J. Hyg., Camb. (1966), **64**, 91
With 1 plate
Printed in Great Britain

A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production

By D. TAYLOR-ROBINSON,* R. H. PURCELL,†
D. C. WONG and R. M. CHANOCK

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

(*Received* 22 *October* 1965)

A property of mycoplasmas (PPLO) is the inhibition of their growth by specific antibody. Edward & Fitzgerald (1954) first demonstrated growth inhibition with antiserum incorporated into the agar medium and Nicol & Edward (1953) used the technique to study mycoplasma strains isolated from the urogenital tract. In order to type mycoplasma strains this technique was modified by several workers. Huijsmans-Evers & Ruys (1956) placed filter-paper disks soaked in specific antisera on the agar surface. Herderscheê, Ruys & van Rhijn (1963) used drops of antisera on the agar and Clyde (1964) evaluated and standardized the filter-paper-disk method. However, these techniques are relatively insensitive for the quantitative measurement of antibody, especially in human sera. A technique which involved sampling and titration of antiserum–organism suspensions was used successfully by Bailey *et al.* (1961) but it is laborious. A similar procedure was used by Jensen (1963) for measuring growth-inhibiting antibody to *Mycoplasma pneumoniae*. A simpler quantitative colour method for *M. pneumoniae* has also been described by Jensen (1964). This is based upon the inhibition of reduction of tetrazolium. Unfortunately, this technique does not have wide application since *M. pneumoniae* is unique among the mycoplasma species that infect man in its ability to reduce readily tetrazolium salts. However, the growth of *M. pneumoniae* and certain other mycoplasma species is accompanied by the production of acid, and the incorporation of phenol red into the medium permits the resultant pH change to be seen. The addition of specific antiserum to the medium inhibits mycoplasma growth and this inhibition can be visualized by failure of the indicator to change colour. This communication describes the development of this technique for the quantitative measurement of growth-inhibiting antibody to the acid-producing mycoplasmas and indicates its practical significance.

* Present address: Common Cold Research Unit, Harvard Hospital, Coombe Road, Salisbury, Wiltshire, England.

† Epidemic Intelligence Service Officer, Epidemic Intelligence Service, Communicable Disease Center, Atlanta, Georgia.

MATERIALS AND METHODS

Mycoplasmas

The following mycoplasma strains were chosen for this study. The FH strain of *M. pneumoniae* was obtained from Liu (1957) and was subcultured over 200 times in this laboratory on agar or in broth medium. The 1428 strain of *M. pneumoniae* was recovered from a marine recruit with atypical pneumonia (Couch, Cate & Chanock, 1964) and was subcultured three times on agar medium. *M. fermentans* (strain G) was obtained from D. G. ff. Edward. The Negroni agent was isolated during the course of attempts to isolate viruses from human leukaemic bone marrow and was originally considered to be a virus (Negroni, 1964). Further studies demonstrated that it was a mycoplasma (Girardi, Hayflick, Lewis & Somerson, 1965). The source of other prototype mycoplasma strains used for the production of antisera has been described previously (Taylor-Robinson, Somerson, Turner & Chanock, 1963).

Media

The medium used for the maintenance of organisms has been described previously (Taylor-Robinson *et al.* 1963). It consisted of seven parts of Difco PPLO agar or broth, two parts of unheated horse serum, one part of 25% yeast extract, 1/2000 thallium acetate, and 1000 units of penicillin G/ml. Henceforth this medium will be termed 'standard medium'. The medium used for the growth inhibition tests and for the growth of organisms to be used in the inhibition tests was the standard medium with 1% glucose and 0·002% phenol red (final concentrations) and the final pH was adjusted to 7·8 with hydrochloric acid. Henceforth this medium will be termed 'standard medium with additives'. Organisms for rabbit immunization were grown in a medium of rabbit infusion broth supplemented with 5% rabbit serum. The preparation of this infusion has been described previously (Taylor-Robinson *et al.* 1963).

Growth of organisms for use in inhibition tests

One hundred ml. amounts of standard broth medium with additives were inoculated with suspensions of mycoplasmas in their early phase of growth and incubated at 34° C. until the pH of the medium decreased by about half a pH unit (i.e. pH 7·8 to about 7·3). The broth cultures were then divided into 1·0 ml. amounts and stored at −70° C. The materials were titrated by making serial tenfold dilutions in the standard medium with additives. These dilutions were incubated at 34° C. in screw-capped vials or microtitre plates. The titre was considered to be the highest dilution which produced a colour change when the test was read at a time when colour changes were no longer progressive.

Rabbit antisera and human sera

Rabbit antisera were prepared as described in detail previously (Taylor-Robinson *et al.* 1963). Briefly, all organisms except *M. pneumoniae* were grown in rabbit infusion broth supplemented with rabbit serum and 20-fold concentrates

were used to inoculate rabbits; *M. pneumoniae* was grown in chick embryo lung and the homogenized suspension of lung used to inoculate rabbits. Paired human sera were obtained from adult male volunteers, before and 3 weeks after they had been inoculated via the oropharynx with the FH strain of *M. pneumoniae*. Sera were obtained also from adult patients, mostly 30–59 years of age and a few older, with non-respiratory illnesses at D.C. General Hospital, Washington, D.C.

Complement-fixation (CF), indirect-haemagglutination (IHA) and tetrazolium reduction inhibition (TRI) techniques

The method used for CF has been reported before (Taylor-Robinson *et al.* 1963). Eight units of antigen were used and the tests were performed by overnight fixation at 4° C. in micro CF plates. The method used for IHA was that described previously (Taylor-Robinson, Canchola, Fox & Chanock, 1964) but modified by the use of sensitized sheep erythrocytes stored at −70° C. (Taylor-Robinson, Ludwig, Purcell, Mufson & Chanock, 1965). The TRI test for measurement of antibody to *M. pneumoniae* was essentially that described by Jensen (1964). Briefly 0·025 ml. amounts of serum diluted in standard medium supplemented with 0·05% triphenyl-tetrazolium chloride were mixed with 0·025 ml. of various dilutions of mycoplasma organisms in the same medium. To this was added a final 0·15 ml. of the same medium. With growth of the organism, colourless tetrazolium is reduced to a red formozan; specific antibody inhibits growth and the resultant colour change.

Growth inhibition or fermentation inhibition (FI) test for acid-producing mycoplasmas

Tests were performed in disposable plastic microtitre plates with U-shaped cups manufactured by Cooke Engineering Company, Alexandria, Virginia. These plates were not treated with ultraviolet radiation before use and no special precautions were taken to prevent bacterial contamination during the performance of the test. Sera were diluted 1/2·5 or 1/5 in standard medium with additives and used after heating at 56° C. for 30 min. In some instances the serum specimens were tested without prior heating. After the addition of 0·025 ml. (1 drop) of standard medium with additives to each cup, serial twofold dilutions of serum were made with spiral wire loops. Mycoplasma suspensions diluted as required in the same medium were then added in 0·05 ml. amounts to each cup and the total volume made up to 0·2 ml. by the addition of 0·125 ml. (5 drops) of standard medium with additives. When guinea-pig serum was used, it was added in the final 0·125 ml. of medium. Controls consisted of cups which contained a mixture of 0·05 ml. of mycoplasma organisms and 0·15 ml. of standard medium with additives. Plates were sealed with 'Scotch Brand' cellophane tape and were not agitated. Incubation was at 34° C. under aerobic conditions unless stated otherwise. Colour changes were observed by placing the plates over a mirror with a fluorescent white light above the plates. An arbitrary system of classifying the colour changes was as follows: no change −(pH 7·8); + (pH *ca.* 7·3); ++ (pH *ca.* 6·8); +++ (pH *ca.* 6·2 or less). The highest serum dilution which prevented a colour change of greater than

50 % when compared with the same dilution of organisms grown in the absence of antiserum was considered the end-point of the serum antibody titration. In some instances, slight colour changes were observed in cups which contained only standard medium. This colour change could be abolished without interfering with changes produced by growth of the mycoplasmas by removing the cellophane tape from the plate and reincubating at 34° C. for a few hours.

Definition of terms used

One colony-forming unit (cfu) is contained in the highest dilution of a mycoplasma suspension that will produce one colony on a PPLO-agar plate.

One colour-changing unit (ccu) is contained in the highest dilution of a mycoplasma suspension that will produce a change of half a pH unit in standard broth medium with additives at a specified time.

Table 1. *Microtitre plate colour change titration of* Mycoplasma pneumoniae

M. pneumoniae suspension		Approx. pH of medium in cup on indicated day of incubation						
Dilution	No. of ccu* at indicated dilution	3	4	5	6	7	8	10
10^{-2}	10^4	7·3	6·8	6·2	6·2	6·2	6·2	6·2
10^{-3}	10^3	7·8	7·8	7·0	6·2	6·2	6·2	6·2
10^{-4}	10^2	7·8	7·8	7·8	7·3	6·5	6·2	6·2
10^{-5}	10	7·8	7·8	7·8	7·8	7·5	6·5	6·2
10^{-6}	1	7·8	7·8	7·8	7·8	7·8	7·8	6·5
10^{-7}	0	7·8	7·8	7·8	7·8	7·8	7·8	7·8
Highest dilution at which pH change of half a unit (1 ccu) occurred on indicated day		10^{-2}	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-6}

* Colour changing units/0·05 ml. of original suspension calculated at day 10.

RESULTS

Microtitre plate titration of mycoplasmas

Tenfold dilutions of *M. pneumoniae* in standard medium with additives were dropped in 0·05 ml. amounts into cups of a microtitre plate and 0·15 ml. of the same medium was added to each cup. The plate was incubated at 34° C. aerobically and the results recorded at various times of incubation are shown in Table 1. On the third day of incubation one ccu was contained in a 10^{-2} dilution, i.e. a 10^{-2} dilution of the *M. pneumoniae* suspension contained in 0·05 ml. produced a colour change of half a pH unit (7·8–7·3). On the tenth day one ccu was contained in the 10^{-6} dilution, i.e. a 10^{-6} dilution of the *M. pneumoniae* suspension was the highest dilution which produced a colour change. Titration of the same *M. pneumoniae* suspension on PPLO-agar plates showed that it contained 3×10^6 cfu/0·1 ml. so that the titre of the suspension in cfu and ccu at 10 days was approximately the same.

Tests with homologous rabbit antisera

M. pneumoniae (FH strain), *M. fermentans* and the Negroni agent were tested with their respective pre-inoculation and post-inoculation rabbit sera which were used without heat inactivation. 'Block' titrations were performed by diluting the organisms in serial tenfold steps and the sera in serial twofold steps. Table 2 shows the maximum fermentation inhibition (FI) serum titres obtained during

Table 2. *Titre of pre-inoculation and post-inoculation rabbit sera in FI tests with homologous mycoplasma organisms**

Mycoplasma used in test	Titre obtained with: Unheated pre-inoculation rabbit serum	Unheated post-inoculation rabbit serum
M. pneumoniae	10	5120 or >
M. fermentans	< 10	5120
Negroni agent	< 10	< 320

* Tests performed with standard medium containing 20% unheated horse serum.

Table 3. *Association between colour change and mycoplasma growth*

Mycoplasma tested	Rabbit serum and dilution (reciprocal) tested	Fluid in cup of microtitre plate: Approx. pH	Mycoplasma cfu/ml.
M. pneumoniae	No serum	6·8*	$3{\cdot}3 \times 10^8$
	Pre-inoculation 10	6·5	$1{\cdot}6 \times 10^8$
	Post-inoculation 10240	7·0	$1{\cdot}8 \times 10^8$
	Post-inoculation 5120	7·3	$6{\cdot}0 \times 10^7$
	Post-inoculation 2560†	7·5	$1{\cdot}4 \times 10^7$
	Post-inoculation 640	7·8	$1{\cdot}4 \times 10^4$
M. fermentans	No serum	7·3‡	$6{\cdot}0 \times 10^6$
	Pre-inoculation 10	7·3	$1{\cdot}0 \times 10^6$
	Post-inoculation 2560	7·5	$1{\cdot}7 \times 10^6$
	Post-inoculation 1280†	7·7	$8{\cdot}0 \times 10^4$
	Post-inoculation 320	7·8	$< 1{\cdot}0 \times 10^2$
Negroni agent	No serum	7·3‡	$9{\cdot}0 \times 10^7$
	Pre-inoculation 10	7·3	$5{\cdot}0 \times 10^7$
	Post-inoculation 20480	7·5	$3{\cdot}0 \times 10^7$
	Post-inoculation 10240†	7·7	$3{\cdot}0 \times 10^6$
	Post-inoculation 2560	7·8	$2{\cdot}0 \times 10^4$

* Fluids in the cups of this test titrated after 4 days incubation at 34° C.
† This dilution represented the serum end-point at the time the test was read.
‡ Fluids in the cups of this test titrated after 3 days incubation at 34° C.

the course of readings taken over several days. Unheated rabbit antisera to *M. pneumoniae* and *M. fermentans* inhibited to high titre the growth of their homologous organisms as indicated by the inhibition of colour change. On the other hand, rabbit antiserum to the Negroni agent at the lowest dilution used did not inhibit

the colour change produced by growth of the organism. As will be shown later, it was necessary to add unheated guinea-pig serum to demonstrate the inhibitory activity of Negroni antiserum. All these tests were performed under aerobic conditions. In an atmosphere of 100 % nitrogen, the growth of *M. pneumoniae* was suppressed, but that of *M. fermentans* was not affected. Under anaerobic conditions the titre of the *M. fermentans* antiserum did not increase. In all further experiments aerobic conditions were employed.

Association of colour change with mycoplasma growth

Microtitre plates which contained *M. pneumoniae*, *M. fermentans* and the Negroni agent were incubated at 34° C. At various time-intervals the cups were sampled, the fluids diluted in tenfold steps and inoculated on to PPLO-agar plates so that the number of cfu within each cup could be determined. Cups which contained rabbit antiserum and mycoplasma organisms were sampled in the same manner. As shown in Table 3 there was a correlation between growth of the organisms and the colour changes produced. On an arbitrary basis we had previously chosen the end-point for the serum titre as the dilution of serum which prevented a colour change of greater than 50 % when compared with the same dilution of organisms grown in the absence of antiserum. It may be seen that at the serum end-point there was at least a tenfold suppression of growth of the three mycoplasmas tested. In addition, it is clear that the organisms must grow within the cups to at least 10^6 cfu/ml. in order to produce a colour change of half a pH unit.

Effect of number of organisms used in the test on the titre of rabbit antiserum

The FH strain of *M. pneumoniae* was tested with pre-inoculation and post-inoculation rabbit sera which had not been previously heat-inactivated. A 'block' titration was performed by diluting the organism suspension in serial tenfold steps and the sera in serial twofold steps. Plate 1 shows the test on the tenth day of incubation at 34° C. when progressive colour changes had ceased to occur. The organism grew to a titre of about 10^6 ccu, so that cups which contained the 10^{-5} dilution of the organism suspension were inoculated with about 10 ccu. At this dilution the pre-inoculation rabbit serum had a reciprocal titre of 10 and the post-inoculation serum a titre of 5120 or greater. With the 10^{-2} dilution (inoculum of 10^4 ccu) the pre-inoculation serum had a reciprocal titre of less than 10 and the post-inoculation serum a titre of 640. On the fourth day, however, when the 10^{-2} dilution had just produced a pH change of half a unit the titre of the post-inoculation serum with this dilution of organisms was 5120 or greater.

'Block' titrations of organisms and antisera of the type shown in Plate 1 were undertaken on several occasions with both *M. pneumoniae* and *M. fermentans*. The results of these kinetic studies are shown in Table 4. The serum titre was relatively unaffected by the size of the inoculum in the test if serum titres were recorded at a time when the pH of the medium in the control titration of the inoculum had just changed half a pH unit. Therefore, when a large number of sera were to be tested, multiple dilutions of organisms were not used; the test was performed with a

mycoplasma dilution which contained 10^3–10^4 ccu and was read when the pH of the medium containing this dilution had just changed half a pH unit.

Effect of heating sera

In the experiment shown in Plate 1, the pre-inoculation rabbit serum at a dilution of 1/10 inhibited the colour change produced by *M. pneumoniae*. Heating the serum at 56° C. for 30 min. abolished this slight inhibitory effect and did not significantly alter the titre of post-inoculation sera, as shown in Table 5. Therefore, in other tests heat-inactivated sera were used.

Table 4. *Effect of size of mycoplasma inoculum used in the test on the growth-inhibition titre of specific rabbit antisera*

Size of inoculum (no. of ccu/ 0·05 ml.)	Growth-inhibition titres* (Rabbit antiserum and homologous organism)								
	M. pneumoniae					*M. fermentans*†			
	Expt. 1	2	3	4	5‡	Expt. 1	2	3	4
$10^{5\cdot5}$	NT§	1280	NT	NT	NT	2560	5120 or >	2560	NT
$10^{4\cdot5}$	5120	2560	5120	2560	2560	2560	5120	2560	640
$10^{3\cdot5}$	2560	2560	NT	5120	5120	1280	2560	640	NT
$10^{2\cdot5}$	5120	2560	5120	2560	NT	1280	2560	NT	1280
$10^{1\cdot5}$	2560	NT	NT	NT	5120 or >	2560	NT	NT	NT

* Titres determined at a time when the medium containing the indicated inoculum had just changed half a pH unit.

† Tests with *M. fermentans* performed with the addition of 6% unheated guinea-pig serum.

‡ Expt. 5 performed with the 1428 strain of *M. pneumoniae*. All other expts. with the FH strain.

§ Not tested.

Table 5. *Titre of unheated and heat-inactivated rabbit antisera in FI tests with homologous mycoplasma organisms*

Treatment of antiserum	Mycoplasma used in test and serum titre obtained			
	M. pneumoniae		*M. fermentans*	
	Test 1	Test 2	Test 1	Test 2
Unheated	5120	5120	5120	2560
Heated at 56° C. for 30 min.	5120	5120	2560	2560

Effect of heating the horse-serum component of the medium

When the horse-serum component of the medium was heated at 56° C. for 1 hr., the ability of *M. pneumoniae* antiserum to inhibit the growth of its homologous organism was lost. This finding indicated that a heat-labile factor in horse serum was required as a co-factor for inhibition of growth of *M. pneumoniae* by specific

antibody. The use of medium heated at 34° C. for 3 days or stored at 4° C. for 2 weeks, did not affect the growth-inhibition titre of *M. pneumoniae* rabbit antiserum.

Effect of unheated and heated guinea-pig serum (GPS)

The result of the experiment described above suggested that the labile factor necessary to demonstrate the growth inhibition of specific antiserum might be complement or a complement-like substance. The possibility that the addition of GPS to the reaction mixture might enhance the titre of rabbit antiserum was investigated. Ten per cent GPS, either unheated or heated at 56° C. for 30 min., in the standard medium with additives was added in the final 0·125 ml. so that the concentration of GPS in each reservoir was approximately 6%. As shown in Table 6, the growth of the FH strain of *M. pneumoniae* was decreased 10,000-fold or more by unheated GPS while heated GPS decreased growth 100-fold. Similar results were obtained with the 1428 strain of *M. pneumoniae*. This inhibition of growth was a reproducible phenomenon. However, the FI titre of the rabbit antiserum was unaffected by the addition of either heated or unheated GPS.

Table 6. *Effect of guinea-pig serum (GPS) on mycoplasma growth and on the titre of homologous antiserum in FI tests*

Expt. no.	Mycoplasma tested	Growth suppression* (ccu) by Unheated GPS	Heated GPS	FI titre of homologous rabbit antiserum after addition to the test† of Medium alone	Unheated GPS	Heated GPS
1	*M. pneumoniae* strain FH	$> 10^4$	10^2	5120	2560	5120
2	*M. fermentans*	$10^{0.5}$	NT‡	5120 or >	2560	NT
3	Negroni agent	$> 10^2$	NT	< 320	2560	< 320
4	Negroni agent	10^5	$10^{0.5}$	320	5120	NT

* Suppression of growth in the presence of 6% final concentration of GPS compared with growth in the absence of GPS.

† 0·025 ml. antiserum + 0·05 ml. mycoplasma + 0·125 ml. standard medium, either alone or containing 10% GPS; therefore, final concentration of GPS about 6%.

‡ Not tested.

Unheated GPS either delayed the colour change produced by *M. fermentans* at any particular dilution of the organism or, at the most, decreased the growth titre by less than tenfold. The FI titre of the *M. fermentans* rabbit antiserum was not increased by the addition of unheated guinea-pig serum to the test. However, the inhibitory effect of antiserum on fermentation produced by a high concentration of organisms was maintained for a longer interval when GPS was added to the reaction mixture than when it was omitted from the medium. In further tests with *M. fermentans* unheated GPS was, therefore, added to the medium. Unheated and heated GPS had an effect on the growth of the Negroni agent similar to that observed with *M. pneumoniae*. However, the FI titre of *M. pneumoniae* rabbit antiserum was not enhanced by the addition of unheated GPS to the test whereas the inhibitory titre of Negroni rabbit antiserum was markedly increased (Table 6).

Heated GPS did not produce a similar effect. Therefore, in further tests with the Negroni agent unheated GPS was used.

In some tests with the mycoplasma organisms and their homologous rabbit antisera it was observed that high dilutions of antiserum inhibited colour change whereas the lowest dilutions, i.e. those containing the highest concentrations of antibody, did not inhibit the colour change. With *M. fermentans* the addition of unheated GPS to the medium suppressed this colour change in cups which contained a high concentration of specific antiserum.

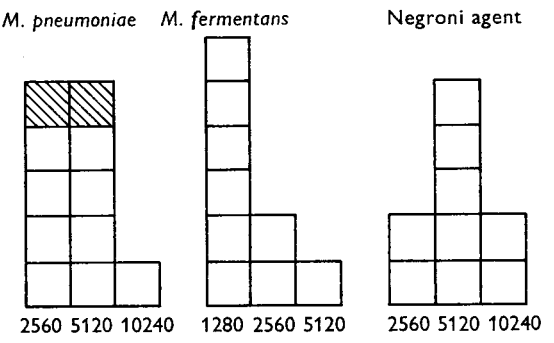


Fig. 1. Rabbit antiserum titres obtained in FI tests with homologous mycoplasma organisms, *M. pneumoniae*, *M. fermentans* and the Negroni agent. Each block represents the serum titre obtained in one test. ▨, 1428 strain; □, FH strain.

Table 7. *Results of cross FI tests*

Rabbit antiserum prepared to	Titre of serum in test with *M. pneumoniae* (FH strain)	*M. fermentans**	Negroni agent*
M. pneumoniae	2560	< 10	< 10
M. fermentans	< 10	1280	< 10
Negroni agent	< 10	< 10	5120
M. hominis type 1	< 10	< 10	< 10
type 2	< 10	< 10	< 10
M. salivarium	< 10	< 10	< 10
M. orale	< 10	< 10	< 10
Oral strain CH 20247	< 10	< 10	< 10
Navel	< 10	< 10	< 10

* Tests with this mycoplasma performed with the addition of 6 % unheated guinea-pig serum.

Reproducibility

Figure 1 shows the results of a number of tests with *M. pneumoniae*, *M. fermentans* and the Negroni agent and their respective rabbit antisera which were performed at different times and by different persons. The same mycoplasma pool and the same hyperimmune serum was used for each test. The sera were initially diluted 1/2·5 or 1/5 and then diluted further in twofold steps by means of wire

loops. Only a two- to fourfold variation in serum FI titre was observed during the course of eleven titrations of *M. pneumoniae* antiserum, nine of *M. fermentans* and nine of Negroni agent antiserum.

Specificity

As shown in Table 7, rabbit antisera to *M. pneumoniae*, *M. fermentans* and the Negroni agent exhibited high homologous titres of growth-inhibiting antibody as measured by inhibition of colour change. On the other hand, these antisera did not inhibit the growth of the heterologous mycoplasma strains. In addition, rabbit antisera to *M. hominis* type 1, *M. hominis* type 2, *M. salivarium*, *M. orale*, the Navel strain of mycoplasma and a newly identified species from the human oropharynx, strain CH 20247 (Taylor-Robinson, Fox & Chanock, 1965), each at a dilution of 1/10, did not inhibit the growth of the three mycoplasma species studied. Although the homologous growth-inhibition titre of each of the last six antisera was unknown, they all possessed high levels of complement-fixing and indirect-haemagglutination antibodies and they all produced zones of inhibition in the disk growth-inhibition test (Taylor-Robinson *et al.* 1964; Taylor-Robinson, Fox & Chanock, 1965).

Table 8. *Antibody titres in paired sera from volunteers inoculated via the oropharynx with* Mycoplasma pneumoniae

	Reciprocal titres of pre- and post-inoculation sera with the indicated organism tested by the indicated technique									
	M. pneumoniae (FH strain)						*M. fermentans*, Negroni agent,			
	IHA		TRI		FI		FI		FI	
Volunteer										
To.	20*	1280†	< 5*	160†	< 5*	160†	< 5*	< 5†	< 5*	< 5†
Be.	20	1280	< 5	320	< 5	320	< 5	< 5	< 5	< 5
Bu.	< 10	40	< 5	160	< 5	160	< 5	< 5	< 5	< 5
Ha.	< 10	640	< 5	80	< 5	160	< 5	< 5	< 5	< 5
McM.	< 10	1280	< 5	640	< 5	320	< 5	< 5	< 5	< 5
Wi.	20	320	< 5	320	< 5	320	5	10	< 5	< 5
Fi.	40	160	< 5	320	< 5	640	< 5	< 5	< 5	< 5
Fe.	10	320	< 5	320	< 5	320	< 5	< 5	< 5	< 5
Va.	< 10	640	< 5	320	< 5	160	< 5	< 5	< 5	< 5
Jo.	320	640	< 5	160	< 5	160	< 5	< 5	< 5	< 5
Bo.	NT	NT	< 5	80	< 5	80	5	20	< 5	< 5
Sm.	NT	NT	< 5	2560	< 5	2560	< 5	< 5	< 5	< 5

* Pre-inoculation serum. † Post-inoculation serum.

Measurement of antibody in human sera

Adult volunteer sera tested against Mycoplasma pneumoniae. Sera were obtained from adult volunteers before and about 3 weeks after inoculation via the nasopharyngeal route with *M. pneumoniae*. They were tested by FI, TRI and IHA with the homologous organism and by FI with *M. fermentans* and the Negroni agent. The results are recorded in Table 8. All the tests with *M. pneumoniae* demonstrated fourfold or greater antibody rises, except IHA in which one of the paired

sera showed only a twofold rise. The results obtained in the FI and TRI tests were in very close agreement, indicating that the two tests were about equal in sensitivity. The occurrence of only one fourfold FI antibody rise to *M. fermentans* and the failure to detect a response to the Negroni agent further demonstrated the specificity of the technique.

Sera from healthy adults tested against Mycoplasma pneumoniae *and* M. fermentans. Sera from ninety-five adults, mostly 30–59 years of age and a few older, were tested. The usefulness of the technique as a means of detecting and measuring antibody in human sera is indicated by the results shown in Table 9. Twenty-two per cent of the sera contained FI antibody at a reciprocal titre of 5 or greater against *M. pneumoniae* and 13·5% contained antibody against *M. fermentans*. Because of the specificity of the test, these data suggest that such persons had been infected with these or very closely related mycoplasma species.

Table 9. *Results of FI tests with* Mycoplasma pneumoniae *and* M. fermentans *and ninety-five sera from adults without respiratory disease*

Organism used in test	No. of sera with reciprocal titre of:									% sera positive, titre 5 or >
	< 5	5	10	20	40	80	160	320	640 or >	
M. pneumoniae (1428 strain)	74	5	6	2	2	3	3	0	0	22
M. fermentans	82	4	2	4	2	0	0	0	1	13·5

DISCUSSION

M. pneumoniae and *M. fermentans* have been shown to ferment glucose probably to lactic acid and it is presumed that the colour change in the test described in this communication is the result of glucose fermentation. Because of this we have referred to the test as one of fermentation inhibition (FI). The biochemical basis for the production of acid by the Negroni agent has not been examined, so that for this and other acid-producing mycoplasmas the description of the test as one of fermentation inhibition may be premature. However, regardless of the mechanism of the acid production, the decrease in pH and consequent colour change of the indicator associated with growth of the organisms is inhibited by the addition of antiserum.

Several factors concerned in the performance of the test have been evaluated. Heat inactivation of sera is a worth-while procedure since it did not decrease the titre of antisera, and with *M. pneumoniae* it eliminated presumably non-specific inhibition produced by the pre-inoculation rabbit serum. The importance of using unheated horse serum in the growth medium cannot be overstressed since the specific inhibitory effect of antisera was markedly reduced when the organisms were grown in medium containing heat-inactivated horse serum. The inhibitory activity of rabbit antiserum was partially restored by the addition of 6% unheated GPS to the test. This indicates that a heat-labile accessory factor present in horse and guinea-pig serum is important for the demonstration of specific antibody activity; whether or not this is a heat-labile component of complement is

unknown. This observation is in agreement with that of Priestley (1952) who worked with *M. mycoides*, but contrary to that of Edward & Fitzgerald (1954) who studied human genital strains. It is possible that the requirement for a heat-labile accessory factor in the inhibition of growth of various mycoplasmas by specific antiserum varies with the mycoplasma studied. The effect of GPS on the growth of mycoplasmas and on the growth-inhibitory activity of their antisera is indicated by a consideration of the three mycoplasmas studied. The growth of *M. pneumoniae* was inhibited by both unheated and heated GPS. The addition of unheated GPS to the test did not enhance the growth-inhibitory activity of specific rabbit antiserum provided that unheated horse serum was present in the medium. The growth of *M. fermentans* was little affected by unheated GPS and the growth-inhibition titre of specific antiserum was not increased. Finally, with the Negroni agent, unheated GPS not only inhibited growth of the organism but was essential for demonstrating the effect of specific antiserum. Thus, the need for unheated horse serum in tests with *M. pneumoniae* and *M. fermentans* and the additional need for unheated GPS in tests with the Negroni agent indicate the varying requirements for a heat-labile accessory factor. The different requirements could reflect a qualitative difference in the factor present in horse or guinea-pig serum or a quantitative difference in requirement for the same factor in horse and guinea-pig serum. These problems will be solved only when the nature of the accessory factor has been determined.

It was demonstrated with *M. fermentans* that the addition of unheated GPS inhibited the 'break-through' of growth in cups which contained a high concentration of specific antiserum. Growth in the presence of a high concentration of antibody may be due to the growth-stimulating effect of serum. Partial and reversible growth inhibition of mycoplasmas in the presence of excess antibody has been observed by Bailey, Clark, Felts & Brown (1963). Regardless of the mechanism of this 'zoning' phenomenon, the fact that it occurs is a pitfall in the 'screening' of sera at low dilution. Growth-inhibiting antibody could be overlooked unless sera are diluted sufficiently and tested over a range of dilutions.

Rabbit antisera prepared against all the human mycoplasma species exhibit low levels of heterotypic cross-reactivity in CF and IHA tests (Taylor-Robinson *et al.* 1963; Taylor-Robinson *et al.* 1964; Taylor-Robinson, Fox & Chanock, 1965). The same sera, however, did not produce heterotypic growth inhibition of the organisms used in the present study; the test we describe therefore appears to be extremely specific. The growth-inhibition technique employing filter-paper disks soaked in antiserum (Huijsmans-Evers *et al.* 1956; Clyde, 1964) is also specific but not applicable to the quantitative measurement of antibody because of its low sensitivity. Although the FI technique has a less generalized application, since it can only be used for acid-producing mycoplasmas, it appears to be at least as sensitive as other serological techniques. Thus, the titres obtained for rabbit sera in FI tests with homologous organisms were as high as the titres obtained previously with the same sera in CF and IHA tests. In addition, various levels of growth-inhibitory antibody to *M. pneumoniae* and *M. fermentans* have been found in random sera from human adults without respiratory illness. The antibody response of adult

volunteers to infection with *M. pneumoniae* measured by FI was not different from the response measured by TRI, indicating that the two techniques have about equal sensitivity.

With the use of standard pools of organisms, the results obtained have been very reproducible. This was true even with the use of widely varying doses of organisms provided that serum titres were recorded at a time when the concentration of organisms used in the test had just produced a colour change of half a pH unit. The fact that a standard mycoplasma inoculum was not essential increased the simplicity of the test even more. This simplicity is in contrast to the laborious nature of the immunofluorescence technique and some other growth-inhibition methods (Bailey *et al.* 1961; Jensen, 1963).

A test which measures growth-inhibiting antibody to mycoplasmas has various applications. First, it should be useful in assessing the immunological status of individuals before experimental or natural infection. Measurement of growth-inhibiting antibody may be a better index of protective immunity than measurement of other antibodies, i.e. CF, IHA. Secondly, the test may be used as a tool in epidemiological studies. In this connexion, the occurrence of antibody to *M. fermentans* in 13·5 % of adult sera diluted 1/5 is of interest since reports of the isolation of this mycoplasma species from man are few (Huijsmans-Evers & Ruys, 1956; Ruiter & Wentholt, 1953). Recently, 'virus-like' particles associated with human leukaemic bone marrow (Murphy, Furtado & Plata, 1965) have been identified as *M. fermentans* in this laboratory. In addition to *M. fermentans* and the Negroni agent, other acid-producing mycoplasmas have been recovered recently in association with human leukaemia and human tumours (Mittelman, Horoszewicz & Grace, 1965). Finally, therefore, the test provides not only an opportunity to study the interrelationship of these acid-producing mycoplasmas but also an opportunity to assess their significance in relation to human disease.

SUMMARY

A fermentation-inhibition test for the measurement of growth-inhibiting antibody to acid-producing mycoplasmas was performed in microtitre plastic plates. *M. pneumoniae*, *M. fermentans* and the Negroni agent were selected for study. Antibody could be titrated since specific antiserum inhibited mycoplasma growth and the concomitant production of acid, thus preventing a change in colour of phenol red which was incorporated in the growth medium. It was essential to use unheated horse serum as a component of the growth medium. The inhibitory effect of specific antiserum was much decreased when the horse serum was heat-inactivated, indicating the need for a heat-labile accessory factor. The additional use of unheated guinea-pig serum was essential for demonstrating the growth-inhibiting effect of specific antiserum on the Negroni agent. The test was reproducible, specific and sensitive. Sixteen-fold or greater antibody rises were demonstrated in paired sera from volunteers infected with *M. pneumoniae*. Tests with ninety-five random adult sera showed that 22 % had antibody to *M. pneumoniae* at a titre of 1/5 or more and 13·5 % had antibody to *M. fermentans*.

We thank Dr M. A. Mufson for sera from persons of different ages, Mr F. T. Caporeal of the Photographic Services Section for the photography and Dr A. B. Sabin for helpful discussion during the course of the work.

REFERENCES

BAILEY, J. S., CLARK, H. W., FELTS, W. R. & BROWN, T. McP. (1963). Growth inhibitory properties of mycoplasma antibody. *J. Bact.* **86**, 147.

BAILEY, J. S., CLARK, H. W., FELTS, W. R., FOWLER, R. C. & BROWN, T. McP. (1961). Antigenic properties of pleuropneumonia-like organisms from tissue cell cultures and the human genital area. *J. Bact.* **82**, 542.

CLYDE, W. A., Jr. (1964). Mycoplasma species identification based upon growth inhibition by specific antisera. *J. Immun.* **92**, 958.

COUCH, R. B., CATE, T. R. & CHANOCK, R. M. (1964). Infection with artificially propagated Eaton agent (*Mycoplasma pneumoniae*). *J. Am. med. Ass.* **187**, 442.

EDWARD, D. G. FF. & FITZGERLAD, W. A. (1954). Inhibition of growth of pleuropneumonia-like organisms by antibody. *J. Path. Bact.* **68**, 23.

GIRARDI, A. J., HAYFLICK, L., LEWIS, A. M. & SOMERSON, N. L. (1965). Recovery of mycoplasmas in the study of human leukaemia and other malignancies. *Nature, Lond.*, **205**, 188.

HERDERSCHEÊ, D., RUYS, A. C. & VAN RHIJN, G. R., (1963). A new oral mycoplasma isolated from the tonsils of two patients suffering from scarlatina. *Antonie van Leeuwenhoek*, **29**, 157.

HUIJSMANS-EVERS, A. G. M. & RUYS, A. C. (1956). Microorganisms of the *pleuropneumonia* group (family of *mycoplasmataceae*) in man. *Antonie van Leeuwenhoek*, **22**, 377.

JENSEN, K. E. (1963). Measurement of growth-inhibiting antibody for *Mycoplasma pneumoniae*. *J. Bact.* **86**, 1349.

JENSEN, K. E. (1964). Antibodies to *Mycoplasma pneumoniae* measured by inhibition of tetrazolium reduction. *Bact. Proc.* p. 70.

LIU, C. (1957). Studies on primary atypical pneumonia. I. Localization, isolation, and cultivation of a virus in chick embryos. *J. exp. Med.* **106**, 455.

MITTELMAN, A., HOROSZEWICZ, J. & GRACE, J. T. (1965). The nucleic acids of PPLO (mycoplasma) 880. *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 596.

MURPHY, W. H., FURTADO, D. & PLATA, E. (1965). Possible association between leukemia in children and virus-like agents. *J. Am. med. Ass.* **191**, 110.

NEGRONI, G. (1964). Isolation of viruses from leukaemic patients. *Br. med. J.* i, 927.

NICOL, C. S. & EDWARD, D. G. FF. (1953). Role of organisms of the pleuropneumonia group in human genital infections. *Br. J. vener. Dis.* **29**, 141.

PRIESTLEY, F. W. (1952). Observations on immunity to contagious bovine pleuropneumonia, with special reference to the bactericidal action of blood. *Br. vet. J.* **108**, 153.

RUITER, M. & WENTHOLT, H. M. M. (1953). Isolation of a pleuropneumonia-like organism (G-strain) in a case of fusospirillary vulvovaginitis. *Acta derm.-vener., Stockh.*, **33**, 123.

TAYLOR-ROBINSON, D., CANCHOLA, J., FOX, H. & CHANOCK, R. M. (1964). A newly identified oral mycoplasma (*M. orale*) and its relationship to other human mycoplasmas. *Am. J. Hyg.* **80**, 135.

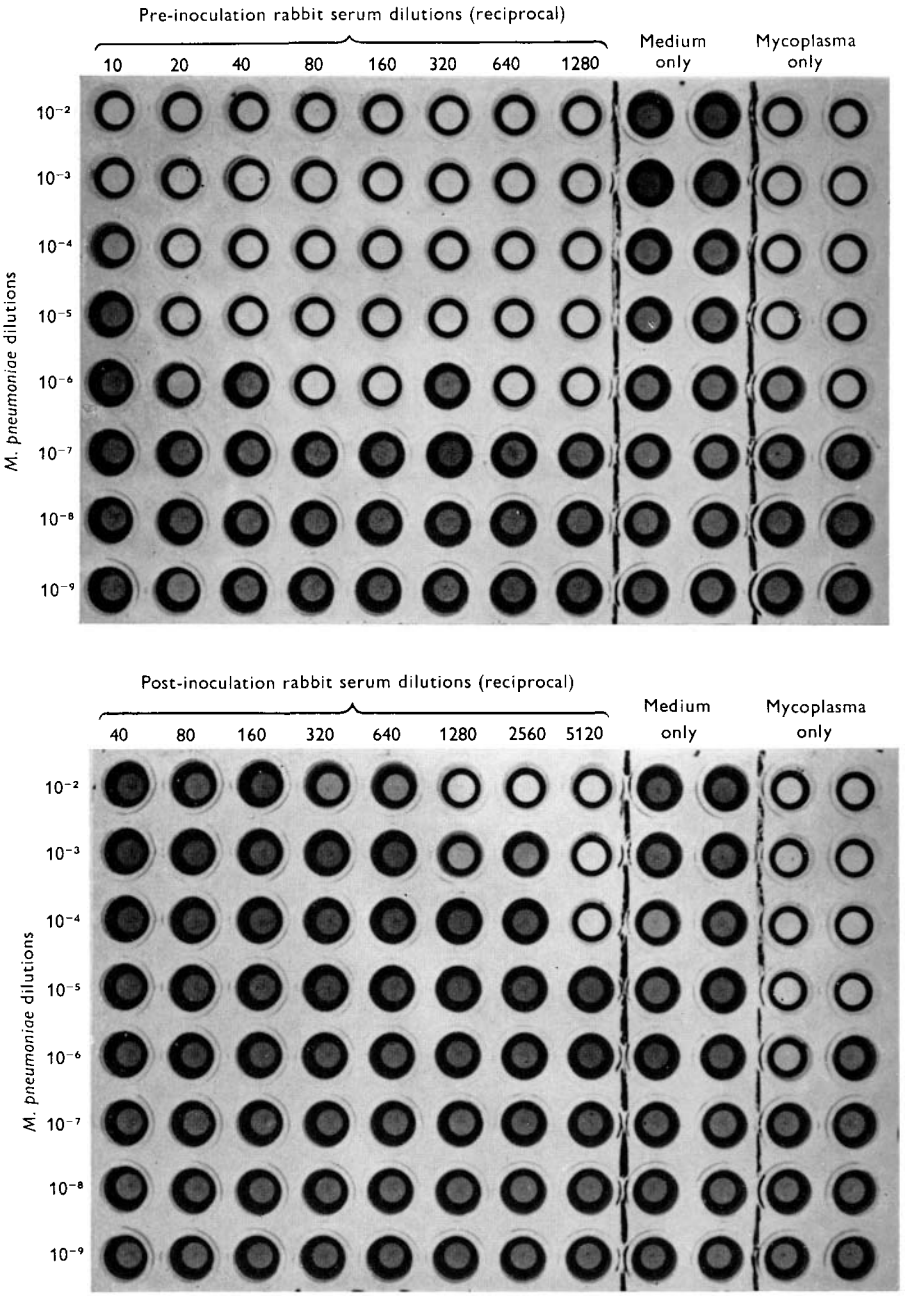
TAYLOR-ROBINSON, D., FOX, H. & CHANOCK, R. M. (1965). Characterization of a newly identified mycoplasma from the human oropharynx. *Am. J. Hyg.* **81**, 180.

TAYLOR-ROBINSON, D., LUDWIG, W. M., PURCELL, R. H., MUFSON, M. A. & CHANOCK, R. M. (1965). Significance of antibody to *Mycoplasma hominis* Type 1 as measured by indirect hemagglutination. *Proc. Soc. exp. Biol. Med.* **118**, 1073.

TAYLOR-ROBINSON, D., SOMERSON, N. L., TURNER, H. C. & CHANOCK, R. M. (1963). Serological relationships among human mycoplasmas as shown by complement-fixaction and gel diffusion. *J. Bact.* **85**, 1261.

EXPLANATION OF PLATE

'Block' titrations in disposable microtitre plates of *Mycoplasma pneumoniae* with pre-inoculation and post-inoculation rabbit sera. Cups which appear dark contain medium of pink colour (pH 7·8); cups which appear colourless contain medium of green-yellow colour (pH < 6·5). Photograph taken on the tenth day of incubation at 34° C. At 10^{-5} dilution of mycoplasma the titre of the post-inoculation serum is 5120 or greater.



https://doi.org/10.1017/S0022172400040377 Published online by Cambridge University Press