J. Hyg., Camb. (1977), **79**, 155
Printed in Great Britain

A selective oleic acid albumin agar medium for the cultivation of *Mycobacterium bovis*

BY J. GALLAGHER AND D. M. HORWILL

Ministry of Agriculture and Food, Veterinary Investigation Centre, Elmbridge Court, Gloucester GL3 1AP

(*Received* 21 *January* 1977)

SUMMARY

The modification of Middlebrook's 7H11 oleic acid albumin agar medium by the addition of fresh bovine serum and lysed sheep red cells to encourage growth of *Mycobacterium bovis* is described. The improved medium was made selective by the addition of antibiotics and a comparative trial of this medium and the guinea-pig test in the isolation of *M. bovis* from badger tissues is reported. A close agreement between the two tests was found; the guinea-pig test detected 95% of all isolations and culture detected 91%.

INTRODUCTION

The guinea-pig biological test has a time-honoured place as the most sensitive means of detecting tubercle bacilli in tissues. While this test has been largely replaced in medical laboratories involved with the isolation of human tubercle bacilli, this enviable situation has not been achieved in laboratories concerned more exclusively with the isolation of bovine strains. Before stopping the routine use of guinea-pig test for diagnosis, Marks (1972) reported that in 2000 tests carried out at the Tuberculosis Reference Laboratory, Cardiff, only one isolation was made by guinea-pig inoculation which was not duplicated by cultural means. This isolate was found to be a strain of *M. bovis*.

Cultural recovery of the more fastidious *M. bovis* has been less successful than culture of *M. tuberculosis* and the guinea-pig remains considerably more reliable than the available cultural methods.

One of the constraints in most routine diagnostic culture methods is that some acid or alkali decontamination of the specimen is required which inevitably reduces the numbers of viable tubercle bacilli. The use of antibiotics to suppress contaminants has been examined as an alternative by Petran & Vera (1971) and Mitchison *et al.* (1972). Trials of the medium developed by the latter authors produced very good results in the isolation of *M. tuberculosis* (Mitchison, Allen & Lambert, 1973; Mitchison & Aber, 1974) but only two 'field strains' of *M. bovis* were encountered, one of which failed to grow on the medium.

We attempted an evaluation of this medium but soon found that primary isolates of 'field strains' of *M. bovis* grew very poorly. However, contaminant suppression was quite effective and so modification of the basal medium to improve growth

was attempted. Here we report the results of those attempts and the findings of a comparative trial of modified medium and the animal test.

MATERIALS AND METHODS

Media

Middlebrook 7H11* medium containing OADC enrichment* was made selective by the addition of 200 units polymixin B†, 100 μg Carbenicillin‡, 50 μg amphotericin B§ and 20 μg trimethoprim‖ per ml as described by Mitchison *et al.* (1972). The amount of malachite green* was increased to 0·0035 % w/v making the medium deep green. Long slopes with no butt were prepared in screw-capped McCartney bottles and selective medium was used within 4 days. Broth suspensions were prepared in Middlebrook 7H9 broth* with added albumin, glucose and Tween 80. Stonebrinks, Lowenstein Jensen and Tarshis blood agar were prepared in the standard manner at the Central Veterinary Laboratory, Weybridge.

Fresh bovine serum was collected from healthy adults at the slaughterhouse and Seitz-filtered before use. Haemolysed blood was prepared by the addition of equal parts of distilled water to whole defibrinated sheep blood obtained aseptically.

Inocula

M. bovis strains were up to third subcultures of 'field isolates' obtained locally from badgers (Muirhead, Gallagher & Burn, 1974; Gallagher, Muirhead & Burn, 1976). Badger strains of *M. bovis* have been shown biochemically, culturally and pathogenically to be identical to cattle strains (Little, Stuart & Burn 1975). The effect of various modifications to basic Middlebrook 7H11 medium, but with no antibiotic additions, was assessed using pure broth cultures as inocula. The modifications are shown in Fig. 1.

A trial of the chosen modified medium made selective by the addition of antibiotics, was then carried out using lesion material or lymph node collections from badger carcasses routinely submitted for tuberculosis examination. Specimens were homogenized in approximately five volumes of saline and samples were treated in two different ways. Quantities of 0·1 ml were inoculated directly on to each of four tubes of medium which were then tightly stoppered to allow accumulation of metabolic CO_2 during incubation (Schaefer, Cohn & Middlebrook, 1955). A further 2 ml of the homogenate was treated with 5 % sulphuric acid for 10 min as described by Marks (1972). Centrifuged deposit was resuspended in 10 % bovine serum in saline as an alternative to bovine albumin used by Kubica, Dye, Cohn & Middlebrook (1963). One guinea-pig was used per sample and inoculated intramuscularly with 1 ml of suspension. A tuberculin test was carried out at 5 weeks and the animals were killed for examination at 6 weeks.

During the early part of the trial the acid treated material was also cultured.

* Bacto, Difco Laboratories, Detroit.
† Aerosporin, Burroughs Wellcome, London.
‡ Pyopen, Beecham, Brentford.
§ Fungizone, Squibb, New York.
‖ Trimethoprim lactate, Burroughs Wellcome, London.

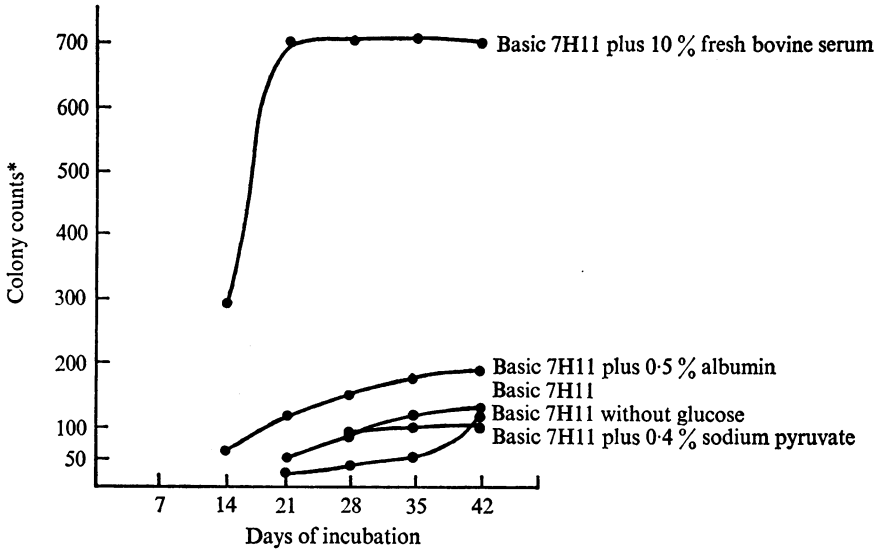

Fig. 1. The effect of various modifications to Middlebrook 7H11 medium on the growth of *M. bovis*. *Counts of three isolates of *M. bovis*: total 12 slopes of each medium.

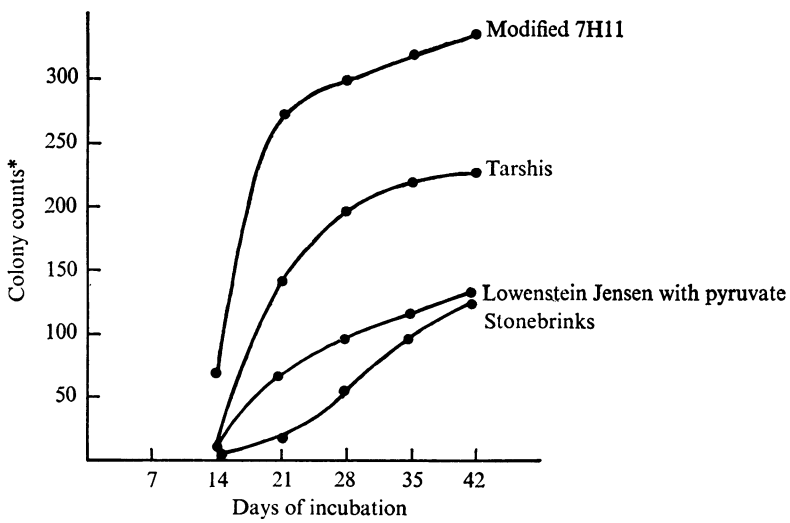

Fig. 2. A comparison of the growth of *M. bovis* on modified 7H11 medium and several conventional media. *Counts of seven isolates of *M. bovis*: total 28 slopes of each medium.

RESULTS

The inability of unmodified Middlebrook 7H11 medium to promote vigorous growth is shown by the results in Fig. 1. The addition of sodium pyruvate (Boissevain, 1943) and the removal of glucose from the medium (Schaefer, 1952) produced no improvement. Doubling the albumin concentration enhanced growths marginally but when 10 % of fresh bovine serum was added luxuriant growths were obtained. Various concentrations of serum were tried and although further

Table 1. *Results of isolation attempts from 465 badger tissue samples using modified 7H11 culture and guinea-pig biological tests*

	Total of isolations	Positive on guinea-pig test	Positive on direct culture	Positive on* indirect culture
*First part of trial	26	25	26†	20
Complete trial	88 (100%)	84 (95%)	80 (91%)	—

* Acid-treated material for guinea-pig inoculation was also cultured in the early part of the trial to determine the effect of the acid on isolation rates.

† One sample negative on both indirect culture and guinea-pig test.

slight improvement was apparent with concentrations above 10%, both contaminant growth and preparation of the medium presented difficulties. However, it was soon appreciated that batches of bovine serum varied greatly in their ability to sustain vigorous growths of *M. bovis*. One particularly good batch had not been taken off the clot very promptly and showed obvious haemolysis. Trace haemolysis in serum has been shown to stimulate growth of leptospira (Savino & Rennella, 1944) and the effect of lysed red cells was therefore assessed in 7H11 medium. The presence of even trace quantities resulted in improved growth but the most beneficial effect was produced by the combination of 10% fresh serum and 0·5% lysed sheep red cells.

The growth of *M. bovis* on 7H11 medium modified by these additions was compared with that on Löwenstein Jensen medium with sodium pyruvate, Tarshis and Stonebrink's media. As can be seen from the results in Fig. 2 growth was more luxuriant on the modified medium. A check was then made to see whether incorporation of antibiotics in the medium had any effect on the growth of *M. bovis*. No growth impairment was discernible.

M. bovis produced a characteristic flat ground-glass growth on the agar and a granular bread-crumb growth in the water of condensation. The agar growths varied in size from 1 to 6 mm diameter and were readily detached from the agar into the water of condensation on tilting the slopes.

During the trial, 465 specimens were examined and 88 isolations of *M. bovis* were made. Seventy-six per cent of isolates appeared by 21 days of incubation and 84% by 28 days. From the results shown in Table 1 it can be seen that organisms were recovered on culture on four occasions when the animal test was negative and the converse on eight occasions. During the first part of the trial, in which material was cultured both directly and following treatment, 26 isolates were recovered by direct culture. Only 20 were isolated after acid treatment and in all these cases colonial counts were lower and isolates took longer to appear.

The main contaminants in badger material, some of which was quite decomposed, were anthracoids, coliforms, staphylococci, streptococci, fungi and miscellaneous Gram-negative organisms. Suppression of these organisms was generally very effective. Only about 5% of tubes were unreadable due to contaminants, mostly fungal.

DISCUSSION

The growth-promoting properties of serum for the cultivation of tubercle bacilli were recognized as long ago as 1939 by Evans & Hanks but have been attributed almost exclusively to the albumin content (Davis & Dubos, 1946, 1947). These authors showed that purified bovine serum albumin protected tubercle bacilli from the toxic effects of free long-chain fatty acids. Esters of these acids are growth promotors, but some contamination with free acids invariably occurs in media in which they are incorporated. Purified bovine albumin is thus now included in commercial media. In a brief note, Dubos (1947) recorded the observation that some batches of unpurified bovine serum albumin not only possessed this protective property but contained a heat-stable non-protein substance having a marked stimulatory effect on growth of tubercle bacilli.

The substance in our serum samples may well be the same as that described by Dubos. The sera used in our trial were not heat-treated, but we have found that some sera do benefit from heat inactivation. The reason for this has not been determined but one possibility is that they have a higher content of lipase. Lipase causes hydrolysis of the fatty acid ester with liberation of toxic free fatty acid (Dubos, Davis, Middlebrook & Pierce, 1946). The mechanism of the stimulatory action of serum and lysed red cells is a matter of current investigation.

In our trial, the modified culture method detected 91 % of all isolates of *M. bovis* and the guinea-pig test 95 %; a difference of only 4 %. This contrasts with Lesslie's finding of differences of 22 % and 24 % in two trials using Stonebrinks medium (1959). Birn (1965) using either Stonebrinks or Tarshis medium found a difference of approximately 12 % in both cases. We found that the avoidance of pretreatment greatly improved isolation rates. Pretreatment with sulphuric acid, considered the least damaging method (Marks, 1972), was shown to result in a lower isolation rate compared with direct culture on the antibiotic medium. Indeed in one case, as a result of acid treatment, the indirect culture and guinea-pig tests were negative, yet direct culture was positive.

Although the number of isolations reported here is not very large, the results are sufficiently promising to warrant consideration being given to the dropping of the routine guinea-pig test for the isolation of *M. bovis* from badger material. We hope that the guinea-pig test may be replaced completely for the isolation of *M. bovis* from other material, with considerable saving of funds, as well as the avoidance of an aesthetically undesirable technique. The inclusion of a further couple of slopes might well result in a cultural sensitivity equal to, or possibly superior to, that of the guinea-pig test.

We are indebted to Dr T. W. A. Little of the Central Veterinary Laboratory, Weybridge, for helpful advice and Mr A. H. Pill, DVO(VI), Gloucester, for constructive comment on this manuscript. We would also like to thank Mrs E. McKenzie, Miss G. Hall and Miss G. Street for skilled technical assistance and Mr G. Turner for the welfare of the guinea-pigs.

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