Acta vet. scand. 1986, 27, 531—539.

From the Institute of Medical Microbiology, University of Aarhus, Denmark, and the National Veterinary Research Institute, Vom, Nigeria.

CHARACTERIZATION AND IDENTIFICATION OF CAPRINE, GENITAL MYCOPLASMAS

By

J. C. Chima, H. Ernø and *M. O. Ojo*

CHIMA, J. C., H. ERNØ and M. O. OJO: *Characterization and identification of caprine, genital mycoplasmas.* Acta vet. scand. 1986, 27, 531—539. — A total of 55 mycoplasma strains, isolated from the vagina of goats, were examined. Three strains, being arginine positive and glucose negative, could not be finally classified. Five isolates were identified as Acholeplasma laidlawii. Fourty-seven strains were phosphatase positive, glucose and arginine negative. Nine of these formed "film and spots" on standard growth medium, and reduced tetrazolium aerobically. Serological examination identified 7 as M. agalactiae, while 2 were M. bovis. The remaining 38 isolates did not reduce tetrazolium aerobically, and did not produce "film and spots" on standard growth medium. All these except one were identified as M. bovigenitalium by immunofluorescence. Their relationship to group 11 of *Al-Aubaidi* is discussed.

ovine/caprine mycoplasmas; classification; M. bovigenitalium; ovine/caprine group 11.

Studies carried out in sheep and goats have shown that Mycoplasma agalactiae, M. capricolum, ureaplasmas, and ovine/caprine group 11 of *Al-Aubaidi* (1972) can be isolated from clinical cases of vulvovaginitis (*Carmichael et al.* 1972, *Singh et al.* 1974, *Cottew et al.* 1974, *Doig & Ruhnke* 1977, *Livingston & Gaur* 1983, *Jones et al.* 1983).

Vulvovaginitis is a fairly common condition among goats in some areas of Nigeria. The purpose of this paper is to characterize and identify a number of mycoplasmas recovered from goats with a clinical diagnosis of vulvovaginitis. Special attention is paid to the relationship between the bovine species, M. bovigenitalium and group 11 of *Al-Aubaidi*. Group 11 has been isolated from goats and sheep, and is biochemically rather

similar to M. agalactiae, but unrelated serologically (*Ernø et al.* 1978). Some strains are reported to react serologically with M. mycoides subsp. mycoides (*Cottew* 1985).

MATERIALS AND METHODS

Mycoplasma strains

In Nigeria, a total of 55 mycoplasmas were isolated from clinical cases of vulvovaginitis in goats. The laboratory investigations of the strains were performed in Denmark.

Media

The standard growth medium was a modified Hayflick medium B (*Ernø & Stipkovits* 1973a).

Sterol requirement

This was examined indirectly by sensitivity to digitonin (*Ernø & Stipkovits* 1973a).

Biochemical characteristics

Tests for fermentation of glucose, hydrolysis of arginine, phosphatase activity, serum digestion, reduction of triphenyltetrazolium chloride and formation of "film and spots" on egg-yolk medium (BY) were performed as described previously (*Ernø & Stipkovits* 1973b). For comparison the formation of "film and spots" was also tested on standard growth medium (B).

Antisera

Hyperimmune rabbit sera against relevant mycoplasmas were prepared as described earlier (*Ernø et al.* 1973).

Serological tests

The indirect immunofluorescence (IMF) technique (*Rosendal & Black* 1972), the agar well modification of the growth inhibition (GI) technique (*Black* 1973) and the growth precipitation (GP) technique (*Ernø & Peterslund* 1983), were used.

RESULTS

Digitonin sensitivity (sterol requirement)

Five strains, biochemical group A, (Table 1) were resistant to digitonin as the inhibition zone was zero or less than 1 mm.

They are consequently members of genus Acholeplasma, not requiring cholesterol for growth. Fifty strains were sensitive to digitonin as zones of inhibition varying from 4 to 12 mm were measured.

Table 1. Biochemical reactions of 55 mycoplasma strains isolated from the vagina of goats.

	Fermentation of glucose	Catabiosis of arginine	Phosphatase activity	Reduction of tetrazolium chloride (aerobic/anaerobic)	Formation of "film and spots"	
					B	BY
Group A: 5 strains of genus Acholeplasma	+	0	+(1) 0(4)	+/+	0	0
Group B: 9 strains of genus Mycoplasma	0	0	+	+/+	+	+
Group C: 38 strains of genus Mycoplasma	0	0	+	0/+	0	+
Group D: 3 strains of genus Mycoplasma	0	+	+	0/+	0	0

+: Positive
0 : Negative
Numbers in paranthesis indicate number of strains

Biochemical characteristics (Table 1)

The acholeplasmas all fermented glucose and reduced tetrazolium chloride aerobically and anaerobically. All were arginine negative. One strain possessed phosphatase activity, while the 4 others did not. Formation of "film and spots" was not detected on any of the media.

Nine of the digitonin sensitive strains (biochemical group B) were glucose and arginine negative, phosphatase positive, reduced tetrazolium chloride aerobically and anaerobically and formed "film and spots" on the standard growth medium as well as the special egg yolk medium.

Thirty-eight strains (biochemical group C) differed from group B in not reducing tetrazolium chloride aerobically, and furthermore did not form "film and spots" on standard growth medium, but did so on the special test medium, BY.

Three strains (biochemical group D) did not ferment glucose, did not form "film and spots", reduced tetrazolium chloride only anaerobically, but hydrolysed arginine and were phosphatase positive.

All 55 strains were serum digestion negative.

Serological characteristics

Regarding the 5 strains of genus Acholeplasma (biochemical group A), positive serological reactions were observed in all 3 tests with antiserum against PG8, the type strain of A. laidlawii. Positive reactions were also seen in the IMF and GI tests when using antiserum against 19-L, the type strain of A. oculi (Table 2).

Table 2. Identification of 5 caprine strains of A. laidlawii.

	Antiserum					
	IMF		GI		GP	
Group A	PG8	19-L	PG8	19-L	PG8	19-L
A. laidlawii (5 strains)	+(5)	+(5)	+(5)	+(2)	+(5)	0(5)

+: Positive
0: Negative
Numbers in paranthesis indicate number of strains

Two strains of biochemical group B reacted positively with antiserum against the type strain of M. bovis (Donetta), in IMF and GP, one in the GI test as well. Both strains reacted also positively with antiserum against PG2, the type strain of M. agalactiae, but in GP only (Table 3). The remaining 7 strains of biochemical group B reacted with antisera against PG2 in IMF and GP tests. In the GI tests, all reactions were negative or insignificantly positive ($\leq$ 1 mm). Six of 7 strains reacted in the GP test also with antisera against M. bovis (Table 3).

In biochemical group C (38 strains), 17 strains reacted in the IMF test with antisera against PG11 only, the type strain of M. bovigenitalium. Fourteen and 10 of the 17 strains reacted

Table 3. Identification of caprine strains of M. bovis (2) and M. agalactiae (7).

Group B	Antiserum					
	IMF		GI		GP	
	PG2	Donetta	PG2	Donetta	PG2	Donetta
M. bovis (2 strains)	0(2)	+(2)	0(2)	+(1)	+(2)	+(2)
M. agalactiae (7 strains)	+(7)	0(7)	0(7)	0(7)	+(7)	+(6)

+: Positive
0 : Negative
Numbers in paranthesis indicate number of strains

also in the GI and GP tests, respectively. Reactions were also seen with antiserum against 2-D in the GI and GP tests, with 3 and 6 isolates, respectively. Strain 2-D is the reference strain of ovine/caprine group 11 of *Al-Aubaidi*. Twenty strains, approximately half of group C, did in the IMF test react with antiserum against both PG11 and 2-D. In the GI test 16 strains reacted with PG 11-antiserum as opposed to 5 strains in the GP test. Of the same 20 strains, 8 and 5 reacted with antiserum against 2-D in GI and GP tests, respectively. One out of 38 strains reacted with antisera against PG11 and 2-D, but in the GI test only (Table 4).

Table 4. Identification of 38 caprine strains of M. bovigenitalium and demonstration of serological relationship to ovine/caprine group 11 of *Al-Aubaidi*.

Group C	Antiserum					
	IMF		GI		GP	
	PG11	2-D	PG11	2-D	PG11	2-D
M. bovigenitalium (17 strains)	+(17)	0(17)	+(14)	+(3)	+(10)	+(6)
M. bovigenitalium (20 strains)	+(20)	+(20)	+(16)	+(8)	+ (5)	+(5)
M. bovigenitalium (1 strain)	0	0	+ (1)	+(1)	0	0
Total	37	20	31	12	15	11

+: Positive
0 : Negative
Numbers in paranthesis indicate number of strains

Group D, consisting of 3 strains being arginine positive, but glucose negative, did not react in the IMF test, using antisera against all recognized mycoplasma species of that biochemical group. However, all 3 strains had antigens in common with at least 2 arginine positive species and 1 strain even with 6 species (M. gateae, M. arginini, M. alkalescens, M. canadense, M. arthritidis and M. equirhinis) as determined by the growth precipitation technique.

DISCUSSION AND CONCLUSIONS

Classification of mycoplasmas includes biochemical and serological methods. In general a satisfactory species-identification may be based on a few biochemical tests (sensitivity to digitonin, fermentation of glucose, hydrolysis of urea and arginine) and 1 or 2 serological methods revealing surface antigens. These tests can be supplemented with a test based on cytoplasmic antigens (*Ernø* 1983). This operational schedule, which is dividing genus Mycoplasma in 4 groups on the basis of the results of the glucose and arginine tests, leaves room for serological or biochemical variants within a given species.

It is worthwhile noticing that to avoid false negative results in the biochemical tests, rather good growth is necessary. Concerning the serological tests used in this work, the IMF and GI tests depend primarily on surface antigens, including the cell membranes. It is a precondition for positive reactions that the antibodies get contact with the proper complementary antigens, and that the membrane antigens are not being covered by non-antigenic material. The GP technique is based on soluble, diffusible antigens, including cytoplasmic compounds released after rupture of the cell membrane. Finally, it is worth noticing that the specificity and sensitivity of the 3 methods used here vary for different species, groups of species and genera.

The 5 strains of genus Acholeplasma are all identified as A. laidlawii (Table 2) as the GP tests were positive with PG8 antiserum only. GP is considered almost species-specific for acholeplasmas, as this method displays fewer cross-reactions between species than GI and IMF tests (*Heitmann & Kirchhoff* 1978). The GP method is specially valuable for differentiation between A. oculi and A. laidlawii (*Ernø & Salih* 1980).

The differentiation and identification of the 2 strains of M.

bovis and the 7 strains of M. agalactiae present no problems (Table 3), since the IMF tests clearly differentiate between the 2 species. The cross-reactions seen in the GP test, between 8 of the 9 strains in this biochemical group, demonstrate a non-species-specific antigenic relationship, a group-specific relationship, reflecting the close genetic relationship between M. agalactiae and M. bovis. The percentage of homology is 40 %, as determined by DNA/DNA hybridization (*Askaa & Ernø* 1976). The low sensitivity of the GI test, and the high sensitivity of the GP test for these 2 species, M. agalactiae and M. bovis, is reflected by the findings that only one of 9 strains reacted clearly positive with the homologous antiserum in GI as compared to 9 positive reactions in GP.

The identification of the 38 strains of biochemical group C is likewise rather straight forward (Table 4). Thirty-seven strains were positive in the IMF test with antiserum against PG 11, the type strain of M. bovigenitalium. Thirty and 15 were also positive in the GI and GP tests. One strain was negative in IMF and GP, but positive in GI. As all 38 strains are biochemically identical to strain PG11, it seems correct to classify them as M. bovigenitalium, although they are of caprine origin. This classification does not imply that the strains are 100 % identical with bovine isolates of the same species. It is possible that they represent a variant of the bovine species.

The results indicate a close relationship between M. bovigenitalium and group 11 as represented by strains PG11 and 2-D. If only antiserum against 2-D had been used, 20 of the strains could have been classified as group 11 of *Al-Aubaidi* on the basis of immunofluorescence tests and biochemical pattern, in some cases even supported by the results of growth inhibition and growth precipitation.

The relationship between the type and reference strains of M. bovigenitalium and group 11 of *Al-Aubaidi* will be further examined (*Christiansen & Chima*) using DNA/DNA hybridization involving 3 of the field strains examined here, namely 1 strain (AMRC-C 2277) of M. bovigenitalium with no cross-reactions to group 11, except for the GP test, another isolate (AMRC-C2291) cross-reacting in IMF and GI tests, but not in growth precipitation and a third (AMRC-C 2260) mycoplasma being positive against PG11 and 2-D antiserum in GP and IMF, but not in GI tests.

The 3 not identified strains of group D will be examined further to elucidate whether they in fact represent one or more new species of the arginine positive group of mycoplasmas. The relation to this group is confirmed with the GP test, demonstrating the occurrence of non-species-specific antigens, common to some of the arginine positive mycoplasmas.

REFERENCES

Al-Aubaidi, J. M.: Biochemical characterization and serological classification of caprine and ovine mycoplasma with special reference to the antigenic relationships of M. mycoides var. capri and M. mycoides var. mycoides. Thesis, Cornell University 1972.

Askaa, G. & H. Ernø: Elevation of Mycoplasma agalactiae subsp. bovis to species rank: Mycoplasma bovis (Hale & al.) comb. nov. Int. J. syst. Bacteriol. 1976, *26*, 323—325.

Black, F. T.: Modifications of the growth inhibition test and its application to human T-mycoplasmas. Appl. Microbiol. 1973, *25*, 528—533.

Carmichael, L. E., T. D. St. George, N. D. Sullivan & N. Horsfall: Isolation, propagation, and characterization studies of an ovine mycoplasma responsible for proliferative interstitial pneumonia. Cornell Vet. 1972, *62*, 654—679.

Cottew, G. S.: Infections with mollicutes in sheep and goats. *In*: I. Gylstorf (ed.): Infektionen durch Mycoplasmatales. VEB Gustav Fischer Verlag Jena 1985, p. 368—386.

Cottew, G. S., L. C. Lloyd, I. M. Parsonson & D. E. Hore: Isolation of a mycoplasma from vulvovaginitis in sheep. Aust. vet. J. 1974, *50*, 576—577.

Doig, P. A. & H. L. Ruhnke: Isolation of ureaplasma from sheep with granular vulvitis. Vet. Rec. 1977, *100*, 179—180.

Ernø, H.: Mycoplasmas related to Mycoplasma mycoides subsp. mycoides. *In* S. A. Hall (ed.): The diagnosis of contagious bovine pleuropneumonia and other infections with Mycoplasma mycoides subsp. mycoides. Document No. EUR 8654, EEC, Bruxelles 1983, p. 33—39.

Ernø, H., J. M. Al-Aubaidi, M. O. Ojo, U. M. Minga & A. Sikdar: Classification and identification of ovine and caprine mycoplasmas. Acta vet. scand. 1978, *19*, 392—406.

Ernø, H., K. Jurmanova & R. H. Leach: Bovine mycoplasmas: A serological study by the metabolic inhibition test. Acta vet. scand. 1973, *14*, 511—523.

Ernø, H. & K. Peterslund: Growth precipitation test. *In*: Methods in Mycoplasmology 1983, *1*, 489—492.

Ernø, H. & M. M. Salih: The growth precipitation test as a diagnostic method for differentiation of mycoplasma and acholeplasma species. Acta vet. scand. 1980, *21*, 469—481.

Ernø, H. & L. Stipkovits: Bovine mycoplasmas: Cultural and biochemical studies. I. Acta vet. scand. 1973a, *14*, 436—449.

Ernø, H. & L. Stipkovits: Bovine mycoplasmas: Cultural and biochemical studies. II. Acta vet. scand. 1973b, *14*, 450—463.

Heitmann, J. & H. Kirchhoff: Acholeplasma species differentiation with the growth precipitation test. Int. J. syst. Bacteriol. 1978, *28*, 96—98.

Jones, G. E., A. G. Rae, R. G. Holmes, S. A. Lister, J. M. W. Jones, G. S. Grater & N. Richards: Isolation of exotic mycoplasma from sheep in England. Vet. Rec. 1983, *113*, 540.

Livingston, C. W. & B. B. Gauer: Occurrence of Mycoplasma sp. (2D) in Texas sheep flocks. Amer. J. vet. Res. 1983, *44*, 868—869.

Rosendal, S. & F. T. Black: Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta path. microbiol. scand. Sect. B 1972, *80*, 615—622.

Singh, N., B. S. Rajyan & G. C. Mohanty: Granular vulvovaginitis (GVV) in goats associated with Mycoplasma agalactiae. Cornell Vet. 1974, *64*, 435—442.

SAMMENDRAG

Beskrivelse og identifikation af caprine, genitale mykoplasmer.

Femoghalvtreds stammer dyrket fra vagina af geder undersøgtes med henblik på identifikation. Tre stammer, der var argininpositive og glukosenegative, kunne ikke endeligt klassificeres. Fem stammer fandtes at være Acholeplasma laidlawii. Syvogfyrre isolater var phosphatase positive, glukose- og argininnegative. Ni af disse dannede "film and spots" på standard vækstmedium og reducerede tetrazolium aerobt. Serologisk undersøgelse viste, at 7 af disse var Mycoplasma agalactiae, og 2 var M. bovis. De 38 resterende stammer dannede ikke "film and spots" på standardmediet og reducerede ikke tetrazolium aerobt. Alle på nær een af disse kunne på grundlag af biokemiske resultater og indirekte immunofluorescens artsbestemmes til M. bovigenitalium. Dette indebærer dog ikke, at de er helt identiske med bovine stammer af samme species. Relationen til ovin/caprin gruppe 11 (*Al-Aubaidi*) diskuteres.

(Received July 18, 1986).

Reprints may be requested from: H. Ernø, the Institute of Medical Microbiology, Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark.