GROWTH, PURIFICATION AND MAINTENANCE OF LEPTOSPIRA ON SOLID MEDIA

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EARLY studies of leptospira have shown that both the viability and virulence of these organisms were maintained better in semi-solid media containing corpuscular elements (small quantities of agar or fibrin) than in modified Korthof medium. Even the addition of small quantities of blood or haemoglobin to the fluid media avoided frequent transfer (every 4–6 weeks) of the many sero-types needed in leptospira reference laboratories. The frequent transfer of a large number of strains consumed much time and material, involved the risk of contamination and even confusion of the sero-types.

In recent years a higher percentage of agar was added to the above-mentioned media for certain reasons : to show that rest-phases of leptospira do exist (Woratz, 1954), to study the oxygen requirements of leptospira for growth (Lawrence, 1951), to demonstrate the growth of leptospira in various types of colonies (Cox and Larson, 1957), or to give evidence that leptospira colonies grow from single organisms (Larson and Edwards, 1958).

The purpose of our studies was to simplify the maintenance of leptospira type collections by growing these organisms as other bacteria on solid media.

We were concerned with finding a standard solid medium for maintenance of leptospira of all known sero-groups (Wolff and Broom, 1954) for long periods, easily recognizing contamination and purifying contaminated cultures from the growth on plates.

The criterion of the suitability of our solid medium consisted of obtaining a macroscopically visible spreading growth of leptospira on the plates in the form of a circle around the spotted inoculum of one loopful of organisms of a well-grown culture. In tubes which were inoculated as stab cultures with the same number of organisms single or double rings were observed.

MATERIALS AND METHODS

The first medium which gave optimum growth on plates and in tubes consisted of the following ingredients :

Basic medium A

Nutrient bacto-tryptose broth (Difco) diluted 1 in 10 in distilled water (containing 0·1 per cent bacto tryptose, 0·03 per cent beef extract and 0·05 per cent saline), 10 per cent inactivated rabbit serum, 1 per cent of 5 per cent rabbit red blood cells lysed in distilled water.

Addition of medium B.—2·6 dichlorphenol indophenol 1 : 30,000 as oxidation-reduction indicator and either vitamin B_{12} (Glaxo) 1 μg. per ml. (Babudieri and Zardi, 1956) or vitamin B_1 (thiamine) in the same quantity.

Before addition of the serum and haemoglobin medium A was adjusted to pH 7·5 and autoclaved for 15 min. at 120°. Plates and tubes closed with rubber bungs were sterilized before use in the same way. The oxidation-reduction indicator was sterilized by Seitz filtration.

The medium was poured in 10 ml. amounts in small petri-dishes (2½ in. diameter) and 3 ml. in narrow tubes (10 mm. diameter). Owing to the long incubation (1–2 weeks) at 30° and the sensitivity of leptospira to dehydration a relatively thick layer of medium (6–10 mm.) was needed in the plates. Both plates and tubes were inoculated with one standard loopful of well grown cultures containing 20,000–40,000 actively motile organisms. The plates were inoculated by spotting (4 cultures in the 4 quadrants) and the tubes as stab-cultures. They were incubated at 30°. In order to demonstrate isolated colonies of leptospira the plates were inoculated by spreading the inoculum in the usual manner. Types of all known sero-groups (Wolff and Broom, 1954) of our leptospira collection (comprising 54 strains) were used in these investigations. They were usually maintained in both the modified fluid (Korthof) and the semi-solid (Noguchi) medium containing 0·1 per cent agar. The transfer occurred every 4–6 weeks. One virulent strain of *Leptospira icterohaemorrhagiae* maintained by guinea-pig passage was also included in these studies.

RESULTS

All strains showed a characteristic growth in the tubes after incubation for 1–2 weeks. The first sign of growth was indicated by reduction of the indicator (blue to pink or red) which usually started at the bottom of the tube. After 7–10 days (rarely after 12–14 days) a heavy growth indicated by single or double rings was seen about 5–10 mm. under the surface of the medium. This confirmed the known fact that optimal conditions for the growth of leptospira are at the level of the minimal oxygen tension. The organisms are micro-aerophilic. The upper part of the medium retained its blue colour, owing to the re-oxidation effect of the oxygen present in the corked tubes. A similar observation was reported by Lawrence (1951), using a different technique (shake-cultures) in a semi-solid medium containing 0·2 per cent agar.

After longer incubation (3 weeks) the ring or rings spread deeper into the medium. Close investigation of various layers of the medium column taken out of the broken tube revealed that at the lowest part, although strongly reduced, there was no growth of leptospira. An advantage of inoculation by the stab-culture technique was early observation of contamination visible along the stab after 1–2 days' incubation. There was growth along the whole column, followed by strong reduction of the indicator (Fig. 1).

In order to check the maintenance of both viability and virulence of leptospira at various temperatures, duplicate cultures grown in properly closed tubes with rubber bungs were stored at room temperature (17°) and in the refrigerator (6°–10°). So far, after 6 months, there has been no difference in the viability of cultures in both series. However, it was noticed that in both series the indicator

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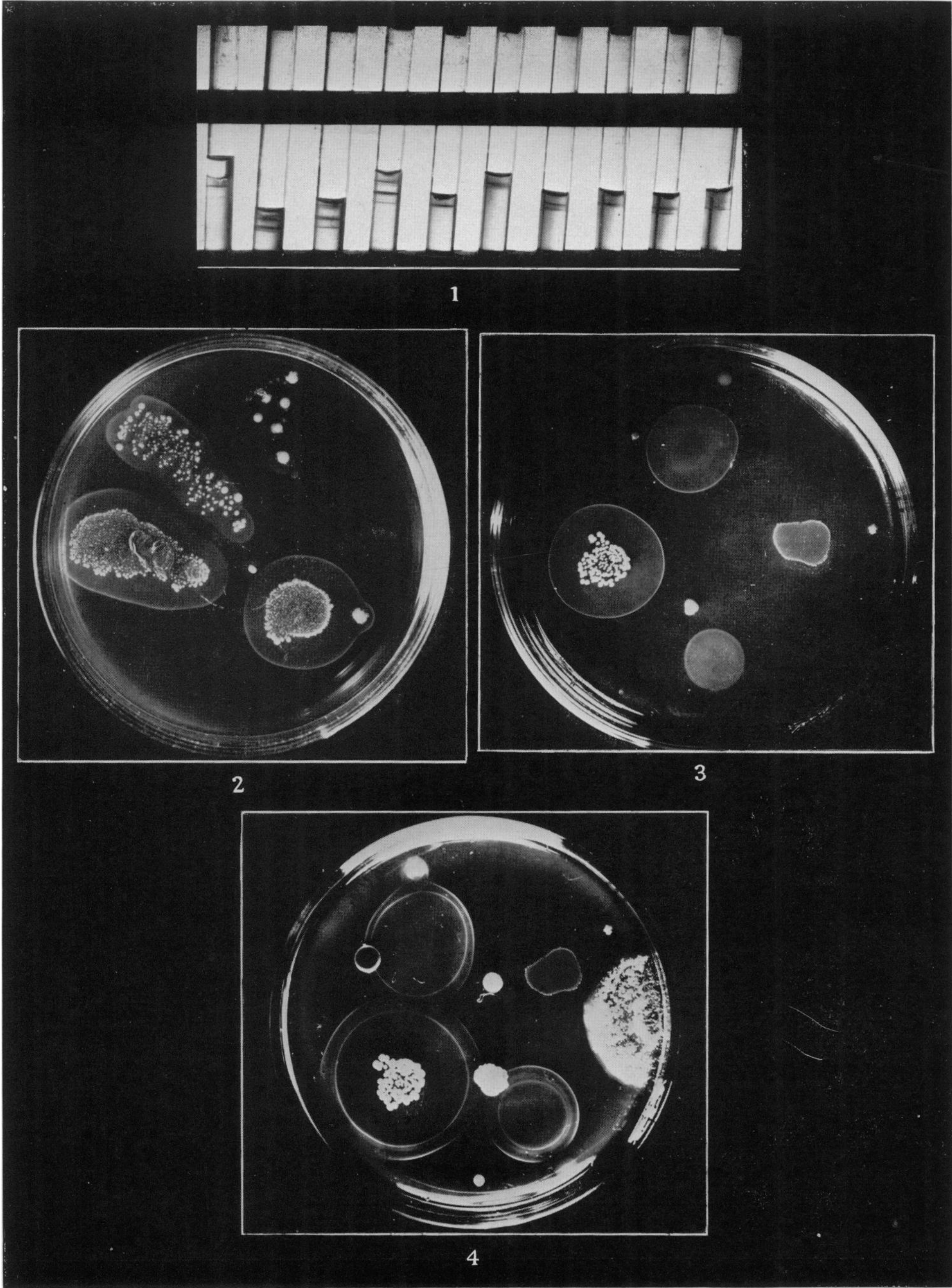
EXPLANATION OF PLATE.

Fig. 1.—Leptospira cultures in solid medium after inoculation as stab-cultures (incubation for 6 days at 30°).

Fig. 2.—Spreading growth of leptospira in circles around the spotted inoculum of one loopful of a well-grown culture, and the hazy appearance of isolated colonies around the restricted growth of contaminants.

Fig. 3.—Growth of 4 different strains after 1 week incubation at 30°. Two pure strains, one contaminated with staphylococci, the 4th strain too heavily contaminated, no growth of leptospira.

Fig. 4.—Secondary ring formation after storage of the same plate for 3 weeks at 17°.



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became re-oxidized. Although the red colour of the medium column had changed to blue, the rings present in the upper part remained clearly visible. A similar observation was reported by Lawrence (1951) in his semi-solid medium and he assumed that this change of the colour reflected the reduced metabolic activity of the still living organisms in the zone of the rings. This assumption was confirmed by our observations that cultures kept for longer periods (6–8 weeks) at 30° showed one or more rings in the upper part of the medium while the reduced red colour of the lower part remained unchanged.

The maintenance of the virulence of one strain of *L. icterohaemorrhagiae* available was examined by guinea-pig experiments. Suspensions of the ring-zones of stab cultures stored at room temperature and in the refrigerator were injected intraperitoneally into young 175–200 g. animals. Cultures 3 months old retained their pathogenicity much better at the lower temperature. The sensitivity of leptospira to dehydration restricted similar tests of cultures grown on plates to only 2 months. The investigations have only been proceeding for 6 months and they were done qualitatively without counting the organisms.

On plates there was a great difference in the rate of growth assessed by measuring the diameters of the macroscopically visible circles. The range of the diameters of different strains varied between 10 and 40 mm. Some strains grew over the whole plate. The growth of large numbers of organisms was observed in the medium and not on its surface. Fig. 2 clearly demonstrates the spreading growth of leptospira in circles and the hazy appearance of isolated colonies in contrast to the white colonies of contaminants. The difference in the rate of growth of leptospira and contaminants (usually staphylococci or yeasts) enabled us to purify the strains easily. The contaminants grew in circumscript colonies (Fig. 3), while a small piece of the medium cut out from the edge of the circles, usually containing a large number of leptospira only, could be transferred in Korthof or Noguchi media.

It was most remarkable that various strains of leptospira even showed growth at temperatures as low as 6°–10°. Growth was quite clearly demonstrated by the formation of new circles at room temperature (Fig. 4) and small circles appeared in the refrigerator. On both plates 4 different strains were inoculated (*L. icterohaemorrhagiae, L. pomona, L. canicola and L. australis* A). Similar observations were made with other strains used for routine investigations, *e.g. L. grippotyphosa, L. sejroe* and *L. australis* B).

There were variations in the requirements for growth promoting compounds (vitamins) by different strains in order to achieve spreading growth on plates. Some strains which were used for our routine investigations grew on plates, though in small circles (10–15 mm. diameter), containing only our basic medium mixed with rabbit or sheep serum and haemoglobin without accessory vitamins. Addition of B_{12} or B_1 vitamin (1 μg. per ml. medium) increased the rate of growth which was visible in the increased size of the circles around the inoculum. The routine strains were : Two *L. icterohaemorrhagiae* (London and Amsterdam), *L. canicola* (Amsterdam), *L. australis* A and B (Brisbane), *L. pomona* (Dunedin), *L. sejroe* and *L. grippotyphosa* (Copenhagen).

It was assumed that these strains required for the spreading growth other stimulating substances. Cytexin (Glaxo) containing B_{12} vitamin and the complex of B vitamins (thiamine, riboflavine, nicotinamide) dissolved according to instructions and added in a volume of 0·8 per cent to the solid medium gave the same results as B_{12} vitamins only.

The first heart-blood cultures of several passages of the virulent strain of *L. icterohaemorrhagiae* which was maintained in guinea-pigs showed good growth in tubes, but gave inconsistent results on plates. Recent investigations revealed that Korthof salt solution could be used as the base in place of diluted broth and 1 per cent Davis granular agar (New Zealand) in place of Difco agar. Rabbit serum, haemoglobin and indicator added to the new base and agar gave less consistent results with both virulent and non-virulent strains.

Although sheep serum is in ample supply in New Zealand it was found to be not suitable on account of the presence of antibodies, also it caused turbidity of the medium when mixed with agar.

SUMMARY

Solid media are described which gave satisfactory results in the maintenance of sero-types of all known groups of leptospira.

Frequently transferred strains in the usual fluid or semi-solid media showed, after 1–2 weeks' incubation at 30° spreading growth in the form of a circle (10–40 mm. diameter) around the spotted inoculum of one standard loopful of a well-grown culture (20,000–40,000 motile organisms). In stab-cultures growth in single or double rings 5–10 mm. under the surface was noticed.

Early growth along the whole stab indicated contamination which was accompanied by strong change in the indicator contained in the medium.

Sero-types of all known groups showed growth in tubes, but on plates the results were irregular.

The difference in the rate of growth of leptospira and contaminants on plates facilitated purification and cultures from single cell colonies of leptospira could be obtained.

Ready made solid medium, stored in corked tubes in the refrigerator could be used for at least 6 months without any change of its suitability for growth, or loss of colour of the indicator.

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