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Modifications of the Growth Inhibition Test and its Application to Human T-Mycoplasmas

F. T. BLACK

Institute of Medical Microbiology, University of Aarhus, DK-8000 Aarhus C, Denmark

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Several factors were found to influence the growth inhibition test. Inoculum size and amount of antiserum are well known variables, but the method of applying the antiserum, the incubation temperature, and the pH of the agar medium also play significant roles. The growth inhibition test modified according to these findings was found to be specific and well suited for the classification and identification of human T-mycoplasmas.

The specific inhibition of mycoplasmas by hyperimmune rabbit serum incorporated in solid medium was first reported in 1954 (9). Since then several modifications of the method have been described (4, 12, 18). Because of its specificity and simplicity, the growth inhibition (GI) test is now one of the most commonly used for the identification and classification of *Mycoplasma* and *Acholeplasma* species.

In contrast to this, T-mycoplasmas have been reported not to be inhibited by their homologous hyperimmune sera, tested with the paper disk technique (16). In 1970, a preliminary report on the application of the indirect hemagglutination, indirect immunofluorescence, and growth inhibition techniques to human T-mycoplasmas was presented by the author (3).

The purpose of this study was: (i) to describe in detail some variables which influence the GI test performed on solid medium; (ii) to compare the sensitivity and specificity of the modified test with the metabolic inhibition (MI) test and to establish serotypes of T-mycoplasmas; and (iii) to determine the suitability of the modified test for the identification of T-mycoplasmas.

MATERIALS AND METHODS

Organisms. Thirty-two T-mycoplasma strains were included in this study. Strain T-960, National Institutes of Health no. 720-002-084, was received from J. G. Tully; strains no. 7, 23, 27, 58, 354, Cook, and Pirillo were supplied by D. K. Ford. All other strains were derived from human source, isolated in this laboratory.

Culture media. T-mycoplasmas were propagated in a modified Shepard medium (S) consisting of 3% Trypticase soy broth (BBL), 20% horse serum, 10% yeast extract (baker's yeast), and 1,500 international units of sodium penicillin per ml. The pH was adjusted to 6.0 unless otherwise stated.

Solid medium was the S medium supplemented with 1.1% Ionagar no. 2 (Oxoid). A 3.4-ml sample of substrate was poured into 6-cm plastic petri dishes.

The serum used for the preparation of the media was obtained from two Norwegian fjord horses, previously tested for their ability to support good growth.

Preparation of antigens. All strains were grown in samples of 2 liters of S medium supplemented with only 0.04% urea, the relatively low urea content preventing the pH from exceeding 7.0. After incubation for 12 to 14 h at 37 C, corresponding to the late log phase, the organisms were harvested in a Sorvall RC 2b high-speed centrifuge at 20,000 rpm for 1 h. The pellet was washed twice in phosphate-buffered saline (PBS) with a pH of 7.2 and finally suspended in PBS with a pH of 7.2 to give a 50-fold concentration of the original volume corresponding to about 3×10^8 colony-forming units (CFU)/ml. The antigen suspension was stored at −20 C.

Preparation of antisera. Three to five albino rabbits weighing approximately 3 kg were used for immunization with each strain. A 0.75-ml sample of antigen suspended in 0.75 ml of Freund complete adjuvant were injected intramuscularly in the upper part of the hind legs eight times at intervals of 3 to 4 days. Three weeks after the last intramuscular injection, two intravenous injections of 1 ml of antigen were given at 3-day intervals. Blood samples were taken before immunization and 10 days after the intramuscular injections. Two weeks after the last intravenous injection, the rabbits were bled out, and the serum was stored at −20 C. Thirty-four of 56 immunized rabbits gave good hyperimmune sera as determined by the GI test.

Fractionation of hyperimmune sera. Gel filtration of hyperimmune sera on Sephadex G-200 columns (90 by 2.5 cm) was carried out by using 3 ml of serum. PBS (pH 7.2) was used for eluation. Trans-

mission in the eluted fluid was determined continuously at 280 nm with an LKB Uvicord. Fractions corresponding to each of the three peaks were pooled and concentrated to the original volume by pressure dialysis against PBS. According to an earlier report (11), antibodies of 19-S type are found in the first peak, those of 7-S type in the second, and albumin in the third.

Growth inhibition test. The S plates were preheated at 37 C for 30 min. One drop (0.01 ml) of mycoplasma culture was allowed to run down the surface of the agar plate to give a uniform distribution of the organisms. After drying at room temperature the antiserum was applied in the middle of the streak, either as an impregnated 6-mm filter-paper disk or in a well. The inoculated plates were then incubated in atmospheric air with 10% CO_2 for 3 days. The plates were examined under a stereomicroscope (Leitz), and zones of inhibition were measured in millimeters from the edge of the disk or well into the beginning of growth. Less than 0.5 mm was registered as no inhibition. Titers were expressed as the highest dilution giving a growth inhibition zone on at least 0.5 mm.

Metabolic inhibition technique. This test was performed according to a previously described method (16).

RESULTS

The following variables were found to influence the GI test.

Inoculum size. With a constant amount of antiserum the size of the GI zone varied with the number of mycoplasmas inoculated. The optimal concentration was found to be 10^4 to 10^5 CFU per ml, which was used in subsequent experiments.

Application of antiserum. Two methods of applying antiserum were compared (Table 1): 6-mm filter-paper disks freshly impregnated with 0.025 ml of antiserum produced significantly smaller zones of inhibition than 4-mm wells containing the same amount of antiserum. This difference was found at both 37 and 27 C, greatest at the lower temperature; the average increase in the inhibition zones was 1.5 and 2 mm, respectively.

A comparison of the diffusion zones obtained by applying methylene blue in the two ways just described revealed a similar difference in size. In subsequent experiments, wells were used instead of disks.

Amount of antiserum. Table 2 shows that increasing amounts of antiserum resulted in larger inhibition zones both at 37 and at 27 C. Similar results were obtained by refilling the wells every hour up to five times and by increasing the diameter of the wells and thus the amount of antiserum. Wells with a diameter of 4 mm and containing 0.025 ml of antiserum were chosen for the standard procedure.

TABLE 1. *Growth inhibition zones produced by 0.025 ml of antiserum applied either on 6-mm filter-paper disks or in 4-mm wells*

Antiserum	Growth inhibition zone (mm)			
	Disk 37 C	Well 37 C	Disk 27 C	Well 27 C
1	0.5	2.5	3	5
2	0.5	2	1.5	3
3	2	3.5	4	6
4	0.5	2	4	6
5	0.5	2	3	5
6	0	1	0	1.5

TABLE 2. *Influence of amount of antiserum on growth inhibition zone at inoculum of 10^5 CFU/ml in 5-mm wells*

Antiserum (ml)	Temp (C)	Growth inhibition zone (mm)
Antiserum 1		
0.01	37	3
	27	6
0.02	37	4
	27	8
0.04	37	5
	27	10
Antiserum 2		
0.01	37	2
	27	4
0.02	37	2.5
	27	5
0.04	37	3
	27	6

Incubation temperature. The incubation temperature had a significant influence on the GI test. Table 3 shows a comparison between growth inhibition zones produced at 37, 30, 27, and 25 C. Two sera were tested. It is seen that lowering the temperature from 37 to 25 C was followed by a twofold increase of the inhibition zones. Although 25 C gave the best results in terms of millimeters, 27 C was chosen as the standard condition because of the slow growth rate at 25 C. Table 4 shows the differences between incubation at 37 and 27 C as expressed in titers. Five sera were tested; four of them showed a fourfold rise and one a twofold rise in titer. Similar differences are also seen in Tables 1, 2, 5, 6, and 8.

A study was carried out to determine the maximum time interval between inoculation of the plates and application of the antiserum

TABLE 3. *Influence of incubation temperature on growth inhibition zone*

Antiserum	Growth inhibition zone (mm)			
	37 C	30 C	27 C	25 C
1	2.5	3.5	5	5.5
2	1.5	2.5	4	4

TABLE 4. *Influence of incubation temperature on GI test expressed in titers*

Antiserum	37 C	27 C
1	4	16
2	8	32
3	64	512
4	16	32
5	4	16

TABLE 5. *Influence of the pH level in agar medium*

Antiserum	Growth inhibition zone (mm)							
	pH 5.5		pH 6.0		pH 6.5		pH 7.5	
	37 C	27 C	37 C	27 C	37 C	27 C	37 C	27 C
1	4	7	3	4.5	2.5	3.5	2	3
2	2.5	8	1.5	6	0.5	3.5	0	2

TABLE 6. *GI effect of 19-S and 7-S fractions compared with whole serum*

Serum	Growth inhibition zone (mm)					
	19-S antibodies		7-S antibodies		Whole serum	
	37 C	27 C	37 C	27 C	37 C	27 C
1	0	2	2.5	5	2.5	5
2	0	0	1	4	1	4

after which GI still occurred. Both at 37 and 27 C, the inhibition zones decreased when the time interval was increased. The maximum intervals were found to be 8 h at 37 C and 16 h at 27 C.

pH of the agar medium. The pH of the agar medium was found to be of essential importance in the GI test. Table 5 shows a comparison between the results obtained at four pH levels. Two different sera were used, and experiments were carried out both at 37 and 27 C. The best results were obtained at pH 5.5. An increase up to pH 7.5 resulted in a corresponding decrease in the inhibition zone. For routine use, pH 6.0 was chosen because the agar medium at pH 5.5 tended to be very soft.

Globulin fractions. Two rabbit hyperimmune sera were fractionated by gel filtration. The 19-S and 7-S fractions were compared with whole immune serum by the GI and MI tests. It is seen from Tables 6 and 7 that almost all the growth- and metabolic-inhibiting antibodies were found in the 7-S fractions and only small or immeasurable amounts in the 19-S fractions.

Further experiments showed that the following variables did not exert any measurable influence on the GI test.

(i) Inactivation of horse serum at 56 C for 1, 2, or 3 h; addition of fresh horse or guinea-pig serum. In a few cases, the guinea-pig serum had an unspecific inhibitory effect on some of the mycoplasmas.

(ii) Freezing and thawing of antiserum from −20 C to room temperature 30 times.

(iii) Performance of the test on medium containing rabbit or swine serum instead of horse serum. These media must be considered suboptimal for the T-mycoplasmas.

(iv) Incubation of the test plates in atmospheric air with 10% CO_2 or in 95% N_2 plus 5% CO_2.

(v) Thickness of agar medium in the petri dishes varying from 2 to 5 mm gave only minor inconstant variations of the results.

(vi) Incubation for longer than 3 days.

Specificity of the GI test. Eight serotypes of human T-mycoplasmas and their homologous antisera were tested to determine the specificity of the GI test at 37 and 27 C.

It is seen from Table 8 that the eight serotypes could easily be separated by the GI test at 37 C. The one-way cross-reaction between the antiserum against serotype II and T-mycoplasma serotype V was the only cross-reaction at all. At 27 C, the test became more sensitive and slightly less specific. In addition to the one-way cross-reaction found at 37 C, antiserum against serotype I reacted with serotype VI, and antiserum against serotype VIII reacted weakly with serotypes IV and V and more distinctly with serotype VII.

Table 9 shows the relationships between the

TABLE 7. *MI titers of 19-S and 7-S fractions compared with whole serum*

Serum	19-S antibodies	7-S antibodies	Whole serum
1	32	512	512
2	32	2048	1024

TABLE 8. *Serological relationships between eight T-mycoplasmas determined by the GI test at 37 and 27 C (in mm)*

Serotype no.	Strain	Antisera against serotypes															
		I		II		III		IV		V		VI		VII		VIII	
		37 C	27 C	37 C	27 C	37 C	27 C	37 C	27 C	37 C	27 C	37 C	27 C	37 C	27 C	37 C	27 C
I	No. 7	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
II	No. 23	0	0	2	3	0	0	0	0	0	0	0	0	0	0	0	0
III	No. 27	0	0	0	0	3	5	0	0	0	0	0	0	0	0	0	0
IV	No. 58	0	0	0	0	0	0	4	8	0	0	0	0	0	0	0	1
V	No. 354	0	0	2	4	0	0	0	0	3	5	0	0	0	0	0	1
VI	Pirillo	0	2	0	0	0	0	0	0	0	0	2	3	0	0	0	0
VII	Cook	0	0	0	0	0	0	0	0	0	0	0	0	3	5	0	2
VIII	T-960	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4

TABLE 9. *Serological relationships between eight T-mycoplasmas determined by MI test*

Serotype no.	Strain	Antiserum against serotypes							
		I	II	III	IV	V	VI	VII	VIII
I	No. 7	8,192	8	32	32	32	128	4	≦2
II	No. 23	≦2	8,192	≦2	128	2,048	16	≦2	≦2
III	No. 27	8	≦2	4,096	≦2	≦2	128	8	64
IV	No. 58	≦2	≦2	16	16,384	≦2	8	8	16
V	No. 354	≦2	≦2	≦2	64	8,192	4	16	32
VI	Pirillo	16	≦2	4	8	≦2	8,192	8	4
VII	Cook	8	32	16	32	64	128	40,960	4
VIII	T-960	≦2	64	≦2	8	≦2	≦2	128	4,096

eight serotypes as determined by the MI technique. The results were largely identical with those obtained by the GI test at 27 C, with the strong one-way cross-reaction between serotypes II and V being the most significant. The other cross-reactions were less distinct than shown by the GI technique.

Sensitivity of the GI test. Sixteen hyperimmune rabbit sera were used in the comparison of the MI and the GI tests. The results are listed in Table 10. The MI test revealed titers varying from 256 to 8,192. Three of the sera with excellent MI titers possessed no measurable GI effect. The 13 other sera presented growth inhibition zones varying from 1 to 9 mm. There was no correlation between the MI titers and the inhibition zones obtained. The GI titers measured for five sera ranged from 16 to 512; again no correlation to the MI titers was found.

Paired sera from 12 patients with nongonococcal urethritis harboring T-mycoplas-

TABLE 10. *Sixteen hyperimmune sera tested by GI and the MI techniques*

GI test (27 C)		MI test (titer)
Millimeters	Titer	
0	ND[a]	1,024
0	ND	2,560
0	ND	5,120
1	ND	640
2	ND	640
2	ND	5,120
2	ND	8,192
4	ND	640
4	16	4,096
5	32	8,192
5	ND	320
5	16	5,120
5	32	5,120
6	ND	2,560
7	ND	256
9	512	640

[a] ND, not done.

mas in their urethra were tested for GI and MI antibodies against the eight serotypes. All sera were negative.

Identification of T-mycoplasmas by the GI test. The eight type sera were used for identification of 24 T-mycoplasma strains isolated from various human sources (Table 11). Twenty-two strains were serotyped with the present type sera. Serotypes I and III were found in 7 out of 10 isolates from patients with nongonococcal urethritis. Serotype III was the most common, making up 25% of the 24 isolations. Two strains could not be serotyped by means of the eight available sera. Preparation of antisera against these two strains is in progress.

DISCUSSION

The GI test was shown to be suitable for serological work with T-mycoplasmas. The principal reason why this test has been reported to be inapplicable (15) probably is the lack of potent antisera. By following the described schedule for immunization, potent growth-inhibiting antisera were obtained from 34 of 56 rabbits.

Several factors were found to influence the results obtained by the GI test.

As stated for other mycoplasmas (4, 7, 9), the inoculum size and the amount of antiserum were critical for the test, and standard conditions were absolutely required.

By application of the same amount of antiserum, using wells instead of filter-paper disks, it was possible to increase the inhibition zones on an average by 2 mm. This difference might be explained by the fact that more antiserum is available for diffusion; the paper disks retain some antiserum. In addition, wells give a slightly higher diffusion rate of the antibodies.

The incubation temperature was found to be optimal at 30 C for the GI test of *M. mycoides* (8). The importance of the incubation temperature was confirmed in this study. Lowering the temperature from 37 to 25 C was followed by a twofold increase in the inhibition zones. This effect might be explained by the slower growth rate at 27 C, which will allow the antiserum to diffuse and react for a longer period of time, as compared with 37 C, before the organisms reach the same stage of multiplication. It seems likely that a certain minimum ratio between the number of organisms and the amount of antibody is necessary for the occurrence of GI (1). This theory may also explain why a large inoculum gives a smaller inhibition zone, and why the maximum interval between inoculation and application of antiserum is greater at 27 C than at 37 C.

The pH of the agar plates had a marked influence on the inhibition zones. When pH was increased from 5.5 to 7.5, the inhibitory effect of the antiserum decreased—an effect which is difficult to explain since suboptimal growth conditions as to the temperature seem to favor the opposite effect.

These experiments failed to demonstrate any effect of heating the antisera or of addition of fresh horse or guinea-pig serum. This confirms previous findings (4, 8, 14, 18). However, others (2, 5, 10, 13, 17) observed an enhanced growth-inhibiting effect on adding fresh horse

TABLE 11. *Serotyping of 24 isolates by GI test*

Source of isolation	No. of isolates	Serotypes								Not typable
		I	II	III	IV	V	VI	VII	VIII	
Urethra in patients with gonorrhea	7	0	1	1	3	0	1	1	0	0
Urethra in patients with NG urethritis	10	3	0	4	0	1	0	1	1	0
Urethra in healthy persons	4	0	2	0	0	0	0	0	0	2
Urethra in patients with Reiter's disease	1	0	0	0	0	0	1	0	0	0
Trachea in a patient with bronchitis	1	0	0	0	0	1	0	0	0	0
Renal pelvis in a patient with pyelonephritis	1	0	0	1	0	0	0	0	0	0

or guinea-pig serum, but these experiments were carried out in liquid medium and therefore not under comparable conditions.

The fact that most of the inhibitory effect was found in the 7-S fractions of the hyperimmune sera was not unexpected because of the long period of immunization.

In accordance with other investigations (4, 6, 8, 14, 18), the GI test showed a high degree of specificity. Eight strains were tested against homologous and heterologous sera at 37 C and only two one-way cross-reactions were found. By lowering the temperature to 27 C, the sensitivity of the test increased, and at the same time it became slightly less specific, as reflected by a few more one-way cross-reactions. By testing the same eight strains by the MI technique, good correlation was obtained although this test was less specific. Comparison of the GI and MI techniques also showed that the MI test is more sensitive, and that no correlation could be demonstrated between the presence of antibodies measured by the two methods, which agrees with a previous study (15).

Eight serotypes of human T-mycoplasmas were established (3).

By using these eight sera the GI test made it possible to identify 22 of 24 strains isolated from human sources. No single strain could be related to any disease or source of isolation, but the small number of cases included in this study do not allow any definite conclusions as to the virulence of the different strains.

It can be concluded that the GI test with the described modifications is suitable for serological identification and classification of human T-mycoplasmas.

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F. T. BLACK

Institute of Medical Microbiology, University of Aarhus, DK-800 Aarhus C, Denmark

Volume 25, no. 4, p. 531, Table 9: Reverse column headings "Serotype no." and "Antiserum against serotype."

Isolation of *Eikenella corrodens* in a General Hospital

STEPHEN H. ZINNER, A. KATHLEEN DALY, AND WILLIAM M. McCORMACK

The Channing Laboratory, Thorndike Memorial Laboratory, Harvard Medical Unit; Department of Medical Microbiology, Boston City Hospital, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02111

Volume 25, no. 5, p. 708, column 2: Add reference 13 as follows:

13. Riley, P. S., H. W. Tatum, and R. E. Weaver. 1973. Identity of HB-1 of King and *Eikenella corrodens* (Eiken) Jackson and Goodman. Int. J. Syst. Bacteriol. **23**:75-76.