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BOVINE MYCOPLASMAS: CULTURAL AND BIOCHEMICAL STUDIES I

By

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ERNØ, H. and L. STIPKOVITS: *Bovine mycoplasmas: Cultural and biochemical studies. I.* Acta vet. scand. 1973, *14*, 436—449. — A survey is given of presently known mycoplasmas of bovine source. Media and methods of cultivation are described. Cholesterol dependence, being the basis of division of Mycoplasmatales into Mycoplasmataceae and Acholeplasmataceae, was examined directly and also indirectly employing sensitivity tests to digitonin and sodium polyanethole sulphonate (SPS). All by now recognized species were found to be correctly classified in Mycoplasma and Acholeplasma, respectively. Of the unnamed "serogroups" 2 should be classified in the latter genus, while 6 serogroups were members of the genus Mycoplasma. Correlation was found between the digitonin test and the direct determination of cholesterol requirement, whereas this was not the case with the SPS test.

bovine mycoplasmas; cultivation; characterization; classification.

The interest in mycoplasmas and mycoplasmosis has been increasing greatly during the last decade resulting in the discovery of many new species or serogroups. The World Health Organization and the Food and Agricultural Organization of the United Nations therefore want to encourage the coordination of international scientific work in this field. To this purpose international reference centres for animal and human mycoplasmas

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have been established. Furthermore, working teams have been created for particular groups of mycoplasmas. The working teams are primarily responsible for developing improved methods for the isolation and characterization of mycoplasmas, and, when possible, for producing reference reagents. A working group has been established for bovine mycoplasmas with Dr. Lindley* as chairman. The increasing interest in mycoplasmas has also led to the establishment of a "Subcommittee on the Taxonomy of Mycoplasmatales" under the International Committee on Nomenclature of Bacteria (*Freundt & Edward* 1967). The subcommittee considered it one of its first aims to suggest minimal requirements to be fulfilled in papers describing new species of mycoplasmas (*Subcommittee* 1972).

According to the subcommittee, classification of an organism as a member of the order Mycoplasmatales should be based primarily on the following criteria: lack of a cell wall, typical colonial appearance, filterability through a 450 nm membrane filter, and absence of reversion to a bacterium under appropriate conditions. Classification into family depends on determination of sterol requirements, as the order Mycoplasmatales is subdivided into Mycoplasmataceae and Acholeplasmataceae, the latter of which differs from the first one in not requiring sterol for growth (*Edward & Freundt* 1970). So far, one genus is recognized in each family, Mycoplasma and Acholeplasma, respectively.

The properties to be determined to establish differences from existing species include cultural and biochemical characteristics as well as antigenicity. Reference is made to a number of tests which are considered particularly important: catabolism of glucose, arginine and urea, production of carotenoids, growth inhibition test and complement fixation test or double immunodiffusion technique. In addition, a series of optional tests are mentioned that may provide further information.

The purpose of the present work is to indicate which methods may be recommended for cultivation of mycoplasmas, other than T-mycoplasmas, from cattle. Furthermore, type or reference strains of known bovine mycoplasmas were examined with special reference to the requirements of the subcommittee.

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The investigations were accomplished to the point of classification into families. Subsequent papers will deal with biochemical and serological data, with the particular purpose of proposing classification of those bovine mycoplasmas that have been tentatively classified as "serogroups" or "serotypes". It is the hope that these publications may be of some help to veterinary laboratories as a guide for routine diagnosis of bovine mycoplasmas and mycoplasmosis. A detailed literature review will not be given, as several reviews have been published during the last few years.

MATERIALS AND METHODS

1. *Strains*

A total of 17 strains (Table 1) were included in the study. Seven of these represent type or reference strains of recognized Mycoplasma and Acholeplasma species or subspecies. Three strains (PG 49, PG 50, PG 51) represent serogroups 6, 7 and 8 of *Leach* (1967). Four strains (B 139 P, B 142 P, B 107 PA, B 144 P) represent serotypes H, I, K and L of *Al-Aubaidi & Fabricant* (1971). Strain M 165/69 isolated from the eye of a calf with infectious bovine keratoconjunctivitis represents a group which, according to preliminary studies, seemed to be unrelated to any known bovine mycoplasma (*Langford & Dorward* 1969). The type strains (PG 2 and PG 3) of 2 ovine organisms (M. agalactiae subsp. agalactiae and M. mycoides subsp. capri) were included in the study because of serological relatedness to the bovine strains M. agalactiae subsp. bovis (Donetta) and M. mycoides subsp. mycoides (PG 1), respectively.

Prior to the experiments all strains were cloned in the following way: a well separated single colony was picked up by suction using a Pasteur pipette. The agar plug with the colony was transferred to approx. 1.7 ml of broth and crushed against the wall of the tube. Tenfold serial dilutions up to 10^{-4} were prepared and inoculated onto plates and incubated at 37°C for 4 days. From a plate with less than 10—20 colonies a single colony was picked up, and the whole procedure was repeated twice. Five cloned cultures were kept at — 70°C and used for subsequent experiments. It has to be mentioned here that the subcommittee recommends another cloning procedure beginning with filtration through a membrane filter with the smallest pore diameter possible, and followed by culture of the filtrate on a solid medium and picking of an isolated colony. This procedure should be repeated at least 2 further times.

2. *Growth experiments*

a. Composition of media. Based upon experience gained during a period of 5 years with several mycoplasma species the authors have chosen to examine growth of unknown strains primarily on 2 standard media, a modified Hayflick medium (B) and medium N. The

Table 1. Type or reference strains of bovine mycoplasmas.

Species or serogroup	Type or reference strain	Supplied by	Isolated by	Cultured from
M. dispar	462/2	NCTC*	Gourlay (1970)	lung
M. bovirhinis	PG 43 (5 M 331)	NCTC	Harbourne, Hunter and Leach (1965)	lung
M. mycoides subsp. mycoides	PG 1	E. A. Freundt (D. G. ff. Edward)	Deposited in NCTC by Laidlaw (1931)	lung
M. mycoides subsp. capri	PG 3	E. A. Freundt (D. G. ff. Edward)	Chu	lung (goat)
Group L (Al-Aubaidi)	B 144 P	J. Fabricant	Moulton (1956)	joint
Group 7 (Leach)	PG 50 (N29)	D. G. ff. Edward	Simmons and Johnston (1963)	joint
M. bovigenitalium	PG 11 (B2)	E. A. Freundt (D. G. ff. Edward)	Edward (1950)	genital tract
M. agalactiae subsp. agalact.	PG 2	E. A. Freundt (D. G. ff. Edward)	Lopez	udder (sheep)
M. agalactiae subsp. bovis	Donetta	D. G. ff. Edward	Hale et al. (1962)	udder
M. arginini	G 230	M. F. Barile	Morris (1968)	brain, scrapie infected mouse
Group 8 (Leach)	PG 51 (M 47/67)	D. G. ff. Edward	Hudson and Etheridge (1963)	nose
Group H (Al-Aubaidi)	B 139 P	J. Fabricant	Al-Aubaidi (1970)	uterus
Group I (Al-Aubaidi)	B 142 P	J. Fabricant	Fabricant (1959)	lung
Unclassified	M 165/69	NCTC	E. V. Langford and W. J. Dorward	eye
A. laidlawii	PG 8	E. A. Freundt	Laidlaw and Elford (1936)	sewage
Group 6 (Leach)	PG 49 (Squire)	D. G. ff. Edward	Langer and Carmichael (1963)	lung
Group K (Al-Aubaidi)	B 107 PA	J. Fabricant	Al-Aubaidi (1970) (contaminated culture)	?

* National Collection of Type Cultures, Colindale Avenue, Colindale, London N.W. 9, England.

latter that had been devised partly on the basis of nutritional studies (*Ernø et al.* 1967) was included because of the obvious advantage of having 2 media differing both in composition of base and additives. Provided a strain will not grow on either of these substrates, several more complex media are tested. In the case of M. dispar, 2 substrates were compared, FF II and GS. Medium FF II was designed to grow M. suipneumoniae (*Friis* 1972), while the GS medium was used in the primary detection of M. dispar (*Gourlay & Leach* 1970).

Medium B

Heart infusion broth (Difco)	90.0	ml
Sterilize by autoclaving		
Horse serum	20.0	ml
Yeast extract (25 %) (*Taylor-Robinson et al.* 1963)	10.0	ml
Thallium acetate (10 % (w/v) solution)	1.0	ml
Penicillin (200,000 i.u. per ml)	0.25	ml
Deoxyribonucleic acid (from calf thymus, Sigma Chemical Company, St. Louis, USA, 0.2 % (w/v) solution)	1.2	ml

pH 7.8

The corresponding solid medium was prepared by replacing heart infusion broth with heart infusion agar (Difco).

Medium N

Bacto brain heart infusion (Difco)	3.7	g
Yeast extract (Difco)	0.5	g
Distilled water	100.0	ml
Sterilize by autoclaving		
Horse serum	20.0	ml
Yeast extract (25 %) (*Taylor-Robinson et al.*)	10.0	ml
Thallium acetate (10 % (w/v) solution)	1.0	ml
Penicillin (200,000 i.u. per ml)	0.25	ml
Deoxyribonucleic acid (Sigma, 0.2 % (w/v) solution)	1.3	ml
Glucose (50 % (w/v) solution)	2.0	ml

pH 7.8

The corresponding solid medium was prepared by adding 1.7 g of ionagar before autoclaving.

FF II broth

Double distilled water	115.0	ml
Brain heart infusion broth (Difco)	0.82	g
Lactalbumin hydrolysate (Difco)	0.20	g
Yeast extract (Difco)	0.08	g
PPLO broth w/o C V (Difco)	0.87	g
Sterilize by autoclaving		
Fresh yeast extract (25 %) (*Taylor-Robinson et al.*)	6.0	ml
Glucose (50 % (w/v) solution)	0.4	ml
Phenol red (0.06 % (w/v) solution)	5.0	ml
Horse serum (kept at room temperature for a week)	16.0	ml
Swine serum (from SPF pigs), inactivated	17.0	ml

Thallium acetate (10 % (w/v) solution)	0.125 ml
Bacitracin (3.5 % (w/v) solution)	1.0 ml
Hanks BSS (10 ×)	4.0 ml
pH 7.6.	

GS agar

Double distilled water	36.0 ml
Lactalbumin hydrolysate (NBC) (5 % (w/v) solution)	10.0 ml
Hartley's* digest broth "B"	20.0 ml
Agarose	0.62 g
pH 8.0	
Sterilize by autoclaving	
Fetal calf serum (56°C for 30 min.)	20.0 ml
Glucose (50 % (w/v) solution)	2.0 ml
Deoxyribonucleic acid (Sigma, 0.2 % (w/v) solution)	1.0 ml
Penicillin (200,000 i.u. per ml)	0.5 ml
Thallium acetate (10 % (w/v) solution)	0.25 ml
Phenol red (0.06 % (w/v) solution)	4.0 ml
Hanks BSS (10 ×)	4.0 ml
pH 7.8.	

b. Gaseous requirements. Plates were inoculated with 0.01 ml of 10-fold serial dilutions from 10^{0} to 10^{-6}, of stock cultures of each strain. The plates were incubated for 10 days under 4 different atmospheric conditions: (1) atmospheric air, (2) candle jar, (3) 100 % N_2, and (4) 95 % N_2 and 5 % CO_2. The plates were examined every second day and growth recorded and coded as shown in Table 2. The principle of this table is that fast and strong growth is indicated by high grades, while slow and weak growth results in low grades. The scale ranges from 1 to 12.

Table 2. Evaluation of growth on agar medium.

Dilution of inoculum	Values for growth				
	Days before appearance of visible colonies				
	1—2	3—4	5—6	7—8	9—10
0	5	4	3	2	1
10^{-1}	6	5	4	3	2
10^{-2}	7	6	5	4	3
10^{-3}	8	7	6	5	4
10^{-4}	9	8	7	6	5
10^{-5}	10	9	8	7	6
10^{-6}	11	10	9	8	7
10^{-7}	12	11	10	9	8

* In preparation of Hartley's digest broth, pancreatic extract is replaced by Trypure (Novo).

c. Growth at different temperatures. Inoculations were performed as described above. The plates were incubated under optimal atmospheric conditions according to the results of the above experiments. Growth was recorded and coded as shown in Table 2.

d. Growth curves. Two ml of broth was inoculated with a single colony. The colony forming units (c.f.u.) per ml were determined every day for 10 days.

3. Filtration experiments

Cultures of all unnamed species or serogroups were filtered through filters with pore diameters of 450 nm (Gelman Metricel GA-6), 200 nm (Gelman Metricel GA-8), 100 nm (Millipore type VC), and 50 nm (Millipore type VM). The cultures were diluted in PBS pH 7.4 with 0.2 % gelatine. Immediately after filtration determination of colony forming units per ml was performed.

4. Morphological studies

The colony morphology was studied by means of a stereomicroscope. All strains were cultivated under optimal conditions. Broth cultures in the logarithmic growth phase were examined by dark field microscopy.

5. Absence of bacterial reversion

All strains were subcultured 5 times using the above media without penicillin or other antibacterial agents.

6. Classification into family

a. Cholesterol requirements. Determination of cholesterol requirement on solid medium was performed according to *Edward* (1971). Three different media were used:

A. Basal medium

Heart infusion agar (Difco)	90.0 ml
Sterilize by autoclaving	
Yeast extract (25 %) (*Taylor-Robinson et al.*)	10.0 ml
Deoxyribonucleic acid (Sigma, 0.2 % (w/v) solution)	1.2 ml
Thallium acetate (10 % (w/v) solution)	1.0 ml
Penicillin (200,000 i.u. per ml)	0.25 ml
pH 7.8.	

B. This medium consisted of medium A supplemented with:

Palmitic acid (0.1 % (w/v) solution)	1.1 ml
Bovine serum albumin (5 % (w/v) solution)	10.0 ml
pH 7.8.	

C. To medium B was added:

Cholesterol (0.05 % (w/v) ethanolic solution)	1.1 ml
pH 7.8.	

Each strain was cultivated in 200 ml of modified medium B*, horse serum (20 ml) being replaced by 1 ml of PPLO serum fraction (Difco).

* See Materials and Methods, section 2 a.

The cultures were centrifuged and washed 3 times in PBS pH 7.4. The sediment was suspended in 10 ml of PBS, and plates with the above media were inoculated with 0.01 ml 10-fold dilutions, from 10^{0} to 10^{-6} of this suspension. The plates were incubated under optimal atmospheric conditions. Growth was rceorded and coded as shown in Table 2.

b. Sensitivity to digitonin and sodium polyanethole sulphonate. The tests were in principle performed as the disc growth inhibition test (*Clyde* 1964). Filter-paper discs (6.35 mm) were soaked with 0.02 ml of a 1.5 % (w/v) ethanolic solution of digitonin (E. Merck A.G., Darmstadt, W. Germany) or 0.02 ml of a 5 % (w/v) solution of sodium polyanethole sulphonate (K and K laboratories, Inc., California) and dried overnight at 37°C. Appropriate medium was dispensed in 5 cm Petri dishes. The plates were dried before use and inoculated with 0.01 ml (calibrated platinum loop) of cultures (10^{5} c.f.u. per ml) using the running drop technique. The discs were pressed gently onto the middle of the inoculated area. The plates were incubated under optimal conditions and the inhibition zone measured after 3—5 days.

RESULTS AND DISCUSSION

1. *Motivation for choice of strains*

Seventeen strains were included in the study. Serotype J of *Al-Aubaidi & Fabricant* (1971) was not examined, because this group is represented by one strain only, isolated from a mixed culture. *Al-Aubaidi & Fabricant* themselves state that "it is impossible to be sure that serotype J is truly of bovine origin". Therefore it seems most logical to exclude serotype J until representatives are in fact isolated from cattle.

Another question has bearing upon the "Donetta-like" strains tentatively classified as M. agalactiae subsp. bovis (*Hale et al.* 1962). This name was used for a group of mycoplasmas causing mastitis in cattle, taxonomy merely being based on pathogenic and biochemical properties. Because of the lack of serological comparison with M. agalactiae (PG 2) *Jain et al.* (1967) proposed the alternative name M. bovimastitidis, but they did not provide any data to show that the proposed new taxon had the properties of a separate species. Based on priority the name M. agalactiae subsp. bovis is used in this paper with strain Donetta as the reference strain.

2. *Growth experiments*

a. Media. Satisfactory growth (10^{7} c.f.u. per ml) was obtained in medium B with all strains except 462/2 (M. dispar). Although

in many cases equally good or better growth was obtained in medium N, medium B is to prefer when possible, because of its more simple composition. In regard to M. dispar, the growth was extremely excellent in FF II broth; a corresponding solid medium was not as good as medium GS, which often gave colonies with the typical fried-egg-appearance. It is evident that only use through a longer period of time can show which media are preferable for primary isolation of mycoplasmas. However, use of the above media will be a good starting point.

b. Gaseous requirements. Fifteen strains grew without significant differences under all 4 atmospheric conditions. One strain, M 165/69, seemed to prefer an atmosphere with added CO_2, either in candle jar or in 95 % N_2 with 5 % CO_2, with a slight preference for the latter condition. One strain, 462/2 (M. dispar) showed best growth in candle jar and did not grow at all when incubated aerobically. It should be borne in mind that the ability to grow under different atmospheric conditions is extremely dependent on medium composition, including pH values. Candle jar incubation is recommended when only one method is used.

c. Growth at different temperatures. The results are summarized in Table 3, where mean values (M) for growth (see Table 2) are listed as well as the maximal numbers of colonies per ml. As it appears from the table, 4 strains (PG 8, B 107 PA, PG 49, and PG 3) reached the same maximum when cultured at 22°C and 37°C. Concerning PG 8 and B 107 PA, also the growth rates were practically identical at 22°C and 37°C, while PG 49 grew faster at 37°C. The faster growth at 37°C was even more pronounced with strain PG 3. For primary isolation it is probably only necessary to incubate at 37°C. Cultivation at 22°C may, however, give a suggestion of whether an isolate belongs to Acholeplasmataceae or Mycoplasmataceae.

d. Growth curves. The growth curves did naturally vary much from strain to strain regarding growth rates, maximum values for c.f.u. per ml as well as survival time. All strains reached a maximum titer of at least 10^7 c.f.u. per ml using optimal conditions for growth; this can be regarded as a satisfactory titer. It may be mentioned here that knowledge of the growth curves is of great practical value for production of antigens as well as for the performance of some biochemical tests.

Table 3. Classification into families.

Species or serogroup	Type or reference strain	Mean values (M) and maximal c.f.u. per ml (T)						Cholesterol requirement	Sensitivity to digitonin	Sensitivity to sodium polyanethole sulphonate
		37°		27°		22°				
		M	T	M	T	M	T		(mm)	(mm)
M. dispar	462/2	3.6	10^5	1.8	3×10^4	0	0	ND*	12.0	7.5
M. bovirhinis	PG 43	5.0	5×10^4	0	0	0	0	+	4.5	3.0
M. mycoides subsp. mycoides	PG 1	7.4	5×10^7	0.2	6×10^2	0	0	+	8.0	9.5
M. mycoides subsp. capri	PG 3	10.0	2×10^9	9.2	10^9	4.6	10^9	+	3.0	3.5
Group L (Al-Aubaidi)	B 144 P	9.0	3×10^8	4.6	3×10^8	0	0	+	6.0	5.0
Group 7 (Leach)	PG 50	10.0	10^9	8.0	10^9	0.8	10^4	+	4.5	4.5
M. bovigenitalium	PG 11	7.6	5×10^7	0.2	3×10^2	0	0	+	10.0	6.0
M. agalactiae subsp. agalact.	PG 2	10.0	10^9	4.0	10^9	0	0	+	7.0	7.0
M. agalactiae subsp. bovis	Donetta	7.2	10^8	1.4	10^5	0	0	+	7.0	6.0
M. arginini	G 230	8.0	3×10^7	5.4	10^7	1.0	10^2	+	7.0	2.0
Group 8 (Leach)	PG 51	9.0	3×10^8	0	0	0	0	+	5.0	5.0
Group H (Al-Aubaidi)	B 139 P	7.0	5×10^6	0.2	7×10^2	0	0	+	7.0	1.0
Group I (Al-Aubaidi)	B 142 P	9.0	10^8	5.2	6×10^7	0	0	+	3.0	0
Unclassified	M 165/69	5.4	10^7	3.8	2×10^5	0	0	+	10.0	5.0
A. laidlawii	PG 8	9.0	2×10^8	7.8	2×10^8	8	10^8	0	0	0
Group 6 (Leach)	PG 49	5.8	5×10^5	5.2	5×10^5	3.8	2×10^5	0	0	0
Group K (Al-Aubaidi)	B 107 PA	9.0	3×10^8	8.4	2×10^8	7.4	2×10^8	0	0	0

* Not determined.

3. Filtration experiments

In all strains examined the smallest reproductive unit was bigger than 100 nm, but smaller than 200 nm.

4. Morphology

Colonies of all strains had the typical fried-egg-appearance, due to central growth into the medium and a peripheral surface growth. This was true also for M. dispar although most colonies of this strain were atypical. The maximal colony size varied from 0.25 mm (M. dispar) to 3 mm (M. mycoides subsp. capri).

By dark field microscopy all strains showed typical pleomorphic morphology, characterized by coccoid bodies and filaments, sometimes branching. In some strains outspoken tendency to clustering of branched filaments could be seen, resulting in coral-stem-like formations.

In regard to morphological studies the subcommittee recommends examination by electron microscopy to demonstrate the lack of a true cell wall. Alternatively, if facilities for electron microscopy are not available, examination of a fluid culture by phase-contrast or dark-field microscopy may be employed. In the present work electron microscopy was not performed, because for practical reasons it was necessary to restrict these investigations to representative strains of new species. Therefore the results of electronmicroscopic studies will be presented in subsequent papers according to the results of serological studies.

5. Absence of bacterial reversion

All strains showed the typical colony morphology after 5 consecutive cultivations on antibiotic-free medium. Consequently the organisms would not appear to represent bacterial L-phase variants.

6. Classification into family

a. Cholesterol requirements. In addition to A. laidlawii 2 unnamed strains, B 107 PA and PG 49, were non-sterol requiring. In regard to the latter strain this is in accordance with the results of *Edward* (1971); the sterol requirement of B 107 PA does not seem to have been studied previously. In the genus Mycoplasma determination of cholesterol dependence could not be accomplished with M. dispar as this species was not able to grow in

either of the 3 substrates used or in serum-free GS medium supplemented with cholesterol, palmitic acid, and bovine serum albumin. All other named species did require cholesterol in agreement with their present classification. Furthermore, 6 unnamed strains B 144 P, B 139 P, B 142 P, M 165/69, PG 51, and PG 50, were cholesterol-dependent. Concerning PG 50, this is in accordance with the results of *Edward*, while the other 5 strains do not appear to have been investigated earlier.

b. Sensitivity to digitonin and sodium polyanethole sulphonate. As it appears from Table 3, 3 strains (PG 8, PG 49 and B 107 PA) were resistant to digitonin, while the remaining strains were sensitive with a zone of inhibition varying from 3 to 12 mm. Four strains (PG 8, PG 49, B 107 PA, and B 142 P) were resistant to sodium polyanethole sulphonate (SPS), while all other strains were sensitive with zones of inhibition varying from 1.0 to 7.5 mm. Indirect tests are desirable in order to determine whether a strain belongs to Acholeplasma or Mycoplasma as the direct method may be very laborious. The digitonin test is based on the fact that cholesterol dependent mycoplasmas are extremely sensitive to digitonin, whereas sterol non-requiring strains are much less sensitive to digitonin induced lysis. In the former strains the sterol retains some of the identity of cholesterol as evidenced by the presence of a 3 β-hydroxy group, thereby being precipitable with digitonin (*Smith & Rothblat* 1960). The SPS test was introduced by *Kunze* (1971) as a diagnostic means for differentiating of Acholeplasma and Mycoplasma. SPS is a synthetic anticoagulant which probably precipitates cholesterol, like digitonin, thereby causing lysis. It was concluded by *Kunze* that the SPS test gave a simple and reliable differentiation between the "Laidlaw group" (Acholeplasma) and "the parasitic group" (Mycoplasma). Judged from the results reported here, the digitonin test is preferable to the SPS test, as one strain, B 142 P, belonging to Mycoplasmataceae was insensitive to SPS.

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SAMMENDRAG

Bovine mykoplasmer: Dyrkningsmæssige og biokemiske undersøgelser. I.

Der er givet en oversigt over de for øjeblikket kendte mykoplasmer af bovin oprindelse. Medier og dyrkningsbetingelser er angivet. Cholesterolafhængigheden, som danner grundlaget for inddeling af Mycoplasmatales i Mycoplasmataceae og Acholeplasmataceae, er undersøgt dels direkte og dels indirekte ved hjælp af sensitivitetsunder-

søgelser over for digitonin og natriumpolyanetholsulfonat (NPS). Alle allerede anerkendte arter fandtes at være korrekt placeret i de 2 eneste slægter, Mycoplasma og Acholeplasma. Af de ikke navngivne „serogrupper" fandtes 2 at tilhøre Acholeplasma, medens 6 serogrupper kunne henføres til Mycoplasma. Der var total overensstemmelse mellem digitonin testen og direkte bestemmelse af cholesterolafhængighed. Dette var ikke tilfældet med NPS testen.

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