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Long-Term Survival of *Leptospira* in a Biphasic Culture Medium Containing Charcoal

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A comparison was made of the survival of 28 leptospiral serotypes in Fletcher semisolid medium and in the same medium containing a basal layer of Fletcher medium plus 0.7% of agar and 0.5% of activated animal charcoal. A year after culture, more motile leptospires were observed by microscope examination in the biphasic medium. Two years after culture, 4 serotypes grown in the biphasic medium and 11 in Fletcher medium did not show motile cells. Nineteen of the serotypes maintained in Fletcher medium and 25 in the biphasic medium for 2 years grew on subculture into Fletcher medium. Subcultures from the biphasic medium showed the characteristic leptospiral ring growth earlier during the incubation period.

At present, a simple, reliable method for the prolonged maintenance of leptospiral cultures which consistently produces satisfactory results has not been reported. The common procedure involves periodic subculturing at 3-month intervals into fresh semisolid media, since storing of leptospiral strains by freezing at −79 C or lyophilization has not been consistently successful (2, 4). Liquid nitrogen refrigeration has been found to be effective in preserving strains by several laboratories (1) but the method is expensive and more suitable to leptospiral reference laboratories which maintain large numbers of *Leptospira* isolates.

Manew (6) reported that *Leptospira* strains could be maintained in a double-layer medium consisting of an agar layer of Korthof medium overlayed with the same medium containing 10% rabbit serum without agar. Leptospires were found to survive up to 8 months, and 87% of the cultures retained their viability. This medium was found to be of value in the control of evaporation. Addition of charcoal to culture media has been used successfully in the adsorption of bacterial metabolites which alter the survival of the mycobacterial organisms (5).

The present study examines the effectiveness of incorporating a basal agar layer containing animal-activated charcoal to Fletcher media (3) for the long-term storage of 28 *Leptospira* serotypes.

MATERIALS AND METHODS

Cultures. The 28 *Leptospira* cultures selected to study the effect of a biphasic culture medium on leptospiral survival were obtained from the reference strain collection, Leptospirosis Unit, Pan American Zoonoses Center, Ramos Mejía, Buenos Aires, Argentina, and included the serotypes indicated in Tables 1 and 2.

All the stock cultures are maintained in Fletcher semisolid medium (3) (Difco) containing 10% pooled rabbit serum. The cultures are routinely subcultured at 3-month intervals, incubated at 30 C until they develop the characteristic ring growth, and then are maintained in the dark at room temperature (about 27 C).

Fletcher medium. Fletcher semisolid medium (3) (Difco), containing 10% pooled rabbit serum, was aseptically dispensed in 6.0-ml amounts into screw-cap test tubes. These tubes were inactivated the following day at 56 C for 1 h.

Biphasic charcoal medium. Three milliliters of Fletcher semisolid medium (Difco) without added rabbit serum and supplemented with 0.7% agar (Difco) and 0.5% animal-activated charcoal (bone) were dispensed into screw-cap test tubes. These tubes were autoclaved for 15 min at 15 lb of pressure (121 C), shaken to uniformly suspend the charcoal particles, and rapidly immersed in a cold-water bath to solidify the agar.

Above this basal layer of solidified charcoal medium, 3.0 ml of sterile Fletcher semisolid medium (Difco) with 10% pooled rabbit serum were aseptically overlayed.

Inoculation of tubes. One tube each of Fletcher medium and the biphasic medium was inoculated with 0.5 ml of a semisolid culture of each *Leptospira* strain studied. The inoculated cultures were incubated at 30 C and examined visually until well formed Dinger zones were evident; microscope examination revealed heavy growth of actively motile leptospires in the absence of contaminating microorganisms. The cultures were then stored at room

temperature in the dark and examined microscopically for motile leptospires 1 year and, again, 2 years later.

Fresh tubes of Fletcher semisolid medium (3) (Difco) with 10% pooled rabbit serum were inoculated with 0.5 ml of each 2-year-old culture to recover the surviving organisms.

RESULTS

The relative survival of the 28 *Leptospira* serotypes in Fletcher semisolid media and modified Fletcher double-layer charcoal media are summarized in Table 1. Actively motile leptospires were observed microscopically in the cultures of all 28 serotypes maintained 1 year in biphasic medium and in 24 of the serotypes grown in Fletcher medium. Estimates on the number of cells per high-dry-field revealed more leptospires in the biphasic medium.

TABLE 1. *Microscopy detection of the motility of 28 Leptospira serotypes maintained in Fletcher semisolid medium and biphasic modified Fletcher media*

Strain	Motility[a]			
	Fletcher medium		Modified Fletcher double-layer medium	
	1 yr[b]	2 yr	1 yr	2 yr
canicola, Hond Utrecht	+	0	+++	+++
sao paulo, Sao Paulo	+	0	++++	++
semaranga, Veldrat Semarang 173	+	+	+++	++
ballum, Castellon 3	++	++	+++	+++
icterohaemorrhagiae, RGA	+	+	+++	+++
copenhageni, M 20	+	+	+++	+++
broomi, Patane	+	0	+++	+
wolffii, 3705	++	+	+++	+++
sejroe, M 84	+	+	+++	+++
autumnalis, Akiyami A	+	+	+++	++
hebdomadis, Hebdomadis	++	+	+++	0
pomona, Pomona	++	0	+++	+++
hardjo, Hardjoprajitno	0	0	+++	++
shermani, LT 821	+	+	+++	++
tarassovi, Mitis Johnson	0	0	+++	++
tarassovi, Perepelicin	+	+	+++	+++
benjamin, Benjamin	+	+	+++	++
andamana, CH 11	+	0	+++	++
djatzi, HS 26	+	+	+++	++
atlantae, LT 81	+	+	+++	+++
grippotyphosa, Moskva V	+	+	+++	+
butembo, Butembo	++	++	+++	++
patoc, Patoc 1	0	0	+++	0
bakeri, LT 79	+	0	+++	++
kennewicki, LT 1026	0	0	++++	0
sentot, Sentot	+	0	++	++
panama, CZ 214 K	+	+	+++	0
alexi, HS 616	+	+	+++	++

[a] Symbols: 0, no motile leptospires seen in two complete scannings of surface area covered by an 18-mm² cover-slip; +, 1 motile organism per several fields using high-power objective 40× and 10× oculars; ++, 1 to 3 motile leptospira per field; +++, several organisms in each field; ++++, many motile organisms.

[b] Time of storage.

TABLE 2. *Recovery of leptospiral strains by subculture in fresh Fletcher media from cultures maintained for 2 years in both Fletcher semisolid media and the modified biphasic Fletcher medium*

Strain	Evidence of ring growth (day)[a]	
	Fletcher semi-solid media[b]	Double-layer charcoal-containing Fletcher medium[b]
canicola, Hond Utrecht	14	6
sao paulo, Sao Paulo	7	6
semaranga, Veldrat Semarang 173	4	5
ballum, Castellon 3	7	6
icterohaemorrhagiae, RGA	8	6
copenhageni, M 20	13	6
broomi, Patane	—	7
wolffi, 3705	—	8
sejroe, M 84	—	5
autumnalis, Akiyami A	6	6
hebdomadis, Hebdomadis	9	—
pomona, Pomona	12	6
hardjo, Hardjoprajitno	—	8
shermani, LT 821	6	6
tarassovi, Mitis Johnson	—	6
tarassovi, Perepelicin	—	6
benjamin, Benjamin	14	7
andamana, CH 11	5	5
djatzi, HS 26	9	5
atlantae, LT 81	12	6
grippotyphosa, Moskva V	12	8
butembo, Butembo	—	9
patoc, Patoc 1	5	5
bakeri, LT 79	6	5
kennewicki, LT 1026	—	—
sentot, Sentot	—	6
panama, CZ 214 K	12	—
alexi, HS 616	9	6

[a] Day in which the characteristic ring growth became evident is indicated; —, indicates absence of leptospiral growth by culture or microscope examination.

[b] Two-year-old cultures maintained in medium.

After 2 years of storage, microscopy evidence of viability was observed in 17 and 24 serotypes grown in Fletcher and biphasic media, respectively. Subculture of all 2-year-old cultures into fresh Fletcher semisolid medium resulted in the recovery of 25 serotypes from those stored in the biphasic medium and only 19 from those maintained in Fletcher medium (Table 2). The characteristic leptospiral ring growth was detected earlier after subculture in those serotypes which had been stored in bi-

phasic medium. The antigenic identity of all the *Leptospira* serotypes studied was not altered during the 2-year study period as determined by cross-agglutination tests using hyperimmune antisera prepared in rabbits.

DISCUSSION

One of the major problems in working with leptospires is the need for the frequent subculture of stock strains for their maintenance. Stuart (7) reported that leptospires kept in Fletcher media at room temperature were viable for 1 and often 2 months and, infrequently, for longer than 3 months after culture. Manew (6) reported that only 2 of 138 cultures grown in this same medium were viable at the end of 6 months. In our laboratory, however, we have frequently recovered cultures which have been maintained in Fletcher media for as long as a year. The present study has revealed that a modification of Fletcher medium by the incorporation of a basal agar layer containing charcoal is superior to the standard medium for the long-term maintenance of leptospiral cultures. Since the satisfactory survival of cultures varies with the particular lot of rabbit serum used (4, 7), the same pool of rabbit serum was used for the preparation of both media in the present study.

Charcoal has previously been added to culture media for the cultivation of tubercle bacilli by Hirsch (5). He found that charcoal was able to supply a growth-promoting substance to *Mycobacterium* culture medium which effectively replaced the requirement for bovine albumin and suggested that the charcoal also facilitated the removal of the toxic products of bacterial metabolism. The results obtained in the present study do not indicate whether the charcoal, the biphasic character of the modified medium, or both, were responsible for this effect.

LITERATURE CITED

1. Alexander, A. D., E. F. Lessel, L. B. Evans, E. Franck, and S. S. Green. 1972. Preservation of leptospiras by liquid-nitrogen refrigeration. Int. J. Syst. Bacteriol. **22**:165–169.
2. Coghlan, J. D., W. H. R. Lumsden, and G. J. C. McNeillage. 1967. Low-temperature preservation of *Leptospira*. Preliminary communication. J. Hyg. **65**:373–379.
3. Fletcher, W. 1928. Recent work on leptospirosis, tsutsugamushi disease and tropical typhus in the federated Malay states. Trans. Roy. Soc. Trop. Med. Hyg. **21**:265–287.
4. Galton, M. M., R. W. Menges, E. B. Shotts, A. J. Nahmias, and C. W. Heath. 1962. Leptospirosis: epidemiology, clinical manifestations in man and animals, and methods in laboratory diagnosis. Communicable Disease Center, Atlanta, Georgia.
5. Hirsch, J. G. 1954. Charcoal media for the cultivation of tubercle bacilli. Amer. Rev. Tuberc. **70**:955–976.
6. Manew, C. 1968. Ein Zweischichtiger Nährboden für die Leptospirenstammhaltung. Zbl. Bakt. I. Abt. Orig. **210**:216–220.
7. Stuart, R. D. 1946. The preparation and use of a simple culture medium for leptospirae. J. Pathol. Bacteriol. **58**:343–349.