Acta vet. scand. 1978, *19*, 392—406.

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CLASSIFICATION AND IDENTIFICATION OF OVINE AND CAPRINE MYCOPLASMAS

By

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ERNØ, H., J. M. AL-AUBAIDI, M. O. OJO, U. M. MINGA and A. SIKDAR: *Classification and identification of ovine and caprine mycoplasmas.* Acta vet. scand. 1978, *19*, 392—406. — The purpose of this investigation was to give a survey of the classification of ovine/caprine mycoplasmas as a basis for the identification of strains isolated from sheep and goats. A total of 13 strains representing 13 species and/or serogroups were biochemically examined, and serological cross-titrations were performed using metabolism inhibition, growth inhibition and immunofluorescence. Serogroup 6 (Al-Aubaidi) was found to be identical with Mycoplasma capricolum.

The results of identification of 57 isolates, sent to the reference centre from different countries, are given.

On the basis of the above investigations and a comparison of some of the classification systems described in the literature, it is concluded that the following species have been isolated from goats and/or sheep: M. agalactiae, M. arginini, M. bovis, M. capricolum, M. conjunctivae, M. mycoides subsp. capri, M. mycoides subsp. mycoides, M. ovipneumoniae, M. putrefaciens, Acholeplasma granularum, A. laidlawii and A. oculi. In addition, both ureaplasmas and strains representing 6 serogroups (groups 5, 7, 10 and 11 of Al-Aubaidi and groups 17 and 18 of Cottew) have been isolated. These serogroups ought to be finally species-classified as soon as possible.

ovine/caprine mycoplasmas; classification; identification.

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For a laboratory wishing to commence the study of the prevalence and significance of mycoplasmas in sheep and goats, problems immediately arise in terms of identification of isolated mycoplasmas. This situation is due to the fact that the literature deals with well-defined species as well as different systems of classification using informal designations as "serogroups" or less suitable words like "serotypes". Two circumstances especially add to the confusion. First, a group designation is often used even if the group in question is already known to be identical with a recognized species; secondly, different systems of classification may overlap each other, with the consequence that serogroup X in one system may be identical with serotype Y in another.

The purpose of this study was primarily to contribute to the clarification of the taxonomic questions, partly by our own biochemical and serological investigations of ovine/caprine mycoplasmas, and partly by studies of the literature. Furthermore, the results of the identification of isolates of ovine and caprine

Table 1. Caprine and ovine mycoplasmas.

Species or serogroup	Type or reference strain	Serogroups of *Al-Aubaidi* (1972)	Serogroups of *Cottew* (1974)
M. mycoides subsp. capri	PG3	3	3
M. mycoides subsp. mycoides	Y-goat	8	8
M. conjunctivae	HRC581		15
M. ovipneumoniae	Y-98	12	12
M. putrefaciens	KS1	4	4
Group 6	Goat 189	6	6
M. capricolum	California kid		
M. arginini	G230	2	2
Group 5	Goat 145	5	5
M. agalactiae	PG2	1	1
Group 7	A1343	7	7
Group 11	2-D	11	11
A. oculi	19-L	9	9
Group 10	2124	10	10
A. laidlawii	PG8		13
Ureaplasmas	—		14
M. dispar	462/2 (10125)		16
Group 17	EHM		17
Group 18	QEW		18

origin, performed at the FAO/WHO Collaborating Centre for Animal Mycoplasmas, will be presented. At the time when this study was planned, it seemed most reasonable to use the classification system of *Al-Aubaidi* (1972) because it is based on a specific test, metabolism inhibition, and because it already comprises as many as 12 serogroups, of which some are recognized species (Table 1). Furthermore, 2 newly classified species, M. capricolum (*Tully et al.* 1974) and M. conjunctivae (*Barile et al.* 1972), were included in the study.

MATERIALS AND METHODS

Strains. A total of 13 reference strains (Table 2) were employed in the biochemical and serological studies. Eleven strains represented serogroups of Al-Aubaidi*, 7 being identical with recognized species (Table 1), while 4 strains (goat 145, goat 189, A1343, and 2-D) represented serogroups 5, 6, 7, and 11. Group 10 was not represented.

* Unless otherwise stated, the term serogroup refers in the following to the classification system of Al-Aubaidi.

Table 2. Caprine and ovine mycoplasmas. Origin of type and reference strains.

Species or serogroup	Type or reference strain	Supplied by	Isolated by	Source
M. agalactiae	PG2	E. A. Freundt (D. G. ff. Edward)	Lopez	Udder (sheep)
M. arginini	G230	M. F. Barile	Morris	Brain (Scrapie-infected mouse)
M. mycoides subsp. capri	PG3	E. A. Freundt (D. G. ff. Edward)	Chu	Pleural fluid (goat)
M. putrefaciens	KS1	J. G. Tully	Adler	Goat
Group 5	Goat 145	A. H. Dardiri	Del Giudice	Joint (goat)
Group 6	Goat 189	A. H. Dardiri	Cordy	Joint (goat)
Group 7	A1343	A. H. Dardiri	Doza	Lung (goat)
M. mycoides subsp. mycoides	Y-goat	A. H. Dardiri	Laws	Peritoneum (goat)
A. oculi	19-L	A. H. Dardiri	Al-Aubaidi	Eye (goat)
M. conjunctivae	HRC581	ATCC	Barile	Eye (goat)
Group 11	2-D	A. H. Dardiri	Carmichael	Genital tract (sheep)
M. ovipneumoniae	Y-98	A. H. Dardiri	Carmichael	Respiratory tract (sheep)
M. capricolum	California kid	J. G. Tully	Cordy	Joint (goat)

Antisera. All immunogen preparations, immunizations and harvesting of rabbit antisera were done as described by *Ernø et al.* (1973).

Biochemical tests. Biochemical tests were performed according to *Ernø & Stipkovits* (1973). The reference strains were tested for sensitivity to digitonin, glucose and arginine catabolism, phosphatase activity, serum digestion and formation of film and spots. Catabolism of xylose, aesculin, arbutin and galactose was examined in the presence of digitonin resistance.

Serological tests. Growth inhibition, metabolism inhibition and immunofluorescence tests were performed; the methods employed have recently been described in detail by *Freundt et al.* (in press).

1. Growth inhibition (Table 4). The agar well modification was employed, using modified Hayflick medium (B) or a medium (C) with a low content of horse serum (2.5 %). The plates were incubated primarily either at 37°, 27° or 22°C. If no growth had occurred after 48 hrs. of incubation at 27° or 22°C, the plates were reincubated at a higher temperature (27° and 37°C) until the growth was visible under the stereomicroscope.

2. Metabolism inhibition tests (Table 5). The tests were generally performed as described earlier (*Ernø et al., Freundt et al.*), utilizing the ability of antibody to inhibit metabolic activities, viz. glucose fermentation, arginine catabolism and reduction of 2,3,5-triphenyl tetrazolium chloride or resazurin. Resazurin (*Jurmanová* 1975) was used as substrate for group 7 and group 11, as the representative strains do not catabolize glucose or arginine, and the reduction of tetrazolium was weak in the medium employed in this study, even when sodium thioglycollate was added.

3. Immunofluorescence tests. The indirect epiimmunofluorescence technique using unfixed colonies was employed except with M. ovipneumoniae. This species is characterized by forming colonies which are washed away from the agar surface when the standard procedure is followed. It was therefore necessary to find a method to fix the colonies to the agar. After some experimentation, the following technique was chosen to obtain good fixation. The agar plates should be stored for at least 1 week before inoculation. After incubation for 3

days at 37°C and 3 days at 22°C, fixation was performed by adding alcohol (95 % (w/v)) to the plates for a period of 90 min. The alcohol was then poured off, and the plates were ready for the test after storage at 22°C for 5 days. Prior to the immunofluorescence staining the colonies were washed in distilled water. The standard procedure was now applied, except that the incubation time with antiserum and conjugate was 60 min. instead of 30 min.

Identification of field strains. The identification procedure was initiated with the digitonin test, followed by tests for glucose fermentation, arginine hydrolysis, phosphatase activity and in some cases serum digestion. The final serological identification was performed by indirect immunofluorescence and confirmed by growth inhibition.

RESULTS

Biochemical reactions

The results of the biochemical examinations, except the digitonin test, are shown in Table 3. Strain 19-L (A. oculi) was the only digitonin-resistant strain. The special biochemical tests for acholeplasmas (*Ernø & Stipkovits* 1973) were used, and 19-L proved to hydrolyse aesculin, but did not ferment galactose, xylose or arbutin.

Glucose and arginine catabolism

The strains of the genus Mycoplasma can be divided into 4 groups according to their reactions:

(1) Five strains were glucose-positive and arginine-negative: Y-goat (M. mycoides subsp. mycoides), PG3 (M. mycoides subsp. capri), HRC581 (M. conjunctivae), Y-98 (M. ovipneumoniae) and KS1 (M. putrefaciens). Strain PG3 and Y-goat were biochemically identical as far as the tests employed here were concerned.

(2) California kid (M. capricolum) and goat 189 (group 6) were glucose-positive and arginine-positive, and identical in the other biochemical reactions tested.

(3) Strains G230 (M. arginini) and goat 145 (group 5) were glucose-negative and arginine-positive. The 2 mycoplasmas were identical in all biochemical reactions tested except for phospha-

Table 3. Caprine and ovine mycoplasmas. Biochemical reactions.

Species or serogroup	Type or reference strain	Fermentation of glucose	Catabiosis of arginine	Phosphatase activity	Digestion of serum	Formation of film and spots	Reduction of tetrazolium chloride (aerobic/anaerobic)
M. mycoides subsp. capri	PG3	+	0	0	+	0	+/+
M. mycoides subsp. mycoides	Y-goat	+	0	0	+	0	+/+
M. conjunctivae	HRC581	+	0	0	0	+	0/+
M. ovipneumoniae	Y-98	+	0	0	0	0	(+)/+
M. putrefaciens	KS1	+	0	+	0	+	(+)/+
Group 6	Goat 189	+	v	+	+	0	+/+
M. capricolum	California kid	+	+	+	+	0	+/+
M. arginini	G230	0	+	0	0	0	0/+
Group 5	Goat 145	0	+	+	0	0	0/0
M. agalactiae	PG2	0	0	+	0	+	+/+
Group 7	A1343	0	0	+	0	0	+/+
Group 11	2-D	0	0	+	0	+	0/+
A. oculi	19-L	+	0	0	0	0	+/+

Strain 19-L (A. oculi) was the only digitonin-resistant strain. The strain reacted to the special biochemical tests for acholeplasmas as follows: aesculin-positive; galactose-, xylose- and arbutin-negative.

v: Variable.
+: Positive.
(+): Weakly positive.
0: Negative.

tase activity, which was positive for goat 145 and negative for strain G230.

(4) Strains PG2 (M. agalactiae), A1343 (group 7) and 2-D (group 11) were glucose- and arginine-negative. They were identical in all biochemical reactions except that strain A1343 did not form "film and spots".

Reduction of tetrazolium chloride

All strains except goat 145 reduced tetrazolium chloride anaerobically. Many strains reduced tetrazolium chloride aerobically; however, Y-98 (M. ovipneumoniae) and KS1 (M. putrefaciens) showed rather weak reactions. HRC581 (M. conjunctivae),

Table 4. Caprine and ovine mycoplasmas. Growth inhibition tests.

Species or serogroup	Type or reference strain	Antisera													Medium	Temperature	
		PG3	Y-goat	HRC 581	Y-98	KS1	Goat 189	Calif. kid	G230	Goat 145	PG2	A1343	2-D	19-L			
M. mycoides subsp. capri	PG3	2	0	0	0	0	0	0	0	0	0	0	0	0	C	27°C	T
M. mycoides subsp. mycoides	Y-goat	0	5	0	0	0	0	0	0	0	0	0	0	0	C	27°C	T
M. conjunctivae	HRC581	0	0	4	0	0	0	0	0	0	0	0	0	0	C	37°C	PI
M. ovipneumoniae	Y-98	0	0	0	2+2	0	0	0	0	0	0	0	0	0	B	37°C	T+ NT
M. putrefaciens	KS1	0	0	0	0	5	0	0	0	0	0	0	0	0	B	37°C	T
Group 6	Goat 189	0	0	0	0	0	6	4	0	0	0	0	0	0	C	27°C	T
M. capricolum	California kid	0	0	0	0	0	6	0	0	0	0	0	0	0	C	27°C	T
M. arginini	G230	0	0	0	0	0	0	0	4	0	0	0	0	0	B	27°C+37°C	NT
Group 5	Goat 145	0	0	0	0	0	0	0	0	2	0	0	0	0	B	27°C+37°C	NT
M. agalactiae	PG2	0	0	0	0	0	0	0	0	0	6	0	0	0	B	37°C	PI
Group 7	A1343	0	0	0	0	0	0	0	0	0	0	2	0	0	B	37°C	NT
Group 11	2-D	0	0	0	0	0	0	0	0	0	0	0	2	0	B	37°C	T
A. oculi	19-L	0	0	0	0	0	0	0	0	0	0	0	0	4+4	B	22°C+27°C	NT+ PI

T: The zone of inhibition was total (measured in mm).
NT: The zone of inhibition was not total, but the number of "break-through" colonies was less than 10.
PI: The zone of inhibition was not total, but a significant reduction in size and number of colonies was seen.

G230 (M. arginini) and 2-D (group 11) failed to reduce tetrazolium chloride aerobically. Goat 145 (group 5) did not reduce tetrazolium chloride either aerobically or anaerobically.

Serological reactions

The results of growth inhibition, metabolism inhibition and immunofluorescence tests appear from Tables 4, 5 and 6, respectively. According to all 3 tests, there were 12 non-reacting serological groups. Goat 189 (group 6) and California kid (M. capricolum) belonged to the same serological group since cross-reactions were observed in all 3 tests.

Identification of field strains

Fifty-seven strains were examined. Of these, 2 could not be identified; 2 were most probably members of the species M. capricolum and 2 most probably M. ovipneumoniae. One strain was related to M. primatum antigenically (growth inhibition and immunofluorescence), but biochemically the strain differed significantly from M. primatum. Fifty strains were identified as follows: A. laidlawii (20), A. oculi (4), A. granularum (1), M. arginini (9), M. mycoides subsp. mycoides* (8), M. mycoides subsp. capri (2), M. capricolum (3), M. ovipneumoniae (2) and M. putrefaciens (1).

DISCUSSION

The biochemical tests that are special for acholeplasmas yielded results that differed from those of *Al-Aubaidi et al.* (1973), who reported that strain 19-L and 4 other strains of A. oculi fermented both galactose and xylose, while in our hands 19-L did not ferment these sugars. The conflicting results may reflect differences in test procedures and growth of the microorganisms.

Strain PG3 (M. mycoides subsp. capri), Y-goat (M. mycoides subsp. mycoides), California kid (M. capricolum) and goat 189 (M. capricolum) digested serum. The capability of digesting serum, therefore, cannot anymore be considered unique for M. mycoides.

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* It should be emphasized that the serological grouping of goat strains (reference strain: Y-goat) into the same subspecies as the bovine strains which cause contagious bovine pleuropneumonia does not necessarily imply pathogenicity for cattle.

Table 5. Caprine and ovine mycoplasmas. Metabolism inhibition tests.

Species or serogroup	Type or reference strain	Antisera													Medium
		PG3	Y-goat	HRC 581	Y-98	KS1	Goat 189	Calif. kid	G230	Goat 145	PG2	A1343	2-D	19-L	
M. mycoides subsp. capri	PG3	512	4	0	0	0	0	0	0	0	0	0	0	0	BT+GPS(5 %)
M. mycoides subsp. mycoides	Y-goat	0	320	0	0	0	0	0	0	0	0	0	0	0	BT+GPS(5 %)
M. conjunctivae	HRC581	0	0	5120	0	0	0	0	0	0	0	0	0	0	BG+GPS(5 %)
M. ovipneumoniae	Y-98	0	0	0	64	0	0	0	0	0	0	0	0	0	BG+GPS(5 %)
M. putrefaciens	KS1	0	0	0	0	1280	0	0	0	0	0	0	0	0	BG+GPS(5 %)
Group 6	Goat 189	0	0	0	0	0	20480	5120	0	0	0	0	0	0	BT+GPS(5 %) +sodiumthio-glycollate (0.1 %)
M. capricolum	California kid	0	0	0	0	0	10240	1280	0	0	0	0	0	0	BT+GPS(5 %)
M. arginini	G230	0	0	0	0	0	0	0	640	0	0	0	0	0	BA+GPS(5 %)
Group 5	Goat 145	0	0	0	0	0	0	0	0	80	0	0	0	0	BA+GPS(5 %)
M. agalactiae	PG2	0	0	0	0	0	0	0	0	0	2048	0	0	0	BT+GPS(5 %)
Group 7	A1343	0	0	0	0	0	0	0	0	0	0	5120	0	0	B+resazurin (0.002 %)
Group 11	2-D	0	0	0	0	0	0	0	0	0	0	0	640	0	B+resazurin (0.002 %)
A. oculi	19-L	0	0	0	0	0	0	0	0	0	0	0	0	512	BG+GPS(5 %)

BT: B medium with tetrazolium.
BG: B medium with glucose.
BA: B medium with arginine.
GPS: Guinea-pig serum.

Table 6. Caprine and ovine mycoplasmas. Immunofluorescence tests.

Species or serogroup	Type or reference strain	Antisera													Medium
		PG3	Y-goat	HRC 581	Y-98	KS1	Goat 189	Calif. kid	G230	Goat 145	PG2	A1343	2-D	19-L	
M. mycoides subsp. capri	PG3	+	0	0	0	0	0	0	0	0	0	0	0	0	C
M. mycoides subsp. mycoides	Y-goat	0	+	0	0	0	0	0	0	0	0	0	0	0	C
M. conjunctivae	HRC581	0	0	+	0	0	0	0	0	0	0	0	0	0	B
M. ovipneumoniae	Y-98	0	0	0	+	0	0	0	0	0	0	0	0	0	B
M. putrefaciens	KS1	0	0	0	0	+	0	0	0	0	0	0	0	0	C
Group 6	Goat 189	0	0	0	0	0	+	+	0	0	0	0	0	0	B
M. capricolum	California kid	0	0	0	0	0	+	+	0	0	0	0	0	0	B
M. arginini	G230	0	0	0	0	0	0	0	+	0	0	0	0	0	B
Group 5	Goat 145	0	0	0	0	0	0	0	0	+	0	0	0	0	B
M. agalactiae	PG2	0	0	0	0	0	0	0	0	0	+	0	0	0	B
Group 7	A1343	0	0	0	0	0	0	0	0	0	0	+	0	0	B
Group 11	2-D	0	0	0	0	0	0	0	0	0	0	0	+	0	B
A. oculi	19-L	0	0	0	0	0	0	0	0	0	0	0	0	+	B

Our results of the biochemical testing of 2-D (serogroup 11) and PG2 (M. agalactiae) confirm those of *Carmichael et al.* (1972), who reported that the 2 organisms are biochemically similar. The only difference between their results and ours is that they found that strain 2-D reduced tetrazolium aerobically, although slowly; in our study, the organism did not reduce tetrazolium chloride aerobically, but did so only in anaerobic atmosphere.

Al-Aubaidi (1972) found that KS1 (M. putrefaciens) was positive for "film and spots", while *Tully et al.* (1974) considered the reaction negative for the same species. In our repeated tests using egg-yolk medium, the "film and spots" formation by KS1 was consistently positive. Strain HRC581 (M. conjunctivae) was positive for "film and spots" after 11 days of incubation, while the other strains formed "film and spots" after only 7 days. *Barile et al.* (1972) reported that strain HRC581 was negative for "film and spots" formation.

Strain goat 189 (serogroup 6) was identical with California kid (M. capricolum) in all characteristics tested apart from the arginine reaction. A positive reaction with goat 189 was observed only when subculturing was carried out in the test medium, or when large inocula were used. California kid was arginine-positive independent of the size of inoculum and subculturing. In general, the division of mycoplasmas into 4 biochemical groups is definitely useful in selecting the antisera that should be used in the final serological identification. It should be borne in mind, however, that biochemical variants of a species may occur.

On the whole, the results of the serological grouping reported here agree with those of earlier workers. *Tully et al.* found a one-way cross-reaction between group 5 and M. arginini in the metabolism inhibition test. In the present study, however, no such cross-reactions were observed, but the titres were also much lower than those reported by *Tully et al.* Strain goat 189 was found serologically identical with strain California kid, and group 6 of Al-Aubaidi is therefore synonymous with M. capricolum.

As far as the identification of field strains is concerned, the most interesting finding is the classification of 1 strain as M. putrefaciens, as this species has hitherto been represented only by the type strain, the origin of which is obscure, even whether

it actually came from a goat or not (*Tully et al.*). It is also worth noting that M. ovipneumoniae was isolated from goats.

In 1974, *Cottew* enumerated 18 ovine/caprine serogroups, regarding T-strains (ureaplasmas) as a single group (Table 1). The list did not include A. granularum, whereas M. dispar, which is a potentially pathogenic species occurring in cattle, was included. It is questionable, though, whether it is correct to include M. dispar; *Cottew* referred to a paper by *Perreau* (1973). However, in our opinion, *Perreau* described the strains as being closely related to M. dispar, but definitive classification was apparently not done. It is therefore, in our view, most reasonable to delete M. dispar from the list of ovine/caprine mycoplasmas until the problem has been further elucidated. Interestingly enough, another bovine species, M. bovis, was isolated from diseased lungs and later proved to be pathogenic for the goat mammary gland (*Ojo & Ikede* 1976).

In summary, the following species have been isolated from goats and/or sheep: M. agalactiae, M. arginini, M. bovis, M. capricolum, M. conjunctivae, M. mycoides subsp. capri, M. mycoides subsp. mycoides, M. ovipneumoniae, M. putrefaciens, A. granularum, A. laidlawii and A. oculi. Furthermore, ureaplasmas and 6 serogroups are recognized (groups 5, 7, 10 and 11 of Al-Aubaidi; groups 17 and 18 of Cottew). The serogroups ought to be finally species-classified as soon as possible. This requires first of all a serological comparison with all known species of the family Mycoplasmataceae.

As previously mentioned, several systems of classification have been established. *Cottew et al.* (1968) used the designations type A, C and N, and these groups are identical with M. agalactiae, M. mycoides subsp. mycoides and M. arginini (*Al-Aubaidi*). *MacKay* (1965) classified his isolates as types A and B; type A is identical with M. ovipneumoniae (*Leach et al.* 1976). *Krauss & Wandera* (1970) used the designations groups I, II and III. The last of these groups does probably belong to the genus Acholeplasma, as growth occurred at 22°C. It may also be mentioned that *Hudson et al.* (1967) employed the name M. capri as a synonym for M. mycoides subsp. capri in accordance with *Villemot & Provost* (1959) and *Turner* (1960) who expressed the view that this group should be regarded as a distinct species.

In addition to the above-mentioned species and serogroups, reports have been published on non-classified strains, for ex-

ample the very interesting work by *MacOwan & Minette* (1976), who isolated strains from outbreaks of caprine pleuropneumonia in Kenya. Similar strains were isolated, also in Kenya, by *Minga* (unpublished results). Studies in this laboratory have shown that the strains are serologically related to M. primatum, but differ significantly from this species in their biochemical reactions. In Iraq, *Al-Shammari & Al-Aubaidi* (1977) isolated 96 mycoplasma strains from sheep and goats; but they have not yet been characterized or classified.

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SAMMENDRAG

Klassifikation og identifikation af ovine og caprine mykoplasmer.

Formålet med dette arbejde er at give en oversigt over klassifikationen af ovine/caprine mykoplasmer som grundlag for identifikation af stammer isoleret fra får og geder. Tretten stammer, repræsenterende 13 arter og/eller serogrupper blev biokemisk undersøgt, og der foretoges serologisk krydstitrering under anvendelse af metabolic inhibition, vækstthæmning og immunofluorescens. Serogruppe 6 (Al-Aubaidi) fandtes at være identisk med Mycoplasma capricolum.

Der redegøres for identifikation af 57 isolater, indsendt til referencecentret fra forskellige lande. Identifikationen baseredes på immunofluorescens og væksthæmning.

På grundlag af resultaterne af ovennævnte undersøgelser samt en gennemgang og sammenligning af nogle af de i litteraturen omtalte klassifikationsstudier konkluderes, at følgende arter er dyrket fra geder og/eller får: M. agalactiae, M. arginini, M. bovis, M. capricolum, M. conjunctivae, M. mycoides subsp. capri, M. mycoides subsp. mycoides, M. ovipneumoniae, M. putrefaciens, Acholeplasma granularum, A. laidlawii og A. oculi. Ydermere kan ureaplasmer forefindes samt stammer repræsenterende 6 serogrupper (Al-Aubaidi's gruppe 5, 7, 10 og 11 samt Cottew's gruppe 17 og 18). Disse serogrupper bør snarest undersøges med henblik på endelig species-klassifikation.

(Received February 14, 1978).

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