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The Use of Fluorescent-Antibody Technique for the Identification of Cultures of *Mycoplasma*

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Summary. Modifications of the fluorescent antibody technique for the identification of cultures of *Mycoplasma* are described. The method has proved completely successful over a period of 3 years.

INTRODUCTION

The identification of cultures of *Mycoplasma* isolated from pathological material can be made using either the growth inhibition test (Clyde, 1964) or the fluorescent-antibody technique (Chanock, Hayflick and Barile, 1962). The growth inhibition test takes 4–6 days, requires a pure broth culture of the organisms and the results are dependent on a reasonably standardized inoculum. With high titre sera, there is sometimes a cross-reaction between strains, although the specific serum will usually produce a larger zone of inhibition than other sera. The fluorescent-antibody test can be carried out on a mixed culture and the result is available within a few hours. The readings are not affected by inoculum size and the test appears to be species specific. However, non-specific fluorescence can cause trouble due to the complexity of the medium on which the strains are cultured. The present paper gives details of a method which has proved satisfactory over a period of more than 3 years.

MATERIAL AND METHODS

Cultures of Mycoplasma

The cultures of *Mycoplasma* used in this study were grown on Difco P.P.L.O. agar (70 ml base) supplemented with 20 ml of swine serum, 10 ml of a low temperature yeast extract, 1·0 ml of 2 per cent deoxyribonucleic acid (sodium salt), 2·0 ml M K_2HPO_4, 0·5 ml of a 10,000 units/ml solution of penicillin and 1·0 ml of 1:80 w/v solution of thallium acetate. Tests were made as soon as definite colonies were present as seen on low-powered microscopy. With fresh isolates of *Mycoplasma pneumoniae*, this was usually 8–14 days after inoculation, but for other strains suitable cultures were usually available 4–6 days after inoculation.

Preparation of impression smears

The colonies were transferred to microscope slides using the method of Clark, Fowler and Brown (1961). Suitable areas containing discrete colonies were selected by microscopic examination. Confluent areas are not satisfactory, as agar remains adherent to the slide after heating and interferes with the test.

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The selected blocks were removed with a fine scalpel blade, inverted on to microscope slides and identified by ringing the underside with a diamond pencil. The slides were then transferred to a holder (see Fig. 1) and immersed in a beaker of tap water at 87°. The flame was withdrawn immediately before the slides were immersed. As soon as the blocks became opaque, they were slipped from the slides by gentle tilting. The rack was then removed from the beaker and the slides washed individually by quick immersion in a second beaker of tap water at 90°. The slides were then placed in a rack to drain. When a batch had been prepared, each slide was examined microscopically to ensure that discrete colonies were present.

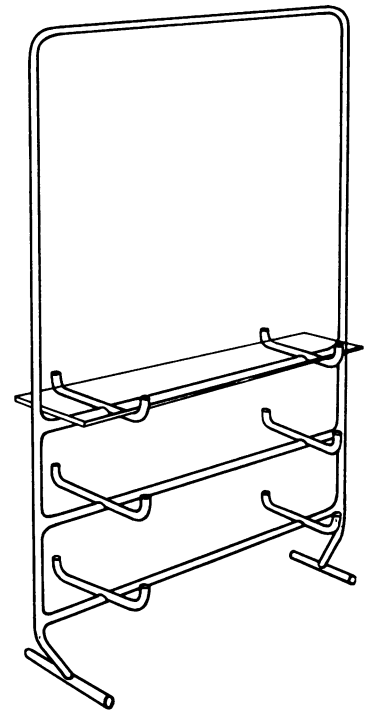

FIG. 1. Diagram of slide holder.

The slides were dried at 37° for 5–10 minutes and then fixed in dry acetone for 10 minutes. False negative results were sometimes obtained if fixation was prolonged beyond this time. The slides were air-dried and, if necessary, were stored at −20° for up to 1 week before use.

Absorption of antisera

The human specific antisera were absorbed with horse liver powder and washed yeast cells before use. The dried horse liver powder was prepared according to the method of Coons, Leduc and Connolly (1955). Baker's yeast was washed three times in phosphate buffer saline, pH 7·2, and stored as a thick suspension, at −20°.

Two hundred milligrams of horse liver powder was weighed into a ¼ oz (7 ml) screw-capped bottle. To this was added 2 ml phosphate buffer saline (pH 7·2) and 2 ml of the washed yeast cell suspension. The mixture was shaken by hand, until the liver powder was

moist, and then centrifuged at 3500 rev/min. for 10 minutes. The supernatant was discarded. To the deposit 1·8 ml phosphate buffer saline and 0·2 ml of serum were added, and stirred with a glass rod. The bottle was then slowly shaken mechanically for 1 hour, avoiding frothing, and then centrifuged at 4000 rev/min for 15 minutes. The supernatant was absorbed once more and recovered by centrifuging at 20,000 rev/min for 15 minutes. The final separation could also be made by spinning at 4000 rev/min and then passing the supernatant through a membrane filter. The treated sera could be stored at −20° for some weeks.

Absorption of anti-human globulin

Difco Bacto FA-Human globulin anti-globulin (goat) conjugated with fluorescein was received as a freeze-dried powder and was re-constituted in distilled water. After reconstitution, the conjugate was divided into 0·3 ml aliquots and stored at −20° to prevent repeated freezing and thawing. As required, the conjugate was thawed at 37° to reduce the chance of dissociation.

The anti-human globulin was absorbed once, as for the absorption of the antisera (see above). The absorbed anti-globulin cannot be stored as dissociation of the conjugate readily occurs, resulting in non-specific fluorescence in the final preparation.

Fluorescent-antibody test

Test slides were placed face upwards on the lid of a plastic box on which had been put a piece of moist filter paper. The base of the box was used as a lid. One drop of the appropriate antiserum was placed on the slides. The sera were spread with Pasteur pipettes to cover the whole of the marked areas. The covered preparations were incubated at 37° for 1 hour. Excess serum was removed by gently pouring phosphate buffer saline over the slides, which were then placed in a slide rack and rinsed in two consecutive baths of buffered saline. The rack was then transferred to a third bath of buffered saline and stirred magnetically at medium speed for 15 minutes.

The slides were then dried, the area of the colonies being gently blotted. Conjugated anti-human globulin was applied to all slides, which were boxed as before and incubated at 37° for 30 minutes. The preparations were washed and dried as before, and mounted in glycerol buffer saline (9 parts of glycerol to 1 part of Bacto FA Buffer, pH 7·2). The edges of the coverslips were sealed with colourless nail varnish. Non-fluorescent glass slides were used but this has subsequently been shown to be unnecessary. The slides were viewed by ultraviolet microscopy using either a 'dry' or 'oil' dark condenser and a ×25 objective.

A known negative serum and another species of *Mycoplasma* were included in each batch of tests.

RESULTS

In positive tests, green fluorescence of the colonies of *Mycoplasma* was observed. This fluorescence was usually limited to the periphery of the colonies, unless very young cultures were used. In negative tests only blue auto-fluorescence of the colonies was seen.

In thirty-four consecutive tests using *M. pneumoniae* and *M. hominis* against a serum known to be positive to *M. pneumoniae* and known negative serum, fluorescence was always present on the *M. pneumoniae* colonies that had been treated with the positive serum.

No fluorescence was seen in the preparations of *M. pneumoniae* treated with the negative serum nor when the *M. hominis* cultures were treated with either serum.

DISCUSSION

The identification of strains of *Mycoplasma* presents a problem to the bacteriologist, since there are no satisfactory biochemical tests by which strains can be differentiated. *M. pneumoniae* ferments glucose and produces a β haemolysin but other strains isolated from man are mainly inactive. Serological techniques are therefore necessary.

Edward and Fitzgerald (1954) demonstrated that the growth of strains of *Mycoplasma* could be inhibited by incorporating specific antiserum in the medium. This property has been utilized in the growth inhibition test of Clyde (1964). It is an extremely useful test, but has the disadvantage of having to grow the strains in liquid medium. Primary isolates of some strains of *Mycoplasma* will grow only poorly or not at all in such medium.

The advantage of fluorescent-antibody test is that it can be made on the original agar culture, even if this culture is not a pure growth, and that the result is available within a few hours. The main disadvantage of the technique is that non-specific fluorescence may make interpretation difficult. The method reported has been found satisfactory over a period of 3 years. Non-specific fluorescence has been eliminated by a double absorption of the antisera and one absorption of the conjugated anti-human globulin with yeast cells and horse liver powder. It was also found necessary to absorb the conjugated anti-human globulin in spite of the fact that it had been put through an ion-exchange column by the makers. This method has given completely satisfactory results in thirty-four tests using *M. pneumoniae* and *M. hominis* with known positive *M. pneumoniae* serum and a negative serum.

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