ASSAY OF MYCOPLASMA REFERENCE ANTISERA BY GROWTH INHIBITION, METABOLIC INHIBITION, GROWTH PRECIPITATION, INDIRECT HEMAGGLUTINATION, AND COMPLEMENT FIXATION TESTS

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A total of 29 antisera against the type strains of *Mycoplasma* and *Acholeplasma* species produced by hyperimmunization of horses or mules were subjected to an extensive homologous and heterologous serological testing using disc growth inhibition (DGI), metabolic inhibition (MI), growth precipitation (GP), indirect hemagglutination (IHA) and complement fixation (CF) tests. With one possible exception all antisera proved highly specific in DGI, GP and MI, and mostly of satisfactory potency also. A fairly high degree of heterologous cross-reactivity was observed on the other hand with the IHA test, although the heterologous titers could be significantly reduced when using formalinized red blood cells rather than fresh cells for the test. The results of the CF test likewise showed an unexpectedly high degree of heterologous cross-reactivity. Further testing of the antisera on a collaborative basis is in progress. However, the antisera and the corresponding cultures of type strains are already available—in limited amounts—to interested research workers, from the following sources: (1) Research Reference Reagents Branch of the National Institutes of Allergy and Infectious Diseases, Bethesda, Maryland 20014, U.S.A , and (2) FAO/WHO Collaborating Centre for Animal Mycoplasmas, Institute of Medical Microbiology, Bartholin Building, University of Aarhus, DK-8000 Aarhus C., Denmark. The latter institution is particularly concerned with providing reagents for research workers outside the Americas.

INTRODUCTION

The current wide interest shown in mycoplasmology in the fields of human, veterinary and plant microbiology has created a growing strong need for the availability of mycoplasma reference reagents, both for practical diagnostic use and for research purposes. For example, the ideal requirement proposed by the Subcommittee on the Taxonomy of *Mycoplasmatales* (10), that before naming a new species it should be compared serologically with all previously named species of *Mycoplasmatales*, cannot possibly be complied with by the research worker unless he has access to serum reference agents.

The initiation of the project reported in the following actually dates back to 1966. It was started at the initiative of and sponsored by the Research Reference Reagents Branch of the National Institutes of Allergy and Infectious Diseases and the Division of Biologic Standards (now Bureau of Biologics, Food and Drug Administration), National Institutes of Health, USA. (Contract Numbers PH-43-68-1516, NIH-69-59, and PH-43-67-682). The project, that was completed by October 31,

1971, included production and testing of both seed and antiserum reagents for mycoplasmas. Since, however, the time allotted for my presentation does not permit a description of the entire project I shall confine myself here to describing the antiserum reagents program.

Materials and Methods

(*a*) *Production of antiserum reagents*

The antisera were produced by immunization of horses or mules using antigens prepared from organisms normally grown in PPLO Broth with 10 per cent added autologous pre-immune horse or mule serum and 2 per cent Yeast Extract Dialysate by the Baltimore Biological Company, Inc. under the responsibility of T. R. Carski, N. F. Robillard, and R. A. DelGuidice. Generally intramuscular inoculations of antigen concentrate, mixed with equal volumes of Freund's Complete Adjuvant, were administered at seven days intervals for three consecutive weeks, Test bleedings were usually obtained for five consecutive weeks, beginning at the time of the third inoculation.

While it was originally planned to produce reference reagents for all named *Mycoplasma* and *Acholeplasma* species, reagents have been produced under this project for only 29 out of the 40 presently recognized species or subspecies. The species and strains concerned will appear from Table I.

(*b*) *Assay of antiserum reagents*

The antisera were subjected in our laboratory to a complete homologous and heterologous cross testing by means of the disc growth inhibition (DGI), growth precipitation (GP), metabolic inhibition (MI), indirect hemagglutination (IHA), and complement fixation (CF) tests. Tests were run in parallel with rabbit antisera produced in our laboratory against the same strains, although of different culture lines, as those used for the preparation of the reference sera.

The sera were inactivated at 56°C/30 min except in the case of the DGI and GP tests. All titrations were performed as microtitrations using the Cook Engineering Company microtiter system.

DGI test—This was performed essentially as described by Clyde (1964) using filter-paper discs (6.35 mm in diameter) soaked with 0.02 ml serum. Agar plates, 5 cm in diameter and about 4 mm deep, were inoculated by the "running drop technique" with 0.01 ml of the test culture containing approximately 10^5 c.f.u./ml. A complete homologous and heterologous testing was first performed on a Hayflick-type medium (BE), unless otherwise required for growth, and at 37°C (referred to in the following as "standard conditions"). In addition, an extended homologous testing was performed using a further three different media (for composition, see legend to Table I) and incubation at both 37°C and 27°C (in the case of *Acholeplasma* species 22°C). If no growth occurred at 27°C after 3–4 days, incubation was continued at 37°C for another 3 days. In case homologous inhibition was obtained only under conditions other than standard conditions a complete heterologous-testing was repeated under optimal conditions for the reagent concerned.

TABLE I

Disc growth inhibition (DGI), growth precipitation (GP), and metabolic inhibition (MI) tests; homologous reactions with NIH antisera

Antiserum prepared against		DGI (mm inhibition)			MI
Species	Strain	At standard condition (Al)	At optimal condition indicated	GP	Reciprocal of titer
M. hominis	PG 21	2.5	8.5 C1	+	4096
M. fermentans	PG 18	7.5	10.0 B, C1	+	4096
M. salivarium	PG 20	6.0	8.0 C1	+	131072
M. orale 1	CH 19299	1.5 (3)	12.5 C3	+	4096
M. orale 2	CH 20247	2.0	5.5 C3	0	8192
M. orale 3	DC-333	10.0 E	—	0	4096
M. pneumoniae	FH	3.0 (8)	8.0 A3	+	4096
M. lipophilum	MaBy	5.0 E	—	0	32
M. mycoides subsp. *myc.*	PG 1	2.0	6.0 B, C3	+	16
M. mycoides subsp. *capri*	PG 3	0	1.5 B2	+	0
M. bovigenitalium	PG 11	1.0 (2.5)	10.0 B3	+	8192
M. bovirhinis	PG 43	4.0	8.5 A3	+	1024
M. agalactiae	PG 2	0	(3.0) D3	+	4096
M. arginini	G 230	1.5	5.5 C3	+	256
M hyorhinis	BTS-7	2.5	4.5 B1	(+)	2048
M. spumans	PG 13	3.5	10.0 A3	+	2048
M. canis	PG 14	0	2.0 C, D3	+	64
M. maculosum	PG 15	3.5	6.5 C1	0	262144
M. arthritidis	PG 6	(3.5)	8.5 C3	+	128
M. pulmonis	PG 34	3.0	9.0 B3	+	512
M. neurolyticum	Sabin A	5.5	9.0 A3	+	16777216
M gallinarum	PG 16	3.5	10.0 A, B2	+	8192
M. iners	PG 30	3.0	10.0 C2	+	16384
M. gallisepticum	PG 31	2.5	6.0 B1	+	262144
M. anatis	1340	2.5 (5)	3.5 B1	+	65536
M. meleagridis	17529	3.0	5.0 C1	+	512
A. laidlawii A	PG 8	0	0 B–D 1–3	+	32768
A. laidlawii B	PG 9	0	0 B–D 1–3	+	256
A. granularum	BTS–39	4.0	3.0 C3	+	32768

Media used for the DGI test

A : Heart infusion agar (Difco); horse serum, 16%; fresh yeast extract, 8%; DNA (Sigma), 0.002%; penicillin G, 50,000 I.U.; *p*H 7.8 (BE medium).

B : as A, with the addition of 10% egg-yolk emulsion (Oxid) diluted 1 : 2 in saline.

C : as A, but with only 2.5% horse serum and without yeast extract.

D : Brain heart infusion agar (Bacto); horse serum, 16%; fresh yeast extract, 8%; yeast extract (Difco), 0.5%; DNA (Sigma), 0.002%; glucose, 0.8%; penicillin G, 50,000 I.U.; *p*H 7.8. (N medium).

E : Heart infusion agar (Difco); PPLO serum fraction, 2 %; L-arginine, 1%; fresh yeast extract, 10%; DNA (Sigma), 0.002%; penicillin G, 50,000 I.U.; *p*H 7.8.

Incubation temperature for DGI test

1 : 37°C (4 days); 2 : 27°C (4 days); 3 : 27°C for 4 days and 37°C for a further 3 days.

Inhibition zone for DGI test

Figures in brackets indicate total plus partial inhibition.

GP test—This method, which was developed in our laboratory by Krogsgaard-Jensen (1972), is in principle a double diffusion in agar based on the interaction between antibody and diffusible components released from growing organisms.

MI test—Tests based on the inhibition of glucose or arginine catabolism, or tetrazolium reduction, were performed according to conventional methods (Purcell *et al.* 1966; Senterfit and Jensen 1966; Taylor-Robinson *et al.* 1966).

IHA test—This was performed as described by Dowdle and Robinson (1964) and further modified by Krogsgaard-Jensen (1971) using the supernatant fluid from sonicated mycoplasma suspensions as antigen (undiluted, or in dilutions of 1:2 or 1:4). The standard procedure was based on the use of tanned fresh sheep red blood cells (r.b.c.) that have been found to increase the sensitivity of the IHA test (Krogsgaard-Jensen 1971). Since, however, some antigens consistently produced hemolysis of fresh r.b.c. during sensitization these were in such cases replaced by formalinized cells (Adler and DaMassa 1967). Fresh r.b.c. were sensitized at *p*H 7.2, formalinized r.b.c. at *p*H 5.5 (Krogsgaard-Jensen 1971). The sera were absorbed with washed, packed r.b.c. (1 ml/1 ml of serum) for 30 min at 4°C.

CF test—Phenolized antigens were produced according to Chanock *et al.* (1962). Two full units of antigen, 2 units of hemolysin, and 2 units of antigen were used for the test. The complement plus serum-antigen mixtures were left overnight at 4°C before adding sensitized sheep r.b.c. The test proper was carried out at 37°C/30 min.

RESULTS

DGI, GP, and MI tests—The results of homologous reactions obtained in these tests are compiled in Table I. It will be seen that the great majority of antisera showed a most satisfactory reactivity in all three tests, rather few sera reacting in two tests only.

It is particularly noteworthy that the growth inhibition obtained under standard conditions, i.e. when performed on the Hayflick medium (BE) and at 37°C, could almost invariably be considerably improved under one or more of the other test conditions used. The conditions yielding better results were usually such which provided suboptimal growth conditions for the test antigen involved, maximal inhibition being observed quite often at the combination of a deficient medium (reduced concentration of serum and no yeast extract) and low temperature. Altogether only two antisera, to *A. laidlawii* A and B, were completely negative in the DGI test under any condition, whereas a third one, the *M. agalactiae* serum, only showed a partial inhibition under a single set of conditions.

The results obtained with the GP test were generally in fair agreement with those achieved by DGI. Since antisera, that are negative in one of these tests, may be positive in the other, the GP test would appear to be a useful supplement to the DGI test and equally simple to perform.

Antibody titers obtained with the MI test were usually rather high, and quite often even exceedingly high. One serum only, the serum produced against *M. mycoides* subsp. *capri,* was negative in this test.

With one exception, all antisera proved highly specific when tested with DGI, GP, and MI. With the DGI test only, one unexpected heterologous reaction

TABLE II

Indirect hemagglutination tests with NIH antisera

Antigens			Reciprocal of IHA titer for antisera to									
			1	2	3	4	5	6	7	8	9	10
1 *M. mycoides* subsp. *mycoides*	PG	1*	1024	512	4	4	0	16	16	4	4	8
2 *M. mycoides* subsp. *capri*	PG	3*	1024	1024	32	8	0	32	8	8	4	8
3 *M. bovigenitalium*	PG	11	16	32	65536	128	128	1024	128	$\geqq$2048	256	1024
4 *M. bovirhinis*	PG	43	0	8	512	131072	4	256	64	$\geqq$2048	256	64
5 *M. agalactiae*	PG	2	4	0	512	32	16384	64	256	512	128	1024
6 *M. arginini*	G	230	0	2	512	256	4	$\geqq$4194304	128	2048	128	256
7 *M. hyorhinis*	PG	29	0	0	0	2	0	8	256	2	4	0
8 *M. spumans*	PG	13	0	0	0	8	2	64	64	$\geqq$4194304	32	32
9 *M. canis*	PG	14	0	0	0	256	0	0	16	0	2048	0
10 *M. maculosum*	PG	15	0	0	256	128	4	256	64	32	64	$\geqq$4194304

*Formalinized guinea-pig r.b.c., others : fresh r.b.c.

occurred, the *M. bovirhinis* serum inhibiting the growth of *M. lipophilum*. The corresponding Aarhus antiserum had no such effect, and neither was there any other serological evidence suggesting a close relationship between these two organisms. With the exception of double crossings between the two subspecies of *M. mycoides* and between types A and B of *A. laidlawii* no heterologous reactions were observed with the GP tests. In MI, heterologous crossings to high titers occurred only between *A. laidlawii* A and B; otherwise heterologous reactions were quite rare and usually at quite negligible levels (2 to 8). Only occasionally they attained titers of 128 to 256, and even then at considerably lower levels than the homologous antibody titers.

IHA test—Contrary to the extreme specificity shown by the DGI, GP, and MI tests a fairly high degree of heterologous cross-reactivity was observed with the IHA test. Since for practical reasons it will not be feasible to present here the very extensive complete cross-testing chart for all 29 sera examined, only representative data for a minor number of sera are shown in Table II. It will be seen that in addition to exhibiting exceedingly high homologous titers, ranging for the total material from 256 to $\geqq$ 4, 194, 304, several antisera reacted with a number of heterologous antigens. However, the heterologous titers were usually, though not invariably, significantly lower than the homologous titers.

In the course of the study the observation was made that the technique used for the IHA test had a rather considerable influence on the extent of heterologous cross-reactivity, this being less pronounced when formalinized rather than fresh r.b.c. were used. Comparative titrations carried out with four antisera using both fresh and formalinized r.b.c. are shown in Table III. It appears that the heterologous reactions occurring for three of these sera, when using fresh r.b.c. were eliminated when replacing the fresh cells with formalinized.

TABLE III

Indirect hemagglutination tests with NIH antisera comparison of titers using formalinized and fresh red blood cells

Antigens	Guinea-pig r.b.c.	Reciprocal of IHA titer for antisera to					
		M. gallinarum	*M. iners*	*M. canis*	*A. laidlawii* A	*A. laidlawii* B	*A. granularum*
M. gallinarum	Form.	$\geqq$242144	0	0	0	0	0
PG 16	Fresh	4194304	256	128	16	32	0
M. iners	Form.	0	8192	8	0	0	0
PG 30	Fresh	$\geqq$2048	8192	1024	16	16	0
M. canis	Form.	0	0	65536	0	0	0
PG 14	Fresh	0	4	2048	0	0	0
A. laidlawii A.	Form.	0	0	0	16384	65536	0
PG 8	Fresh	128	128	64	13768	$\geqq$2048	128

TABLE IV-A

Homologous and heterologous IHA titers of NIH M. iners *antiserum using formalinized and fresh r.b.c.*

Antisera		Antigen : PG 30	
		Guinea-pig red blood cells	
		Form.	Fresh
M. iners	PG 30	8192	8192
M. hominis	PG 21	0	128
M. fermentans	PG 18	0	16
M. salivarium	PG 20	0	16
M. orale 1	CH19299	0	2
M. orale 2	CH20247	8	8
M. orale 3	DC-333	0	128
M. pneumoniae	FH	0	8
M. lipophilum	MaBy	0	16
M. mycoides subsp. *mycoides*	PG 1	0	8
M. mycoides subsp. *capri*	PG 3	0	32
M. bovigenitalium	PG 11	0	1024

TABLE IV-B

Heterologous IHA titers of NIH M. iners *antiserum using formalinized and fresh r.b.c.*

Antisera		Antigen : PG 30	
		Guinea-pig red blood cells	
		Form.	Fresh
M. bovirhinis	PG 43	2	256
M. agalactiae	PG 2	0	16
M. arginini	G 230	0	256
M. hyorhinis	BTS-7	0	16
M. spumans	PG 13	8	$\geqq$2048
M. maculosum	PG 15	16	512
M. arthritidis	PG 6	0	128
M. pulmonis	PG 34	4	64
M. neurolyticum	Sabin A	0	128
M. gallisepticum	PG 31	0	256
M. anatis	1340	0	32
M. meleagridis	17529	1024	$\geqq$2048

The advantage of using formalinized r.b.c. was further confirmed by an experiment testing one of the antigens (*M. iners*) included in Table III, against all of the remaining antisera. As will be seen from Tables IVA and IVB this antigen showed a particularly extensive cross-reactivity using fresh r.b.c. With formalinized cells, on the other hand, heterologous titres were either totally eliminated or reduced to quite insignificant titres as compared to the homologous titer of 8192 that remained unchanged.

CF test—Representative results are shown in Table V. Homologous titers in the total material ranged from 16 to $\geqq$ 2048. Here again, a high degree of heterologous cross-reactivity was found, sometimes even reaching the degree of homologous reactivity.

TABLE V

Complement fixation tests with NIH *antisera*

Antigens		Reciprocal of CF titer for antisera to									
		1	2	3	4	5	6	7	8	9	10
M. mycoides subsp. *mycoides*	PG 1	**256**	128	16	256	4	128	128	32	64	64
M. mycoides subsp. *capri*	PG 3	32	**256**	0	128	2	32	32	32	32	32
M. bovigenitalium	PG 11	16	32	**128**	64	8	64	64	256	64	128
M. bovirhinis	PG 43	8	2	4	**256**	0	8	16	0	16	16
M. agalactiae	PG 2	16	16	8	64	**512**	128	64	256	32	64
M. arginini	G 230	8	4	4	32	2	**128**	32	32	32	32
M. hyorhinis	PG 29	32	32	16	256	2	128	**2048**	64	128	64
M. spumans	PG 13	16	16	64	128	4	256	128	**512**	64	128
M. canis	PG 14	16	4	16	512	4	128	128	32	**2048**	128
M. maculosum	PG 15	8	4	4	32	2	64	32	32	32	**1024**

DISCUSSION

Though not complete, the present survey probably represents one of the most extensive comparative studies performed of *Mycoplasma* and *Acholeplasma* species and their antisera. This pertains both to the number of species involved and to the number of different tests employed.

From the results obtained, it may be concluded that the suitability of the antisera as international reference reagents was largely confirmed. With a few possible exceptions this holds true both with respect to the potency and as regards the specificity of the sera.

For the purpose of identifying and classifying new isolates of mycoplasmas by means of these sera, the combined use of DGI, GP, and MI or at least two of these tests, should be recommended. Very likely the IHA test may also serve this purpose, provided formalinized erythrocytes be used throughout. On the other hand, most of the antisera appear to be less suitable for diagnostic use in the CF test, at least when this be carried out by the standard technique used in this laboratory.

It may be mentioned, however, that the cross-reactivity exhibited by the analogous rabbit antisera produced in our laboratory was almost invariably considerably less pronounced than was that of the NIH antisera. This was particularly true for the CF test, and to some extent also for the IHA test.

The study reported here admittedly needs extension in order to meet in any respect international standards. Firstly, the antisera should be tested for heterologous reactivity against the type strains of the remaining ten species not included in this project. In addition, each serum reagent should be tested against a number of strains other than the type strain of the species concerned.

Actually, this final testing is already in progress, being performed on a collaborative basis by the five Working Teams that have been established under the FAO/WHO Animal Mycoplasma Characterization Board, viz. the teams for avian, bovine, caprine, porcine and laboratory animal mycoplasmas.

Very likely, a few additional years are needed to complete this second phase of the test programme. It is the intention, however, of the Research Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases to release, in the near future, the reference reagents for distribution among interested workers. The pertinent test data already obtained will be included in a forthcoming supplement to the NIH Catalogue of Research Reagents (Cunningham and Pennington 1970).

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