
Unclassified	caprine F38	MacOwan and Minette (1976)
Unclassified (related to *M. mycoides*)	canine HRC689	Rosendal (1975)

METHODS

Mycoplasmas

The equine mycoplasmas N3 and N11 were those isolated and described by Allam & Lemcke (1975). The mycoplasmas with which the equine strains were compared are listed in Table 1.

Biochemical tests

Tests for fermentation of glucose, hydrolysis of arginine, sensitivity to digitonin, phosphatase activity and liquefaction of coagulated serum were carried out as described by Freundt, Ernø & Lemcke (1979). Ability to digest casein was determined using skim-milk agar, as described by Cottew & Yeats (1978). Tests for haemolytic activity, using an overlay technique, and tests for tetrazolium reduction were carried out according to the methods of Aluotto *et al.* (1970). Sterol-dependence was determined by the procedure of Edward (1971).

Survival at 45 °C was determined by performing viable counts on cultures held for various times at this temperature (Cottew & Yeats, 1978). Cultures were diluted in the growth medium to give an initial count of about 10^6 colony-forming units (c.f.u.) per ml.

Antisera

Antisera against strains PG1, Y-goat, PG3, PG50, B144P, F38, California kid and HRC689, used for immunofluorescence (IMF) and growth-inhibition (GI) tests at the FAO/WHO Collaborating Centre for Animal Mycoplasmas were prepared as described by Ernø, Jurmanova & Leach (1973). Antiserum was prepared in rabbits against strains N3, N11, Gladysdale and G1/61 as described by Allam & Lemcke (1975). Rabbit antisera against strains PG1, PG3 and PG50 used at the Lister Institute were the gift of Dr R. H. Leach (Mycoplasma Reference Laboratory, Norwich). All rabbit antisera were prepared against mycoplasmas grown in fluid media containing rabbit serum, to reduce the formation of antibody against foreign serum proteins.

Serological tests

Disc growth-inhibition (GI) tests were carried out by the method of Clyde (1964) and running drop/agar-well GI tests as described by Black (1973). At the

FAO/WHO Collaborating Centre, tests were carried out on B medium (Ernø & Stipkovits, 1973), and at the Lister Institute on DNA agar (Allam & Lemcke, 1975).

Indirect immunofluorescence (IMF) tests were performed on colonies grown on B medium by the procedure described by Rosendal and Black (1972). The standard dilution of conjugate was chosen on the basis of one heterologous and two homologous chessboard titrations. All antimycoplasmal sera were used at a dilution of 1:20 on the basis of titrations using the standard dilution of conjugate (1:30) (Ernø, 1977). The results were recorded as + or −.

Methods for complement-fixation (CF) and double immunodiffusion (DID) tests were those described by Hollingdale and Lemcke (1972). None of the sera used in DID tests reacted with constituents of the medium.

RESULTS

Mycoplasma strains N3 and N11 grew rapidly, producing heavy growth in fluid media and large colonies, often exceeding 1 mm in diameter, on solid media. This type of growth resembled that of *M. mycoides* subsp. *capri* or the large-colony type of *M. mycoides* subsp. *mycoides* such as Y-goat (Cottew & Yeats, 1978), rather than that of the small-colony type represented by PG1, Gladysdale or G1/61. In regard to biochemical activities, N3 and N11 fermented glucose, failed to hydrolyse arginine or produce phosphatase, reduced tetrazolium aerobically and anaerobically, and produced β-haemolysis of sheep and horse erythrocytes. The strains were also sensitive to digitonin and showed a requirement for cholesterol. These characteristics were shared by all the strains examined, with the exception of HRC689, which was phosphatase positive, and F38, which reduced tetrazolium only anaerobically. Strains N3 and N11 liquefied coagulated serum as did the isolates Y-goat, PG3, PG50, B144P, F38, *M. capricolum* California kid, and HRC689. Serum liquefaction by the PG1, Gladysdale and G1/61 strains of *M. mycoides* subsp. *mycoides* was absent. Of the five strains tested for ability to digest casein, N3, N11, PG50 and *M. capricolum* strain California kid were positive whereas *M. mycoides* subsp. *mycoides* strain Gladysdale was negative.

Of the three strains tested for survival at 45 °C, cultures of N3 and N11 showed declines in their viable counts of less than 10-fold after 48 h at this temperature. In contrast, viable counts of *M. mycoides* subsp. *mycoides* strain Gladysdale showed a decline of at least 1000-fold within 12 h.

The results of IMF and GI tests carried out by the agar-well procedure at the FAO/WHO Collaborating Centre showed that N3 and N11 reacted with antisera to the PG1 and Y-goat strains of *M. mycoides* subsp. *mycoides* (Table 2). Neither N3 nor N11 cross-reacted in either test with antisera against *M. mycoides* subsp. *capri* PG3, PG50, B144P, F38, *M. capricolum* California kid, or HRC689. In reciprocal tests, PG1 and Y-goat reacted with N3 and N11 antisera although only weakly in growth inhibition, as the zones of inhibition were approximately 2 mm. In IMF both strains reacted with N3 antiserum, exhibiting strong fluorescence, while a weaker reaction was obtained with N11 antiserum.

Since these results suggested that an antigenic relationship might exist between

Table 2. *Comparison of strains N3 and N11 with* Mycoplasma mycoides *and proteolytic mycoplasmas by indirect immunofluorescence and agar-well growth-inhibition tests**

		Antiserum									
		M. mycoides subsp. *mycoides*		*M. mycoides* subsp. *capri*	*M. capricolum*	Bovine serogroup 7		Unclassified			
Test	Strain	PG1	Y-goat	PG3	Calif. kid	PG50	B144P	F38	HRC689	N3	N11
IMF	N3	+	+	–	–	–	–	–	–	+	+
	N11	+	+	–	–	–	–	–	–	+	+
GIT	N3	+	+	–	–	–	–	–	–	+	+
	N11	+	+	–	–	–	–	–	–	wk +	+

* Carried out at FAO/WHO Collaborating Centre for Animal Mycoplasmas.
IMF + fluorescence with antisera diluted 1/20; – no reaction with antisera diluted 1/20.
GIT + growth-inhibition zones > 2 mm; wk + growth-inhibition zones ≤ 2 mm; – no inhibition of growth.

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Table 3*a*. *Comparison of strains N3 and N11 with* Mycoplasma mycoides *by agar-well growth-inhibition tests**

	Antiserum					
	M. mycoides subsp. *mycoides*			*M. mycoides* subsp. *capri*	Equine	
Antigen	PG1	Gladysdale	G1/61	PG3	N3	N11
N3	2·5 p.i.	2 p.i.	1·5 p.i.	2·5 p.i.	**4**	4
N11	2 p.i.	3 p.i.	2 p.i.	3 p.i.	5	**5**
PG1	**10**	9	9†	0	3	5
Gladysdale	NT	**11**	12†	0	3	7
G1/61	NT	8 p.i.	**9**	NT	2 p.i.	4 p.i.
PG3	0	0	0	**7**	0	0

* Carried out at Lister Institute.

† Small inhibition zones (< 2 mm) given by corresponding preimmunization serum.

Inhibition zones in mm; p.i., partial inhibition; NT, Not tested; homologous reactions in bold type.

Table 3*b*. *Comparison of strains N3 and N11 with* Mycoplasma mycoides *by double immunodiffusion tests**

	Antiserum				
	M. mycoides subsp. *mycoides*		*M. mycoides* subsp. *capri*	Equine	
Antigen	PG1	G1/61	PG3	N3	N11
N3	1† (0)	3 (3)	7 (6)	**8**	7 (5)
N11	0	2 (2)	6 (5)	7 (7)	**5**
PG1	**3**	5 (5)	4 (4)	6 (4)	4 (2)
Gladysdale	3 (3)	5 (5)	3 (3)	6 (4)	4 (2)
G1/61	3 (3)	**5**	4 (4)	6 (4)	4 (2)
PG3	1 (0)	4 (4)	**8**	6 (4)	6 (4)

* Carried out at Lister Institute.

† Number of precipitin lines.

Homologous reactions in bold type; there were no reactions with the corresponding pre-immunization sera; in parentheses, number of lines showing reactions of identity with lines given by homologous antigen.

the two equine strains and *M. mycoides* subsp. *mycoides*, further comparisons were made at the Lister Institute by GI, DID and CF tests. Both N3 and N11 cross-reacted with antisera against three strains of *M. mycoides* subsp. *mycoides* and the PG3 strain of *M. mycoides* subsp. *capri* in GI tests carried out by the agar-well method (Table 3*a*), although inhibition was usually only partial and less than that observed with the homologous strains. In reciprocal GI tests carried out by the same method, the three strains of *M. mycoides* subsp. *mycoides*, Gladysdale, PG1 and G1/61, were inhibited by antisera to N3 and N11, but PG3 was not. No cross-reactions were observed between *M. mycoides* and the equine mycoplasma in GI tests carried out by the classical disc method, thus showing that the agar-well method was more sensitive. The results of DID tests showed extensive cross-

Table 3*c*. *Comparison of strains N3 and N11 with* Mycoplasma mycoides *by complement-fixation tests**

	Antiserum			
	M. mycoides subsp. *mycoides*		*M. mycoides* subsp. *capri*	Equine
Antigen	Gladysdale	G1/61	PG3	N3
N3	640	320	640	**10240**
N11	1280	320	640	10240
Gladysdale	**5120**	1280	40	40
G1/61	5120	**1280**	160	160
PG3	320	10	**2560**	320

* Carried out at Lister Institute.
Homologous titres in bold type.

reactions between the equine mycoplasmas and both subspecies of *M. mycoides* (Table 3*b*). Fusion of some of the lines in reactions of complete identity suggested the presence of common antigens. In CF tests (Table 3*c*), N3 and N11 reacted to high titre with both the antisera to *M. mycoides* subsp. *mycoides* against which they were tested and with antiserum against *M. mycoides* subsp. *capri*; antiserum titres with the equine strains showed at most an eightfold difference from the homologous titre. Reactions of the *M. mycoides* strains with N3 antiserum were, however, lower than the homologous titre by a factor of at least 32.

In confirmation of the results obtained at the FAO/WHO Collaborating Centre, no cross-reactions were observed in either direction between the equine mycoplasmas and the Group 7 strain, PG50, in agar-well GI tests. In CF tests, PG50 did not react with N3 antiserum at 1/20, the lowest dilution tested, and N3 reacted with PG50 antiserum at a titre of 1/80 compared with the homologous titre of 1/1280.

DISCUSSION

The results suggest that the equine mycoplasmas N3 and N11 are antigenically related to *M. mycoides* subsp. *mycoides*. Their relationship to *M. mycoides* subsp. *capri* is more equivocal. No cross-reactions were observed in GI and IMF tests in one laboratory. However, using a different antiserum, a one-way cross-reaction was observed in GI tests in the other laboratory. Cross-reactions also occurred between the equine mycoplasmas and PG3 in DID and CF tests; these tests are recognized as being less species-specific than GI and IMF tests (Freundt, Ernø & Lemcke, 1979).

The earlier failure to detect the relationship of the equine strains to *M. mycoides* subsp. *mycoides* can be attributed to the exclusive use of inhibition tests for serological identification (Allam & Lemcke, 1975). Since these mycoplasmas develop rapidly and produce a heavy growth, serological tests which depend on inhibition of growth or metabolism may not be satisfactory for detecting cross-reactions at the levels found in this study. In metabolism-inhibition tests, for example, end-points of serum titrations may be rapidly obscured by overgrowth

of the mycoplasmas. Moreover, the disc GI test which was originally used still failed to show the cross-reactions which were revealed by the more sensitive agar-well method. These observations demonstrate the desirability of including in identification procedures techniques such as immunofluorescence which do not depend on inhibition of growth. Even immunofluorescence is far from ideal in relation to the *M. mycoides* group; the end-point titres are usually rather low and the results depend very much on the age and size of the colonies. The test is therefore semi-quantitative; while false positives are unlikely, false negatives can be obtained even when using small, young colonies, which are most suitable for the test.

Cottew & Yeats (1978) and Cottew (1979) recently suggested that two categories should be recognized in the subspecies *mycoides*, a 'large-colony' type comprising Y-goat and related strains and a 'small-colony' type comprising strains pathogenic for cattle. If these suggestions are accepted, N3 and N11 would be more closely related to the 'large-colony' type of *M. mycoides* subsp. *mycoides* on the basis of colony size, ability to digest coagulated serum and casein, and survival at 45 °C. Although N3 and N11 are not completely identical with strain Y-goat, the reference strain of the large-colony, proteolytic type of *M. mycoides* subsp. *mycoides*, they would undoubtedly have been classified as such if they had been isolated from goats.

In view of these findings, it is improbable that strains N3 and N11 are pathogenic in cattle, but it would be unwise to exclude the possibility that they are pathogenic for goats. On the other hand, it seems unlikely that the occurrence in the equine respiratory tract of mycoplasmas related to *M. mycoides* has any significance in the pathogenesis of respiratory infection in horses. So far, N3 and N11 are the only isolates of this type recovered during several major surveys of mycoplasmas in horses (reviewed by Lemcke, 1979). Both strains were recovered from nasopharyngeal swabs, but from horses in two different stables in widely separated places. Both animals had a nasal discharge, one purulent, but subsequent infection experiments failed even to establish N3 in the respiratory tract of ponies or minimal-disease foals (Hooker, Butler & Burrows, 1977; Poland & Lemcke, 1978). Naturally occurring CF-antibody against N3 was detected in a high proportion of thoroughbred horses (Hooker & Butler, 1976, 1978), but such antibody may not have been specifically directed against this mycoplasma, since organisms of this type have been isolated so infrequently from horses. It is not known whether the horses from which N3 and N11 were isolated were in contact with goats, although the latter are sometimes used as companion animals for horses.

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