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THE GROWTH PRECIPITATION TEST AS A DIAGNOSTIC METHOD FOR DIFFERENTIATION OF MYCOPLASMA AND ACHOLEPLASMA SPECIES

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ERNØ, H. and M. M. SALIH: *The growth precipitation test as a diagnostic method for differentiation of Myocplasma and Acholeplasma species.* Acta vet. scand. 1980, *21*, 469—481. — The purpose of this paper is to evaluate the growth precipitation (GP) test for routine identification. The test was performed as described by *Heitmann & Kirchhoff* (1978) which is a modification of the method of *Krogsgaard-Jensen* (1972). On the basis of examination of 82 strains, using indirect immunofluorescence (IMF) and growth inhibition (GI) as well as GP tests it is concluded that the GP test seems to be very useful for species identification in the genus Acholeplasma, as this method displayed fewer cross-reactions between species than the other 2 tests. When applied to the genus Mycoplasma, however, the GP test is not species-specific, due to cross reactions observed within the group of arginine positive and within the group of glucose and serum digestion positive species. In the genus Mycoplasma the method can only be used as a screening tool, and final identification is in general based on growth inhibition and immunofluorescence.

mycoplasmas; antigenic relationship; identification; growth precipitation.

Serological examination of microorganisms may be used for identification at the species or subspecies level, or to demonstrate common antigens in closely related species or even in genetically non-related organisms. Within the order Mycoplasmatales, species differentiation is best afforded by the growth inhibition (GI), metabolism inhibition (MI) or immunofluorescence (IMF) tests for the genus Mycoplasma, while for the genus Ureaplasma the same tests are used for subspecies classification and identification. Speciation within the genus Acholeplasma may be performed

with the same methods, but in our experience it is often necessary to make a semiquantitative evaluation of the serological results in order to make a proper identification, as cross reactions seem to occur frequently between established species.

The growth precipitation (GP) test (*Krogsgaard-Jensen* 1972) was initially regarded at this laboratory as a highly specific test for identification of strains of genus Mycoplasma (*Freundt et al.* 1973), but gradually the method was used less and less, due to the greater reliability of the immunofluorescence technique, and due to the fact that cross reactions seemed to occur to a greater extent than expected (*Ernø & Jurmanová* 1973). It was therefore most interesting to learn that *Heitmann & Kirchhoff* (1978) concluded that a modified GP test was suitable for typing acholeplasma strains, as a high degree of specificity of this method was indicated by the results of testing 43 strains representing all 7 species of genus Acholeplasma. The purpose of this paper is to further evaluate the method for routine diagnostic purposes, in regard to both acholeplasmas and mycoplasmas.

MATERIALS AND METHODS

Exp. 1. Specificity of the GP test within genus Acholeplasma (Table 1).

Strains. The strains were the type strains of the 7 currently recognized species in the genus Acholeplasma and 39 field strains of A. laidlawii, A. granularum and A. oculi. The field strains had been identified previously by means of the agar well modification of the growth inhibition test (*Black* 1973, *Freundt et al.* 1979) and the indirect immunofluorescence technique (*Rosendal & Black* 1972, *Freundt et al.* 1979) The identification was usually based on semiquantitative assessment of the zones of inhibition or intensity of fluorescence, respectively.

Antisera. The antisera were raised in rabbits against the type strains as described earlier (*Ernø et al.* 1973).

Growth precipitation test. This was performed by the method of *Heitmann & Kirchhoff* (1978), which was modified from that of *Krogsgaard-Jensen* (1972), especially with regard to preparation of antigens. An agar block (1×1 cm) with dense growth of mycoplasmas was placed in a tube with 2 ml of liquid medium. The tube was shaken vigorously and left at room temperature for approx. 1 h. The liquid culture was then used as antigen. Filter paper discs, 6 mm in diameter, were impregnated with 0.025 ml of antiserum and placed on the agar surface at a distance of 1 cm from a well containing 0.05 ml of antigen. The plates were incubated at 37°C in a humid atmosphere of air with 8 % CO_2. Reactions were recorded every day for a period of 2 weeks. The method of *Heitmann & Kirchhoff* was

used in all experiments, as this technique has the advantage over that of *Krogsgaard-Jensen*'s that the liquid culture used contains dissolved antigens as well as a considerable number of colony forming units.

Exp. 2. Specificity and sensitivity of the IMF, GI and GP tests within the genus Acholeplasma (Table 2).

Strains. The type strains and 39 field strains of A. laidlawii, A. granularum and A. oculi (the same as in Exp. 1) were used because cross reactions were known to occur between these species.

Antisera. Type strain antisera as in Exp. 1.

Methods. The type strains were cross-titrated using the agar well modification of the GI test (*Black*), the IMF test as described by *Rosendal & Black*, and the GP test. The field strains were examined with the same methods, using undiluted sera in GI and GP tests, whereas antiserum was diluted 1:20 for the immunofluorescence technique.

Exp. 3. Specificity of the GP test within arginine positive species of genus Mycoplasma (Table 3).

Strains and antisera. The type strains of 10 arginine positive mycoplasma species and the corresponding hyperimmune sera were used.

Exp. 4. Specificity of the IMF, GI and GP tests within arginine positive species of genus Mycoplasma.

Strains and antisera. The type strains of M. gateae, M. alkalescens and M. arginini were cross-tested using undiluted hyperimmune sera in the GI and GP tests, and serum dilutions of 1:20 in the IMF test. Six field strains of M. arginini were also examined (Table 4). The type strains of M. gallinarum and M. iners were cross-tested, and in addition 16 isolates of M. gallinarum were tested against both antisera.

Exp. 5. Specificity of GP test in relation to serum digestion positive species and groups of genus Mycoplasma.

Strains and antisera. The type or reference strains of all serum digestion positive species and serogroups were cross-tested. Two caprine field strains of M. mycoides subsp. mycoides (ovine/caprine serogroup 8 of *Al-Aubaidi* 1972) as well as an isolate belonging to the F38-like group of caprine mycoplasmas (*Kaliner & MacOwan* 1976) were examined with the same antisera.

Exp. 6. Antigenic relationship between M. ovipneumoniae and M. dispar as illustrated by the GP test.

Strains and antisera. The type strains of M. ovipneumoniae (Y-98) and M. dispar (462/2) as well as 18 field strains of M. ovipneumoniae were examined using antisera against the said 2 species.

RESULTS

1. *Specificity of the GP test within the genus Acholeplasma (Table 1).*

One-way cross reactions were observed between strain PG8 (A. laidlawii) and antisera against BTS-39 (A. granularum) and 19-L (A. oculi).

Of the 27 field strains of A. laidlawii, 25 precipitated against homologous antisera only. Two strains cross-reacted with either A. granularum or A. oculi antiserum.

Six field strains of A. granularum reacted with homologous antiserum only, while 1 strain cross-reacted with A. laidlawii antiserum.

Five isolates of A. oculi did not show any cross reactions at all.

2. *Specificity and sensitivity of the IMF, GI and GP tests within the genus Acholeplasma.*

It appears from Table 2 that growth inhibition is more specific and sensitive than growth precipitation as judged by the results of cross-testing the type strains. However, the cross reactions observed with the latter technique are only seen when undiluted antiserum is used. Indirect immunofluorescence is the least specific test, as only non-significant differences in titers were demonstrated between strains PG8 and 19-L, representing A. laidlawii and A. oculi.

The examination of the 39 field strains revealed that the GP test was more specific than growth inhibition, as only 3 cross reactions were observed with the former as compared to 15 with the latter technique. Forty-two cross reactions occurred with the IMF test.

3. *Specificity of the GP test within arginine positive species of genus Mycoplasma (Table 3).*

Cross reactions were observed between 6 species (M. alkalescens, M. columbinum, M. gateae, M. hominis, M. opalescens and M. spumans).

M. gallinarum, M. maculosum, M. orale and M. salivarium did not cross-react with any of the 9 heterologous antisera used.

The cross reactions observed were in some cases one-way crosses, and the number of cross reactions varied from 1 to 4, within these 10 species.

Table 1. Specificity of the GP test within the genus Acholeplasma.

Species	Strains	Antiserum						
		PG8	BTS-39	19-L	S-743	PG49	C112	C1
A. laidlawii	PG8	+ (5 days)	+ (5—7 days)	+ (7—9 days)	0	0	0	0
	27 strains isolated from cattle, goats, sheep or birds	+ (27) (5 days)	+ (1) (5 days)	+ (1) (5 days)	0	0	0	0
A. granularum	BTS-39	0	+ (5 days)	0	0	0	0	0
	7 strains isolated from cattle, goats or swine	+ (1) (5 days)	+ (7) (5 days)	0	0	0	0	0
A. oculi	19-L	0	0	+ (5 days)	0	0	0	0
	5 strains isolated from goats or sheep	0	0	+ (5) (5 days)	0	0	0	0
A. axanthum	S-743	0	0	0	+ (5 days)	0	0	0
A. modicum	PG49	0	0	0	0	+ (5 days)	0	0
A. equifetale	C112	0	0	0	0	0	+ (5 days)	0
A. hippikon	C1	0	0	0	0	0	0	+ (5 days)

Numbers in parentheses indicate GP positive strains, and time of appearance of precipitates is also given.

Table 2. Specificity and sensitivity of the IMF, GI and GP tests within the genus Acholeplasma.

Strains	Antiserum								
	IMF			GI			GP		
	PG8	BTS-39	19-L	PG8	BTS-39	19-L	PG8	BTS-39	19-L
A. laidlawii (PG8)	256 (T)	0	256 (T)	8 (T)	0	0	16 (T)	1 (T)	1 (T)
27 strains from different animal species (A. laidlawii)	+ (27)	+ (23) 0 (4)	+ (4) 0 (23)	+ (27)	+ (1) 0 (26)	+ (12) 0 (15)	+ (27)	+ (1) 0 (26)	+ (1) 0 (26)
A. oculi (19-L)	128	0	1024	0	0	8	0	0	2 (T)
5 strains from different animal species (A. oculi)	+ (3) 0 (2)	0 (5)	+ (5)	+ (2) 0 (3)	0 (5)	+ (5)	0 (5)	0 (5)	+ (5)
A. granularum (BTS-39)	0	2048	0	0	64	0	0	8	0
7 strains from different animal species (A. granularum)	+ (6) 0 (1)	+ (7)	+ (6) 0 (1)	0	+ (7)	0	+ (1) 0 (6)	+ (7)	0 (7)

IMF: Immunofluorescence.
GI: Growth inhibition.
GP: Growth precipitation.
+: Positive.
0 : Negative.
T: Titres as obtained with the type strains.

Table 3. Specificity of the GP test within arginine positive species of genus Mycoplasma.

Mycoplasma strains		Antiserum									
		PG51	MMP-1	PG16	CS	PG21	PG15	MH5408	CH19299	PG20	PG13
M. alkalescens	(PG51)	+	0	0	+	0	0	0	0	0	0
M. columbinum	(MMP-1)	0	+	0	0	0	0	0	0	0	0
M. gallinarum	(PG16)	0	0	+	0	0	0	0	0	0	0
M. gateae	(CS)	+	+	0	+	0	0	+	0	0	+
M. hominis	(PG21)	+	0	0	0	+	0	0	0	0	0
M. maculosum	(PG15)	0	0	0	0	0	+	0	0	0	0
M. opalescens	(MH5408)	0	0	0	0	0	0	+	0	0	0
M. orale	(CH19299)	0	0	0	0	0	0	0	+	0	0
M. salivarium	(PG20)	0	0	0	0	0	0	0	0	+	0
M. spumans	(PG13)	+	0	0	0	0	0	0	0	0	+

+: Precipitation.
0 : No visible precipitation lines.

4. *Specificity of the IMF, GI and GP tests within arginine positive species of genus Mycoplasma* (*Table 4*).

The type strains of M. alkalescens, M. arginini and M. gateae did not cross-react at all in IMF or GI tests, while they all cross-reacted in the GP test. Six field strains of M. arginini cross-reacted in the GP test with M. gateae and M. alkalescens.

Similarly M. gallinarum and M. iners were clearly separated by IMF and GI tests, but a one-way cross reaction was observed between the type strain of M. iners and M. gallinarum antiserum

Table 4. Specificity of the IMF, GI and GP tests within arginine positive species of genus Mycoplasma.

Species and strains		Antiserum								
		IMF			GI			GP		
		PG51	G230	CS	PG51	G230	CS	PG51	G230	CS
M. alkalescens	(PG51)	+	0	0	+	0	0	+	+	+
M. arginini	(G230)	0	+	0	0	+	0	+	+	+
M. gateae	(CS)	0	0	+	0	0	+	+	+	+
6 strains from sheep, goats or pigs (M. arginini)		0	+	0	0	+	0	+	+	+

in the growth precipitation test. The common antigen was demonstrated in 3 of 16 field strains of M. gallinarum, as these 3 strains, identified by IMF and GI tests, cross-reacted with M. iners antiserum in the GP test.

5. *Specificity of the GP test in relation to serum digestion positive species and groups of genus Mycoplasma* (*Table 5*).

The growth precipitation test was clearly not species-specific, as cross reaction occurred not only between strains related to M. mycoides (PG1, PG3 and PG50), but also between this group and Calif. kid (M. capricolum) as well as the F38-like strain (AMRC-C 1424).

Table 5. Growth precipitation: Cross-reactions between serum digestion positive species and groups of genus Mycoplasma.

		Antiserum					
Species/serogrup	Strains	PG1	Y-goat	PG3	PG50	Calif.kid	F38
M. mycoides subsp.mycoides	PG1*	+	+	+	+	+	+
M. mycoides subsp.mycoides (ovine/caprine serogroup 8)	Y-goat	+	+	+	+	+	+
M. mycoides subsp.mycoides (ovine/caprine serogroup 8)	AMRC-C 1467	+	+	+	+	+	+
M. mycoides subsp.mycoides (ovine/caprine serogroup 8)	AMRC-C 1468	+	+	+	+	+	+
M. mycoides subsp.capri	PG3	+	+	+	+	+	+
Bovine serogroup 7	PG50	+	+	+	+	+	+
M. capricolum	Calif.kid	+	+	+	+	+	+
F38-like	AMRC-C 1424	+	+	+	+	+	+

* Strain PG1 is serum digestion negative.

6. *Antigenic relationship between M. dispar and M. ovipneumoniae as examined by the GP test.*

All 18 field strains of M. ovipneumoniae were positive in the GP test using the species-specific antiserum, while precipitation never occurred using M. dispar antiserum. Cross reactions did not occur between the type strains either.

DISCUSSION AND CONCLUSIONS

The growth precipitation test seems, according to our results and as demonstrated earlier by *Heitmann & Kirchhoff* (1978), to be very useful for species identification in the genus Acholeplasma. This is at any rate true for the group as represented at present by the 7 species, but one cannot exclude the possibility that new species may cross-react to a higher degree than seen so far. The one-way cross reactions observed between the type strains of A. laidlawii, A. granularum and A. oculi demonstrate that these strains do possess at least 1 common antigenic determinant. However, the determinant does not always seem to elicit an antibody response as measured by GP. The number of cross reactions may incidentally depend on the rabbits used for immunization as well as the immunization procedure, as it is a general immunological principle that the greater the number of injections, the greater is the number of different antibodies if, as in this case, the inoculum consists of many antigenic determinants.

As the GP test is insensitive, in so far as it requires high concentrations of antigen and antibody in the right proportions, one-way cross reactions as well as false negatives are to be expected. Good growth is necessary, and therefore optimal media must be used, but even different batches of the same media formulation may vary from time to time with regard to their influence on precipitate production.

The cross reactions between A. laidlawii and A. oculi seen in the growth inhibition test and in the indirect immunofluorescence tests are surprising as no cross reactions were demonstrated in the GP test. One might ask then, whether it is certain that A. laidlawii and A. oculi are in fact 2 different species. In the description of A. oculi (*Al-Aubaidi et al.* 1973) cross reactions were not observed using either the metabolism test or double immunodiffusion. Furthermore DNA/DNA hybridization experiments showed that the 2 species hybridize only to a degree of

22—25 % (*Askaa* 1974) which leaves no doubt that they are 2 distinct species. It seems therefore that strains of 2 different species may possess a sufficient number of surface antigens to give the impression, when compared by a technique usually considered species-specific, that they are representatives of only 1 species. The serological cross reactivity between some of the acholeplasma species can be accounted for by the identity of lipids (*Al-Shammari & Smith* 1979).

The growth precipitation test is not species-specific when applied to the genus Mycoplasma. The cross reactions observed within the group of arginine positive mycoplasmas and within the group of glucose and serum digestion positive species or subspecies clearly demonstrate that the test is not useful for identification. Neither M. alkalescens nor M. capricolum would have been recognized as new species, if the growth precipitation test was considered species-specific. On the other hand it appears from the work of *Freundt et al.* (1973) that one may examine a large number of species without revealing any cross reactions between them. Although growth precipitation is not species-specific, the test is still of great practical value when used as a preliminary test before final identification with techniques considered to be species-specific. In our experience, the time of appearance of precipitation does not give a clear indication with regard to identification, as heterologous reactions may occur before, concurrently with, or after the homologous reactions. It is to be remembered in this context that homologous/heterologous refers to mycoplasma/antiserum and not to antigenic determinants/antibody molecules.

The fact that the serum digestion negative strain PG1 cross-reacted with the 4 serum digestion positive strains demonstrates that the antigenic relationship is not exclusively due to identical proteolytic enzymes. The results are furthermore in accordance with the findings of *Kaliner & MacOwan* (1976) who concluded that strain F38 had a minimum of 2 antigens in common with strains of both subspecies of M. mycoides, as demonstrated by the agar gel double diffusion test.

The GP test is of value in distinguishing between species which for some reason or other are difficult to identify in routine diagnostic work or which are of particular interest in terms of antigenic relationships or the absence of them. This is based on the assumption that, if 2 strains do not have common anti-

gens that can be shown in growth precipitation, they cannot be of the same species assuming that the correct conditions for precipitation are present. Examples are M. dispar and M. ovipneumoniae (*Perreau* 1973, *Ernø et al.* 1978) or M. suipneumoniae and M. flocculare (*Friis* 1977). A positive reaction, however, indicates an antigenic, and possibly a genetic relationship, but not necessarily at the species level.

It may be concluded that the GP test reveals common antigens and seems for the time being to be almost species-specific in the genus Acholeplasma, while in the genus Mycoplasma cross reactions occur to such a degree that the test may only be used as a screening tool, and final identification of strains of this genus is in general based on growth inhibition and immunofluorescence tests. However, even these tests do not show complete species specificity, as for example that bovine group 7 is inhibited by antisera against M. bovigenitalium (*Cottew* 1970, *Ernø & Jurmanová* 1973). Whether the observed difference between the genus Mycoplasma and the genus Acholeplasma, in relation to the specificity of GP test, is true or apparent, requires further investigation with a larger number of field strains of genus Acholeplasma. We cannot exclude the possibility that some of the known differences between acholeplasma and mycoplasma species, for example the localization of enzyme systems — membrane or cytoplasmic — may explain the greater specificity within Acholeplasma than within Mycoplasma.

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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.

Al-Aubaidi, J. M., A. H. Dardiri, C. C. Muscoplatt & E. H. McCauley: Identification and characterization of Acholeplasma oculusi spec. nov. from the eyes of goats with keratoconjunctivitis. Cornell Vet. 1973, *63*, 117—129.

Al-Shammari, A. J. N. & P. F. Smith: Lipid and lipopolysaccharide composition of Acholeplasma oculi. J. Bact. 1979, *139*, 356—361.

Askaa, G.: Mycoplasmatales: Biokemiske, serologiske og genetiske undersøgelser. Ph. D. Thesis, University of Aarhus 1974.

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

Cottew, G. S.: Mycoplasmas isolated from cattle in Australia. Aust. vet. J. 1970, *46*, 378—381.

Ernø, H. & K. Jurmanová: Bovine mycoplasmas: Serological studies by double immunodiffusion, growth precipitation and growth inhibition. Acta vet. scand. 1973, *14*, 524—537.

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

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.

Freundt, E. A., H. Ernø, F. T. Black, A. Krogsgaard-Jensen & S. Rosendal: Evaluation of reference reagents for mycoplasmas. Ann. N. Y. Acad. Sci. 1973, *225*, 161—171.

Freundt, E. A., H. Ernø & R. M. Lemcke: Identification of mycoplasmas. *In* T. Bergan & J. R. Norris (eds.): Methods in Microbiology. Acad. Press, London 1979, *13*, 378—434.

Friis, N. F.: Mycoplasma suipneumoniae and Mycoplasma flocculare in the growth precipitation test. Acta vet. scand. 1977, *18*, 168—175.

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

Kaliner, G. & K. J. MacOwan: The pathology of experimental and natural contagious caprine pleuropneumonia in Kenya. Zbl. Vet.-Med. B 1976, *23*, 652—661.

Krogsgaard-Jensen, A.: Mycoplasma: Growth precipitation as a serodiagnostic method. Appl. Microbiol. 1972, *23*, 553—558.

Perreau, P.: Mycoplasmes de la chèvre apparentes á l'espéce Mycoplasma dispar. (Goat mycoplasma closely related to Mycoplasma dispar species). Rev. Élev. 1973, *26*, 13—25.

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.

SAMMENDRAG

Vurdering af vækstpræcipitationsteknikken som serologisk metode specielt med henblik på artsidentifikation af mykoplasmer.

Formålet med dette arbejde er at vurdere vækstpræcipitationsteknikken (GP) specielt med henblik på rutineidentifikation af stammer af slægterne Myco- og Acholeplasma. Testen blev udført som beskrevet

af *Heitmann & Kirchhoff* (1978), en modifikation af den oprindelige metode udformet af *Krogsgaard-Jensen* (1972). På basis af undersøgelse af 82 stammer, under anvendelse af indirekte immunofluorescens, vækshæmning og GP konkluderes, at sidstnævnte er meget anvendelig til speciesidentifikation indenfor slægten Acholeplasma, idet vækstpræcipitation var mere artsspecifik end de 2 andre tests. Derimod fandtes metoden ikke at være anvendelig til artsdifferentiering indenfor slægten Mycoplasma, idet der hyppigt optrådte krydsreaktioner både indenfor argininspaltende arter og i den gruppe af glukosespaltende og serumdigestion-positive arter, som bl. a. rummer de stammer, der er årsag til ondartet lungesyge hos køer, henholdsvis geder.

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