

A Solid Medium Test for Measuring Growth Inhibition and Neutralization of *Mycoplasma mycoides* by Immune Bovine Serum

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SUMMARY

The growth of *Mycoplasma mycoides* var. *mycoides* was inhibited and neutralized by immune serum from cattle which had recovered from infection with *M. mycoides*. Although mycoplasmal neutralization is not necessarily the same as virus neutralization, it is suggested that this terminology be retained since it is accepted and is descriptively appropriate. The effect of immune serum on colony growth was determined by incubating test serum in plastic cylinders on nutrient agar with pre-incubated microscopic *M. mycoides* colonies. Optimal inhibition and neutralization occurred when serum and colonies were incubated at 30° before final incubation at 37°. Inhibition of growth and neutralization also occurred at 37° and 21°; inhibition but no neutralization occurred at 5°.

INTRODUCTION

Blood from cattle affected with contagious bovine pleuropneumonia (CBPP) has been reported to be bactericidal to *Mycoplasma mycoides* var. *mycoides* (Priestley, 1952). Inhibition (Edward & Fitzgerald, 1953; Bailey *et al.* 1961; Cottew, 1963) and 'neutralization' (Edward & Fitzgerald, 1954) of growth of *Mycoplasma* species by hyperimmune sera incorporated in liquid media have also been reported. The possibility that these phenomena may have been caused by agglutination does not appear to have been excluded.

The specific inhibition of *Mycoplasma* species by hyperimmune rabbit serum incorporated in the solid growth medium was reported by Edward & Fitzgerald (1953). This phenomenon has been used to elucidate the serological relationship of several *Mycoplasma* species (Huijsmans-Evers & Ruys, 1956; Clyde, 1964) and to detect specific antibodies in sera of individuals infected with *M. pneumoniae* (Herderschee, 1963).

A review of the literature about 'neutralization' and inhibition of *Mycoplasma* species by antisera indicates that these phenomena, when observed in liquid media, have not been clearly differentiated from agglutination, that neutralization has not been demonstrated on solid media, and that factors which affect the sensitivity of these phenomena have not been fully investigated. A concurrent report presents a study of mycoplasmal inhibition and neutralization in liquid media (Gourlay &

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Domermuth, 1966). The work described here was designed to elucidate further the nature of mycoplasmal neutralization and inhibition by immune sera on solid medium.

METHODS

Organism. A highly virulent strain of *Mycoplasma mycoides*, referred to as Gladysdale (Turner, 1961), was used as the test organism in this work. Seed culture was grown in modified Newing's tryptose broth (Gourlay, 1964) at 37°. The maximum viable titre (colony count) was reached in 72 hr. At this time, the culture was divided into small samples and stored at -70° until used.

Immune sera and control. A pool of immune sera was prepared from grade cattle (crosses between Zebu and European cattle) which had recovered from subcutaneous infection with the Gladysdale strain of *Mycoplasma mycoides* (Gourlay & Domermuth, 1966). This *M. mycoides* antiserum was selected as a test serum because the donor animals had proved to be immune to subsequent subcutaneous challenge with *M. mycoides*. Pooled freeze-dried normal bovine serum (Nutritional Biochemicals Corporation, batch number 5920) was obtained from the U.S.A., a country free from contagious bovine pleuropneumonia. Both pools of sera were tested for *M. mycoides* antibodies by the complement-fixation test (c.f.t.; Gourlay, 1965). The immune serum had a c.f.t. titre of 1/1280 and the normal serum had no titre to *M. mycoides*.

Preparation of nutrient agar plates for application of test sera. Solid medium (Gourlay, 1964), modified to contain 1000 i.u. penicillin/ml., thallium acetate 0.5 mg./ml. and agar 1.1% (w/v), was dispensed to a depth of 4.0 mm. in small Petri plates (6.0 cm. internal diameter). The agar surfaces were inoculated by flooding with 1.0 ml. of a 1/10 dilution of seed culture (see above). The inoculum was allowed to remain on the surface of the agar for 15 min., excess of inoculum then poured off and the plates inverted and incubated for 7 or 14 hr at 37°. After this initial period of incubation, the plate tops were flamed and the plates returned to an upright position. Six plastic cylinders (4.5 mm. internal diameter × 6.0–7.0 mm. height) were then inserted 2.0–3.0 mm. in the agar of each plate. The plastic cylinders were sterilized before use by boiling for 20 min. in thallium acetate solution (2 mg./ml.) followed by boiling in sterile distilled water, and then vacuum-dried.

Determination of neutralization and/or inhibition of Mycoplasma mycoides by test sera. The two test sera, anti-*M. mycoides* (immune) and *M. mycoides* negative (normal), described above were heated at 56° for 45 min. and penicillin (1000 i.u./ml.) added to each. Serial doubling dilutions of serum, 1/1 (undiluted) to 1/128 were made in a liquid nutrient medium, prepared by omitting the agar from the solid medium described above. Two drops of each serum dilution were then delivered into each plastic cylinder. Two sets of plates with each of these dilutions were incubated at 5°, 21°, 30° and 37° for 24 hr and two similar sets for 72 hr. After incubation, the test sera were removed from one complete set of plates by flooding each plate with 10 ml. of the nutrient liquid medium which was used as serum diluent. The plastic cylinders were then removed, and the washing medium was removed by decantation and replaced with fresh liquid medium. The plates were then allowed to stand overnight at 21° (ambient), the liquid medium removed, and the plates inverted before and during final incubation. Final incubation of these and identical unwashed plates was at 37° for 24 hr (plates previously incubated with test serum and colonies at 30° and

37°) or 48 hr (plates previously incubated with test serum and colonies at 5° and 21°). After this final incubation, the remaining plastic cylinders were removed and all plates washed for 10 min. with M/15 KH_2PO_4 containing 0.04% (w/v) sodium azide. The plates were then stained with neutral red (equal parts of 0.5% neutral red in 95% ethanol and M/15 KH_2PO_4 plus 0.85% NaCl in distilled water; fresh stain prepared weekly) for 5 min., washed with the $\text{KH}_2\text{PO}_4 + \text{NaN}_3$ solution, and examined with a dissecting microscope (magnification $\times 12.5$) to determine the effect of the test sera on colony growth.

Absorption of test sera with Mycoplasma mycoides. Test sera were twice absorbed with freeze-dried *M. mycoides* (20 mg./ml.) for 1 hr at 37°, followed by 3 hr at 21° and 18 hr at 5°. After absorption the sera were twice centrifuged at 2000g for 30 min. The supernatant sera were retained and tested for neutralizing and inhibitory activities as described above except that only the most sensitive form of the test was used (30° incubation of test sera and mycoplasma colonies).

Effect of complement on neutralizing and inhibitory activities of test sera. One per cent of 5% (v/v) of guinea-pig serum (complement-fixing activity of 100 minimal haemolytic doses as determined for the complement-fixation test; Gourlay, 1965) was added to the solid medium and to test serum dilutions. Inactivated (56° for 45 min.) control sera were similarly added to control plates. The effect of these components on neutralization and inhibition was determined by the most sensitive test procedure as above.

Examination for growth-inhibitory antibodies in serum of normal cattle and of cattle which had been infected with M. mycoides. One hundred samples of sera with no c.f.t. titre against *M. mycoides* and 10 sera from cattle endobronchially infected with *M. mycoides* were tested for inhibitory antibodies by the most sensitive test procedure.

RESULTS

The appearance of plates prepared for the application of test sera. Plates inoculated in the manner described produced an average of 25 colonies/mm.². When incubated at 37°, the organisms in the inoculum developed into colonies which grew into the agar and after 6 hr could not be washed away with test serum or nutrient medium. Such colonies were therefore usable for the test. They became visible, therefore unusable for the test, at 16 hr. As judged by continued incubation and observation of 7 hr (37°) pre-incubated colonies, slow growth of *M. mycoides* occurred at 30° and no growth occurred at 21° during the 2-week observation period.

Determination of neutralization and inhibition of Mycoplasma mycoides by test sera. Absence of growth of colonies in the presence of test serum is called inhibition; absence of growth of colonies after washing the test serum from the agar is called neutralization. The term neutralization is used to conform with previously used and accepted terminology (Edward & Fitzgerald, 1954); however, this terminology does not necessarily conform in meaning to that used in virology.

Growth of *Mycoplasma mycoides* colonies was inhibited by *M. mycoides* antiserum in all variations of the test system used (Fig. 1).

Neutralization occurred when colonies and antiserum were incubated together at 21°, 30° and 37°, but not when incubated at 5° (Fig. 1). No neutralization or inhibition was produced by normal serum; partial and complete neutralization and inhibition

of growth were produced by immune serum. Younger (7 hr) colonies were more sensitive to the inhibitory and neutralizing effects of serum than older (14 hr) colonies (Fig. 1).

Partial inhibition and neutralization were produced by immune serum dilution 1/64, complete inhibition by dilution 1/32 and complete neutralization by dilution 1/2 (Fig. 1). The appearance of uninhibited and partially inhibited colonies is shown in Pl. 1. Colonies which are completely inhibited or neutralized do not grow to visible size.

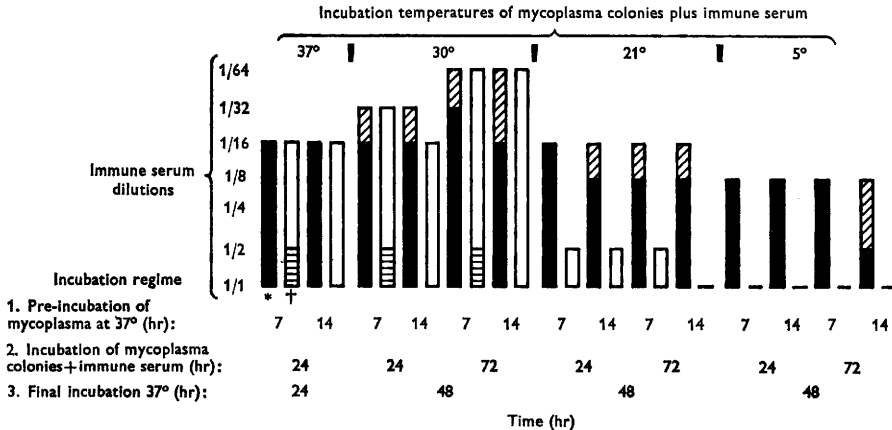


Fig. 1. Effect of physical variables on neutralization and inhibition of *Mycoplasma mycoides* colony growth by homologous antiserum from *M. mycoides* immune cattle. * Serum retained during final incubation: ▨, partial inhibition; ■, complete inhibition. † Serum removed during final incubation: □, partial neutralization; ▩, complete neutralization.

Absorption of immune serum with Mycoplasma mycoides. The neutralizing and inhibitory activities of immune serum were completely removed by absorption with *M. mycoides*.

Effect of complement. The presence or absence of complement produced no detectable change in the neutralizing or inhibitory capacity of immune serum.

Growth-inhibitory antibodies in serum of normal cattle and of cattle infected with M. mycoides. The growth of *Mycoplasma mycoides* was not inhibited by sera from 100 normal cattle but was inhibited by sera from all 10 infected cattle.

DISCUSSION

A sensitive method of detecting *in vitro* neutralization and inhibition of *Mycoplasma mycoides* var. *mycoides* by immune bovine serum has been developed. The method, while utilizing the basic method of Edward & Fitzgerald (1953), has been modified by using plastic cylinders as test serum reservoirs to maintain appropriate serum concentrations. This modification restricts the dilution of serum and permits several sera to be tested on each Petri plate, thus causing the test to be of greater potential value in the diagnosis of contagious bovine pleuropneumonia and other mycoplasmal infections which produce antisera of low inhibitory titre. The use of a temperature of 30° instead of the customary 37° for incubating organisms and sera also significantly increased the sensitivity and thus the applicability of the test.

'Neutralization' of *Mycoplasma* species by immune serum has been long regarded as a unique characteristic of the Mycoplasmataceae (Edward & Fitzgerald, 1954). The data presented in the present report support and strengthen Edward & Fitzgerald's (1954) findings about 'neutralization', as the present information was obtained by observing the effect of immune serum on microscopic colonies rather than in liquid medium where agglutination may have been partially or wholly responsible for diminution of mycoplasmal titre (see Gourlay & Domermuth, 1966). That all inhibitory antibody was washed from the agar before the final incubation was effectively demonstrated, as no neutralizing effect was observed on colonies incubated with immune serum at 5° (Fig. 1). If an effective antibody concentration had been present after the removal of immune serum by washing, this would have been readily apparent and some inhibition of colonies would have occurred during the final incubation at 37°.

The maximum inhibition and neutralization observed in these tests occurred at 30°, a point very near 27°, which was reported to be the lower growth limit for *Mycoplasma mycoides* (Dujardin-Beaumetz, 1900). This observation suggests that maximum neutralization and inhibition are favoured by slow growth of the test organisms; however, the fact that some neutralization was observed at 21° indicates that the phenomenon was not completely growth-dependent as might be deduced from studies in liquid medium. The reason for this apparent discrepancy is not known.

It would be of interest to determine what antigen(s) are responsible for the production of inhibitory antibody and the nature of the mechanism of inhibition. In addition, the relationship of inhibition to immunity should be elucidated as this knowledge is of great potential value to understanding the pathogenesis of contagious bovine pleuropneumonia. The serious nature of the non-specificity of conventional diagnostic tests for contagious bovine pleuropneumonia has only recently been elucidated (Shifrine & Gourlay, 1966); since conventional mycoplasmal growth inhibition tests indicate that inhibition is species-specific (Clyde, 1964), it is possible that the test described here will eliminate false positive reactions when used in the diagnosis of this disease.

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EXPLANATION OF PLATE

Inhibition and neutralization tests on *Mycoplasma mycoides* colonies.

Fig. 1. Normal serum: colonies are uninhibited and normal in appearance.

Fig. 2. Immune serum: colonies are partially inhibited by immune serum as evidenced by reduction in size. When colonies are completely inhibited or neutralized, no colony growth is visible.

