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The metabolic inhibition test for mycoplasmas based on phosphatase production

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SUMMARY

A metabolic inhibition test based upon phosphatase production is described. This is of value for those *Mycoplasma* species which produce phosphatase but which cannot be examined by the previously published metabolic inhibition techniques.

INTRODUCTION

The metabolic inhibition test (MIT) is one of the most useful methods of determining antibody titres in serum and for identifying *Mycoplasma* species. The test is based upon inhibition of growth by specific antisera, and results demonstrated by inhibition of acid production (Taylor-Robinson, Purcell, Wong & Chanock, 1966), arginine hydrolysis (Purcell, Taylor-Robinson, Wong & Chanock, 1966*a*), urea metabolism (Purcell, Taylor-Robinson, Wong & Chanock, 1966*b*) and tetrazolium reduction (Jensen, 1964).

In recent years many unidentified mycoplasmas have been isolated from different animal species. Some of these do not produce acid, hydrolyse arginine, metabolize urea or reduce tetrazolium.

This communication describes the development of a metabolic inhibition technique based upon phosphatase production.

MATERIALS AND METHODS

The medium used was basically that described by Taylor-Robinson, Williams & Haig (1968), consisting of seven parts of Difco PPLO broth, two parts of unheated horse serum, one part of 25% yeast extract, thallium acetate 1/2000 and 1000 units penicillin G/ml. To this basic medium was added 0·01% phenolphthalein diphosphate.

Antisera production

Antiserum to the mycoplasma strains was produced as described by Morton & Roberts (1967). A suspension of the organism in 0·2 ml of phosphate buffered saline and complete Freund's adjuvant was injected into each hind-foot pad of a rabbit, and 0·5 ml intramuscularly into four sites on the shoulders and thighs. Three weeks later, four injections of 0·5 ml of the mycoplasma suspension alone were injected into four sites on the shoulders and thighs. The serum was collected after another 7 days.

Table 1. *Origin of mycoplasma strains*

Strain	Animal sp.	Site of isolation
CH	Chinese hamster	Conjunctiva
PUMA	Puma	Throat
LL2	Lion	Throat
LA	Lion	Throat
Species		
M. fermentans (PG28)	Man	—
M. felis (CO)	Cat	—

Mycoplasmas

The strains used in this study are shown in Table 1. Unidentified strains were isolated at the Laboratory Animals Centre. The named mycoplasma species were received from Dr M. Barile (Bethesda). Mycoplasmas were grown in broth medium for 2–5 days at 35 °C and then stored in 1 ml volumes at −70 °C.

Metabolic inhibition test

Frozen suspensions of organisms were thawed and diluted in tenfold steps. The tests were performed in microtitre U-shaped plates as described previously (Taylor-Robinson *et al.* 1966). A volume of 0·025 ml of broth medium was added to each cup. A similar quantity of serum was added to the first cup and twofold serial dilutions were then made. After this, 0·125 ml of medium and 0·05 ml of broth containing organisms was added to each cup, making a total of 0·2 ml. This test was repeated for each tenfold dilution of organisms. Sets of 12 control cups were set up, each set containing 0·15 ml of broth medium and 0·05 ml of one of the mycoplasma dilutions in each cup. The plates were sealed with cellophane tape and incubated at 35 °C under aerobic conditions. Twice a day, one drop of N sodium hydroxide was added to one control cup of each organism dilution. As soon as this addition caused the control to turn pink (showing that the organism was producing phosphatase) one drop of N sodium hydroxide was added to all test cups. The serum titre was recorded as the highest serum dilution which prevented a colour change with sodium hydroxide.

The test was compared with the glucose fermentation and arginine hydrolysis inhibition tests by carrying it out on mycoplasma species which are glucose-fermenting or arginine-hydrolysing as well as phosphatase-producing. Strains LA, CH and PUMA were also examined in the phosphatase test against antisera to all strains listed in Table 2.

RESULTS

The results are shown in Table 2. The serum titres in the phosphatase production test correlated closely with both the glucose fermentation and arginine hydrolysis tests. Strain LL2 does not ferment glucose or hydrolyse arginine. No strains cross-reacted with antisera to the other strains tested above a titre of 10.

Table 2. *Metabolic inhibiting antibody titre with homologous antigen*

Mycoplasma strain	Test	Culture dilution 10^1	10^2	10^3	10^4	10^5
PUMA	Arginine	128	256	512	512	
	Phosphatase	128	256	512	2048	
CH	Glucose	64	128	512	1024	4096
	Phosphatase	1024	1024	1024	4096	4096
LA	Glucose	128	128	255		
	Phosphatase	256	512	512		
M. felis	Glucose	128	256	512	1024	
	Phosphatase	256	1024	2048	2048	
M. fermentans	Glucose	1024	2048	4096	4096	
	Phosphatase	512	1024	4096	4096	
* LL2	Phosphatase	128	256	512	512	

* Non-glucose-fermenting or arginine-hydrolysing strain.

DISCUSSION

The MIT based upon phosphatase production is simple to perform and shows a clear colour change. The value of this test is that it provides a MIT for those strains of mycoplasmas, such as some of the feline isolates, which do not react in the published MI techniques.

The organism suspension should be diluted optimally to prevent initial overgrowth of the organism and the test read as soon as a pink colour is obtained, as the serum titre is lower if the plate is incubated for a longer period, though this drop is not as much as with the acid production test. The optimal dilution of organisms was based on the highest dilution which gave a clear colour change after at least 3 days incubation, and usually contained 10–100 viable organisms.

These considerations also apply to the established MI techniques.

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