J. Hyg., Camb. (1968), **66**, 319
With 1 *plate*
Printed in Great Britain

A modification of the growth-inhibition test and its use for detecting *Mycoplasma mycoides* var. *mycoides*

By G. DAVIES* and W. C. S. READ*
East African Veterinary Research Organization, Muguga, Kenya

(*Received* 27 *December* 1967)

INTRODUCTION

The growth-inhibition test is, by now, a well recognized method of identifying mycoplasma species. It has not, however, been widely used for the routine identification of *Mycoplasma mycoides* var. *mycoides*, the causal agent of contagious bovine pleuropneumonia (CBPP). This paper describes a modification of the test designed to facilitate the screening of large numbers of mycoplasma ioslates for *M. mycoides* var. *mycoides* and experiments to check the specificity of the test when used for this species.

MATERIALS AND METHODS

Liquid media

Tryptose serum broth (Newing & Macleod, 1958) as modified by Gourlay (1964) was used. Preparation of this broth has been fully described by Brown, Gourlay & Macleod (1965). The broth included penicillin (100 i.u./ml.) and thallium acetate (1 part in 2000) as bacteriostatic agents. The dextrose component was omitted from some batches of broth as *M. mycoides* var. *mycoides* maintains its viability for a longer period in such a medium.

Solid medium

Tryptose serum agar (Gourlay, 1964) was used. This was prepared in two parts:

A. Tryptose agar

Bacto tryptose (Difco)	2·00 % (w/v)
Sodium chloride	0·50 % (w/v)
Glycerol	0·50 % (v/v)
Anhydrous di-sodium phosphate (Na_2HPO_4)	0·25 % (w/v)
Distilled water to	100·00 %

B. Pig serum and additives

Inactivated pig serum	30 ml.
Dextrose	5 ml. (10 %, w/v)
Bacto yeast extract (Difco)	1 ml. (10 %, w/v)
Crystalline penicillin G (Glaxo)	100,000 units
Thallium acetate	1 ml. (1 % solution)

Part A was prepared and kept in stock until needed.

Part B was prepared immediately before use and filtered through Seitz E.K.

* Seconded to the East African Veterinary Research Organization from the Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Weybridge, Gt Britain.

pads before being added to the melted part A in the ratio of 100 vols. part A to 36 vols. part B. Care was taken to use fresh pig serum and to avoid heating it for more than 30 min. at 56° C. as *M. mycoides* var. *mycoides* has been found to be extremely sensitive to changes in the serum component of solid media. In some batches of solid medium the penicillin and thallium acetate were omitted.

Antisera

These were prepared in rabbits using the method of Lemcke (1964). All sera were heated to 56° C. for 30 min. before use.

Precipitin tests

These were carried out as described by Turner (1962) using supernatant from centrifuged broth cultures as antigen and rabbit antisera as antibody.

The growth-inhibition test

The growth-inhibition test used for identification was modified to deal with large numbers of strains.

Tryptose serum agar plates were dried and the back of the plates marked off into squares $\frac{1}{2} \times \frac{1}{2}$ in. The cultures to be tested were inoculated into tryptose broth devoid of dextrose; incubated cultures showing a fine opalescence were used for the test. One loopful of each culture was spread over the surface of the medium so as to cover the area of a marked square. The squares were labelled with the identity of the cultures and the plates allowed to dry at room temperature for 15–30 min. A loopful of *M. mycoides* var. *mycoides* antiserum was placed on the medium in the centre of each square using a 2–3 mm. diameter platinum loop bent at right angles to the stem, and allowed to dry on the plates at room temperature before incubation at 37 °C. in a moist atmosphere. Plates were examined after 48–72 hr. incubation and cultures that showed a central zone of inhibition of growth (see Plate 1) were recorded as *Mycoplasma mycoides* var. *mycoides*.

RESULTS

The specificity of the growth-inhibition test for identification of Mycoplasma mycoides *var.* mycoides

Differentiation of Mycoplasma mycoides *var.* mycoides *from other* Mycoplasma *species*

Rabbit antisera, produced against the Gladysdale strain of *M. mycoides* var. *mycoides*, and normal rabbit serum were tested against cultures of the following organisms, using a growth-inhibition test: *M. mycoides* var. *mycoides* (Gladysdale strain), *M. mycoides* var. *capri* (Longley strain (Longley, 1951)), *M. laidlawii* A. (PG 8), *M. bovirhinis* (PG 47), *M. agalactiae* var. *bovis* (*M. bovimastitidis*) (Donetta; PG 45), unnamed mycoplasma (Squire; PG 49), unnamed mycoplasma (N 29; PG 50), unnamed mycoplasma (D 12; PG 51).

The normal rabbit serum failed to produce a zone of inhibition of growth in any of these cultures. *M. mycoides* var. *mycoides* antiserum produced a clear zone of inhibition in the culture of *M. mycoides* var. *mycoides*, but no zone of inhibition with any of the other cultures.

The response of various strains of M. mycoides *var.* mycoides *to the growth-inhibition test*

Four strains were examined:

(1) T_1 (46th passage): isolated in Kajiado, Kenya, and used as a vaccine strain in east Africa. (Piercy & Knight, 1957) (Brown *et al.* 1965).

(2) KH_3J (85th passage): isolated in the Sudan and used as a vaccine strain there and in West Africa.

(3) Archers Post (2nd passage): isolated in Northern Kenya (Bygrave, Moulton & Shifrine, 1968).

(4) Gladysdale: isolated in Australia and since then passaged continuously in cattle as infected lung material.

Rabbit antiserum to the Gladysdale strain of *M. mycoides* var. *mycoides* was used on the above strains in the growth-inhibition test. In each case a clear zone of inhibition of growth was caused by this serum. No colonies grew within the zone and normal rabbit serum produced no effect. This suggests that *M. mycoides* var. *mycoides* can be identified by this test irrespective of the strain or the number of subcultures it has undergone.

The identification of Mycoplasma mycoides *var.* mycoides *in a mixed culture*

Twenty-four-hour broth cultures of *M. mycoides* var. *mycoides* and *M. bovirhinis* were mixed in the following ratios: 3:1, 1:1 and 1:3. The mixed cultures were plated and tested with serum prepared against *M. mycoides* var. *mycoides* (Gladysdale strain). In each case a distinct zone of inhibition of growth was visible even though there was a fine growth (presumably of *M. bovirhinis*) within the zone.

The effect of the concentration of organisms on the clarity or size of the zone

Tenfold dilutions of a 24 hr. culture of *M. mycoides* var. *mycoides* were made in tryptose broth. Each of these dilutions was plated and tested for inhibition of growth by *M. mycoides* var. *mycoides* antiserum as before. Each dilution of culture which produced growth on the plates (up to 10^{-5} dilution) showed a zone of inhibition of growth. The zones did not vary in size and in each case there was no visible growth within the zone. This suggests that, using this modification of the test, the concentration of organisms is not critical.

Comparison of the results obtained with the growth-inhibition test and the precipitin test

A total of 110 mycoplasma strains isolated from animals involved in vaccine trials and animals slaughtered during a field outbreak of CBPP were examined using the above two tests. The results recorded in Table 1 show complete correlation between the two tests. In addition, the precipitin test was carried out, using anti-*M. mycoides* serum, on the seven bovine mycoplasma serotypes tested above *M. mycoides* var. *mycoides* was the only one to give a positive reaction.

Table 1. *Comparison of two tests to distinguish* Mycoplasma mycoides *var.* mycoides

		Growth-inhibition	Precipitin
Experiment A (14 animals infected with Gladysdale strain *M. mycoides*)	+ve	68	68
	−ve	15	15
Experiment B (8 animals infected with T_1 strain *M. mycoides*)	+ve	14	14
	−ve	4	4
Field outbreak in Northern Kenya	+ve	9	9
	−ve	0	0

(The figures indicate the number of strains tested.)

DISCUSSION

The growth-inhibition test for identifying mycoplasma species was developed by Edward & Fitzgerald (1954) from an observation by Priestley (1952). It appears to be more specific than the complement fixation and indirect haemagglutination tests (Clyde, 1964) and is consistent with nucleic acid homology (Reich *et al.* 1966). Various techniques have been tried but the filter-paper-disk method developed by Huijmans-Evers & Ruys (1956) and used in human mycoplasma identification by Clyde (1964) is the one universally used. The disadvantages of this technique are the large amounts of serum used and the liability of the sera to bacterial contamination. In addition, the size of the zone of inhibition is influenced by the density of the colonies on the plate (Clyde, 1964). Stanbridge & Hayflick (1967) have attempted to overcome the first two disadvantages by freeze-drying paper disks previously saturated with antisera.

In this laboratory we have been screening very large numbers of mycoplasma strains for *M. mycoides* var. *mycoides* from cultures made from the carcasses of cattle used in vaccine trials for CBPP. It became apparent that there was an extensive mycoplasma flora in these cattle and the modification of the growth-inhibition test described in this paper was developed to identify these strains. Our experience has been that it reduces the number of manipulations to a minimum and thus reduces the risk of bacterial contamination of the sera. It is also economical with serum. The zones of inhibition produced are very distinct (see Plate 1) and as they do not depend to any great extent on diffusion of the serum their size remains constant irrespective of the density of growth on the plate.

The growth-inhibition test has been used for the identification of *M. mycoides* var. *mycoides* by workers at Farcha (unpublished work, reported at the FAO meeting, Khartoum, 1967). It has also been used by Leach (1967) to differentiate the various mycoplasmas isolated from cattle. The present work confirms Leach's findings that the test distinguished between *M. mycoides* var. *mycoides* and the other known bovine mycoplasmas. (The only bovine mycoplasma not examined was *M. bovigenitalum*, which was unavailable.)

There is no published work on the results of the growth-inhibition test when used with various strains of *M. mycoides* var. *mycoides*. The present work suggests

that strains react identically to the test regardless of whether they are recently isolated or attenuated strains.

The precipitin test has been used by workers in Australia (Turner, 1962), particularly to identify *M. mycoides* var. *mycoides* antigens in lung lesions where cultural examination was impossible. The present results show complete agreement between the two tests.

We have used the growth-inhibition test for epidemiological studies in CBPP and it has distinguished mycoplasma other than *M. mycoides* var. *mycoides* in cultures from the respiratory tract of cattle which had typical CBPP lesions and from which *M. mycoides* var. *mycoides* was isolated. Mycoplasmas other than *M. mycoides* var. *mycoides* can also be isolated from the macroscopically normal lungs of experimental cattle in East Africa and these findings suggest that it is essential to use an identification test such as the growth-inhibition test on all mycoplasma strains from such cattle. Until now it has been customary to rely on the characteristic 'thread' growth of *M. mycoides* var. *mycoides* in fluid media to identify the organisms, but Turner (1959) has pointed out that the thread phase is only seen in cultures derived from small inocula and Razin, Cosenza & Tourtellotte (1967) have demonstrated thread phases in a number of other mycoplasmas.

SUMMARY

A modification of the growth-inhibition test for identifying *Mycoplasma* species is described. The modification simplifies the screening of a large number of strains for one species. Experiments showed that it was effective and specific when used to identify *M. mycoides* var. *mycoides*. Its use in studying the epidemiology of contagious bovine pleuropneumonia is discussed.

This work was financed in part by the United States of America Agency for International Development under the terms of the CCTA/AID Joint Project 16 for research on contagious bovine pleuropneumonia.

We wish to thank Dr R. H. Leach for the various bovine mycoplasma cultures.

REFERENCES

Brown, R. D., Gourlay, R. N. & Macleod, A. K. (1965). The production of T_1 broth culture contagious bovine pleuropneumonia vaccine. *Bull. epizoot. Dis. Afr.* **13**, 149.

Bygrave, A. C., Moulton, J. E. & Shifrine, M. (1968). Clinical, serological and pathological findings in an outbreak of contagious bovine pleuropneumonia. *Bull. epizoot. Dis. Afr.* (in the press).

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

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

F.A.O. Report. (1967). Report of the third meeting of the FAO/OIE/OAU Expert Panel on contagious bovine pleuropneumonia. Khartoum.

Gourlay, R. N. (1964). Antigenicity of *Mycoplasma mycoides*. I. Examination of body fluids from cases of contagious bovine pleuropneumonia. *Res. vet. Sci.* **5**, 473.

Huijmans-Evers, A. G. M. & Ruys, A. C. (1956). Micro-organisms of the pleuropneumonia group (family of Mycoplasmataceae) in man. II. Serological identification and discussion of pathogenicity. *Antonie van Leeuwenhoek* **22**, 377.

LEACH, R. H. (1967). Comparative studies of mycoplasma of bovine origin. *Ann. N.Y. Acad. Sci.* **143**, 305.

LEMCKE, R. M. (1964). The serological differentiation of *Mycoplasma* strains (pleuropneumonia-like organisms) from various sources. *J. Hyg., Camb.* **62**, 199.

LONGLEY, E. O. (1951). *Contagious Bovine Pleuropneumonia—a Study of the Disease in Nigeria.* Colon. Publ. no. 7, London: H.M.S.O.

NEWING, R. & MACLEOD, A. K. (1958). *Rep. E. Afr. vet. Res. Org.* 1956–7 Available from the Librarian, E.A.V.R.O.

PIERCY, S. E. & KNIGHT, G. T. (1957). The preparation, titration and challenge of avianised bovine pleuropneumonia vaccines. *Bull. epizoot. Dis. Afr.* **5**, 161.

PRIESTLEY, F. W. (1952). Observations on immunity to CBPP with special reference to the bacteriocidal action of blood. *Brit. vet. J.* **108**, 153.

RAZIN, S., COSENZA, B. J. & TOURTELLOTTE, M. E. (1967). Filamentous growth of mycoplasma. *Ann. N.Y. Acad. Sci.* **143**, 66.

REICH, P. R., SOMERSON, N. L., ROSE, J. A. & WEISSMAN, S. M. (1966). Genetic relatedness among mycoplasmas as determined by nucleic acid homology. *J. Bact.* **91**, 153.

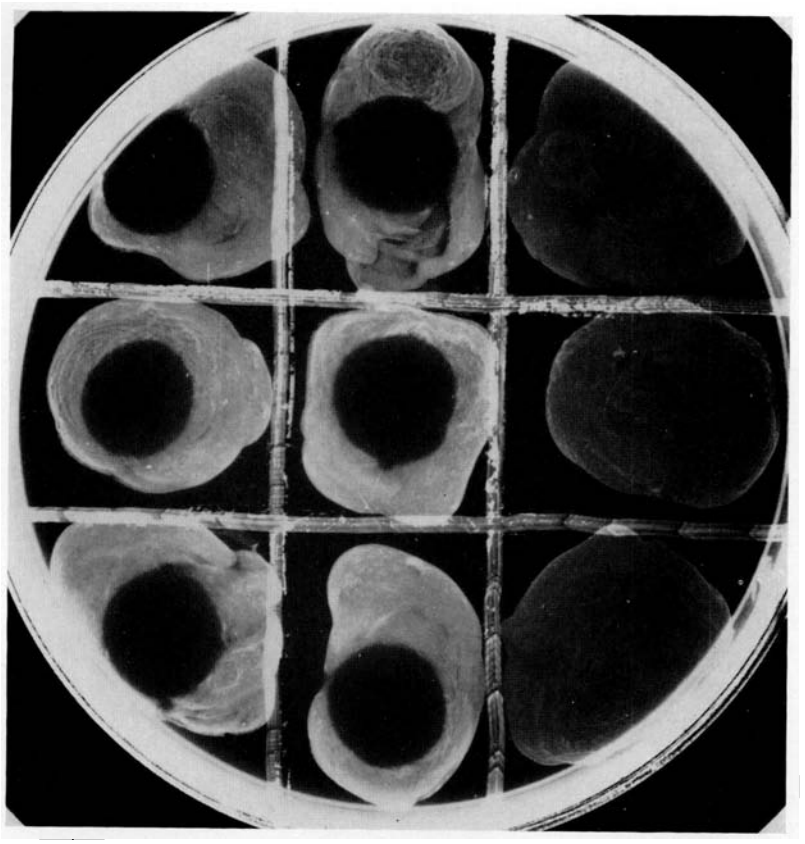
STANBRIDGE, E. & HAYFLICK, L. (1967). Growth-inhibition test for identification of mycoplasma species utilising dried antiserum-impregnated paper discs. *J. Bact.* **93**, 1392.

TURNER, A. W. (1959). *Infectious Diseases of Animals* (by Stableforth and Galloway), p. 440. London: Butterworths Ltd.

TURNER, A. W. (1962). Detection of *Mycoplasma mycoides* antigen and antibody by means of precipitin tests as aids to diagnosis of bovine contagious pleuropneumonia. *Aust. vet. J.* **38**, 335.

EXPLANATION OF PLATE

Mycoplasma strains isolated from the respiratory tract of a cow. The six cultures on the left show zones of inhibition of growth which identifiy them as *M. mycoides* var *mycoides*. (Stained neutral red.)



G. DAVIES AND W. C. S. READ

(*Facing p.* 324)

https://doi.org/10.1017/S002217240004119X Published online by Cambridge University Press