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Low-temperature preservation of *Leptospira*, preliminary communication

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INTRODUCTION

One of the main difficulties in running a service for the diagnosis of leptospirosis in man and animals is the maintenance of cultures of the various serotypes and strains for use as antigens. Present methods demand subculture in fluid medium at regular intervals of from 1 to 3 weeks, which is laborious and time consuming. Also, diminution in the viability of strains sometimes occurs and this may result in their loss. To avoid these difficulties, a reliable method of preparing a preserved stock of serotypes from which one could draw would be of value. Preservation of leptospires by freeze-drying has not proved satisfactory (Wolff, 1960), although Annear (1956, 1958) was able to recover leptospires in culture 2 years after drying them in high vacuum on sterile frozen-dried plugs of starch-peptone. These 'desiccates' stored in ampoules at 4° C. were more successfully preserved than those kept at room temperature. Maintenance of leptospires in tubes of semi-solid medium as recommended by Kirschner (1958) is more reliable in our experience but demands considerable storage space. So also does their maintenance in long narrow-bore tubes, such as tissue culture tubes, which minimize evaporation (using liquid medium to which 2 to 3 drops of fresh guinea-pig blood are added), a method recommended by L. H. Turner (WHO/FAO Leptospirosis Reference Laboratory, London). Weinman & McAllister (1947) described a method of preserving pathogenic protozoa by freezing suspensions of them in amounts up to 3 ml. in sealed Pyrex glass tubes, 100×13 mm. The tubes were cooled to $-15°$ C. and then stored at '$-70°$ C.' They included in their study a brief description of this treatment applied to several virulent strains of *Leptospira icterohaemorrhagiae* and to the saprophytic *Leptospira biflexa*; they obtained satisfactory growth in cultures from 9 out of 18 preparations after maintenance in a frozen state for periods ranging from 12 to 901 days. Wolff (1960) reported favourably on a similar method whereby a culture of leptospires in fluid medium was quickly frozen in a thin layer against the inner surface of a test tube (shell frozen) and then stored at low temperatures. He found that the organisms did not survive at $-32°$ C., whereas at about $-70°$ C., 39 of 48 strains were successfully subcultured after 1–7 years' storage. The virulence of the organisms was also maintained. Tarasevich, Bulk & Mudrova (1963) preserved four strains of pathogenic leptospires by a method similar to that of Weinman & McAllister (1947).

Their cultures grown in distilled water with 5% rabbit serum were stored in 1 ml. amounts at −78° and −30° C. for periods ranging from 24 hr. to 6 months; the actual times of storage at the two specified temperatures are not made clear.

Polge & Soltys (1957) modified the method of Weinman & McAllister (1947) for the preservation of trypanosomes by adding glycerol to the suspension as a freezing protectant and by using a slow rate of cooling. By these means they obtained good survival rates for 8 months. The method has since been widely applied to the preservation of protozoal material. Detailed descriptions of apparatus and methods are available (Cunningham, Lumsden & Webber, 1963; Lumsden, Robertson & McNeillage, 1966). These workers developed methods that are very simple to operate and economical of space, as the suspensions of organisms are stored in glass capillary tubes.

Material preserved at low temperatures differs fundamentally from 'strain' material maintained in continuous reproduction by serial passage in that selection of the reproducing population is avoided and stabilization of biological characters may therefore be expected. This matter is discussed by Lumsden & Hardy (1965), who propose the term 'stabilate' for material preserved in this way. They define the term stabilate as 'a population of an organism preserved in a viable condition on a unique occasion'. The concept and methods had obvious application to the preservation of *Leptospira* and the present paper describes preliminary studies to this end.

MATERIALS

Twenty-seven different strains representing 11 serotypes have been studied. Details of these are given below. The nomenclature follows the World Health Organization's (1965) classification as far as possible. New isolates or strains of unknown origin are indicated by inverted commas.

Serotype	Strains
icterohaemorrhagiae	Wijnberg; Kantorowicz; 'O'Connor'; 'Downes'; 'Kirschner'
canicola	Hond, Utrecht IV; 'Welsh'; 'Cochrane'; 'Barnes'
ballum	Castellón 3; S 102
autumnalis	Akiyami A
bratislava	'H 10'; 'H 30'; 'H 41'; 'H 43'; 'H 102 A'
pomona	Pomona
grippotyphosa	Andaman CH 31
hardjo	Hardjoprajitno
sejroe	M 84; Mallersdorf II
saxkoebing	Mus 24
bataviae	van Tienen; 'Kearslie'; 'Young'; 'Kuching'; the last three were virulent strains of Sarawak origin

Some of these strains were provided by Prof. J. Wolff, Institute for Tropical Hygiene and Geographical Pathology, Amsterdam, and some by Dr L. H. Turner, London School of Hygiene and Tropical Medicine.

Strain 'Downes' was isolated in blood culture from a case of Weil's disease in a farm worker, strains 'Welsh' and 'Cochrane' from cases of canicola fever in pig farm workers and strain 'Barnes' from the urine of a pig on a farm associated

with another human case of canicola fever. These four strains were isolated in the Edinburgh area. Strains H10, H30, H41, H43 and H102A were isolated from the kidneys of hedgehogs, the first three caught near Edinburgh and the others near Compton, England (sent by Dr A. McDiarmid, Agricultural Research Council, Institute for Research in Animal Diseases, Compton, Berkshire). Strain designated 'Kirschner' was supplied by Dr Kirschner, Otago University, New Zealand.

METHODS

The leptospires were grown in small screw-capped bijou bottles (A. R. Horwell, Kilburn, London) containing 3 ml. modified Stuart's medium (Bryan, 1957) until they reached their maximum density, usually in 5–7 days. Before preservation, each culture was tested for freedom from contaminants by plating on blood agar. Since no antibiotics were used it was essential to apply strictly aseptic technique throughout the procedure.

The apparatus used for preservation was essentially the same as that described and illustrated by Cunningham *et al.* (1963) and Lumsden *et al.* (1966). The capillary tubes (lymph tubes, 4 in. long by 1 mm. outside diameter) were supplied by Messrs Plowden and Thompson, Stourbridge, England. The storage unit was made by Messrs E. K. Bowman, London, England; it can accommodate up to 12,000 tubes in 100 individually numbered compartments. This makes the locating of required material a simple procedure. The unit is filled with methanol and stored in a dry ice cabinet (Lumsden & Webb, 1961).

Glycerol was added to the culture to be preserved to give a final concentration of about 7·5% by volume. The mixture was transferred to the capillary tubes held *in a rack (Cunningham et al. 1963) by means of a Pasteur pipette and teat.* The suspension flows into the tubes readily by capillary action and when a tube is about half filled the pipette is withdrawn and transferred to the next one and so on. When all the tubes have been so treated, the rack is tilted backwards and forwards so that the suspension comes to occupy the centre of each tube; the ends of the tubes are then sealed in a micro-burner. Care should be taken to ensure that the tubes are completely sealed. The tubes are taken from the rack and transferred to a cork-stoppered tube (120 mm. × 15 mm.) containing absolute methanol, which is then placed in an insulating jacket of Onazote (Expanded Rubber and Plastics, Ltd., Croydon, England) of walls 25 mm. thick and deposited in the dry ice cabinet. The rate of cooling of the material under these conditions has been defined by Lumsden *et al.* (1966): $-60°$ C., is reached in about 50 min. After 18–24 hr in the dry ice cabinet, when the temperature of the material will be approximately $-79°$C., the test tube is transferred quickly to a bath of methanol at $-79°$ C. for manipulation of the capillaries contained in it into the containers for permanent storage.

When a particular strain is required, one capillary tube is withdrawn from the appropriate compartment in the storage unit and wiped free of alcohol with a sterile pad of cotton-wool. Each end of the tube is carefully marked with a glass cutter and broken off and passed quickly through a gas flame. The contained

suspension, now thawed, is blown out into bottles containing 3 ml. Stuart's medium. This is done aseptically by means of a perforated rubber teat attached to one end of a 5 cm. length of glass tubing plugged with cotton-wool (Fig. 1). The other end of the tube is fitted with a soft rubber stopper of the kind used for vaccine

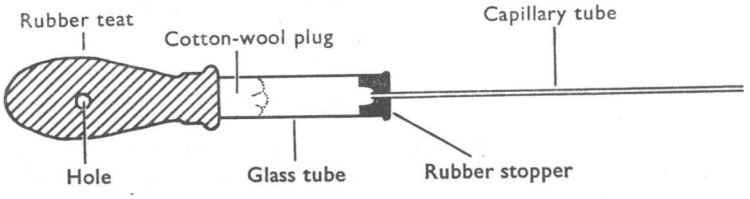

Fig. 1. Device used for transferring contents of capillary tube aseptically to culture medium.

Table 1. *Recovery rate of* 21 *strains of* Leptospira *preserved by freezing during the period* 7 *February–*9 *June* 1964

		No. of months frozen									
Serotype	Strain	4	5	6	7	8	12	18	24	27	Total
canicola	Welsh	1/1	—	2/2	3/3	—	1/2	—	—	0/4	7/12
ballum	S 102	1/1	—	2/2	1/3	—	—	—	—	—	4/6
canicola	Hond Utrecht IV	—	—	3/3	2/2	2/3	—	—	—	0/4	7/12
saxkoebing	Mus 24	—	2/3	1/3	0/1	—	—	—	—	—	3/7
ictero-haemorrhagiae	Wijnberg	—	—	1/3	4/4	1/1	—	2/2	—	4/4	12/14
ictero-haemorrhagiae	Kantorowicz	—	3/3	—	—	—	—	—	0/4	—	3/7
pomona	Pomona	—	—	2/2	—	—	—	—	0/4	—	2/6
bratislava	H 10	—	—	2/2	—	—	—	—	—	0/4	2/6
sejroe	M 84	—	2/2	1/1	2/3	—	—	—	—	—	5/6
sejroe	Mallersdorf II	—	2/2	—	—	—	—	—	0/4	—	2/6
grippotyphosa	Andaman CH 31	—	2/2	3/3	1/1	—	—	—	—	4/4	10/10
ictero-haemorrhagiae	Downes	—	3/3	2/2	1/1	—	2/2	—	—	—	8/8
hardjo	Hardjo-prajitno	—	—	2/2	—	—	—	2/2	—	—	4/4
bratislava	H 102 A	—	2/2	—	—	—	—	0/2	—	—	2/4
ballum	Castellón 3	—	2/2	3/3	—	—	—	—	—	—	5/5
autumnalis	Akiyami A	—	2/2	—	—	—	—	—	—	—	2/2
ictero-haemorrhagiae	Kirschner	—	—	2/2	—	—	—	2/2	—	—	4/4
bataviae	van Tienen	—	2/2	—	—	—	0/2	—	—	—	2/4
canicola	Cochrane	1/2	—	—	—	—	—	—	—	—	1/2
canicola	Cochrane	1/2	—	—	—	—	—	—	—	—	1/2
bratislava	H 30	2/2	—	—	—	—	—	—	—	—	2/2

Percentage number of cultures satisfactory, 68.

or dental anaesthetic tubes. A small hole is pierced in the rubber stopper so that the end of the capillary tube can be inserted into it (a number of these tubes with

stopper are sterilized in a container ready for use). By applying finger pressure to close the hole in the teat and to compress the teat, the suspension in the lymph capillary can be blown out of the other end into the medium.

RESULTS

Between 7 February and 9 June 1964, capillaries of 21 different strains of *Leptospira* were frozen and then stored for periods ranging from 4 months to $2\frac{1}{4}$ years (Table 1). Every one of the strains was recovered after freezing, although not all of them survived for the same length of time.

Table 2. *Recovery rate after preservation by freezing of eight cultures aged* 3–8 *weeks*

Serotype	Strain	Age (weeks)	Period of storage months	Recovery rate: Living organisms seen in culture	Recovery rate: Satisfactory multiplication in culture
icterohaemorrhagiae	Kearlsey	4	2	2/2	2/2
			19	2/2	0/2
		8	5	2/2	0/2
			6	—	0/2
			7	—	0/2
			19	0/2	0/2
bataviae	Young	3	2	2/2	2/2
			19	2/2	0/2
		3	5	2/2	0/2
			6	—	0/2
			7	—	0/2
			19	1/1	0/2
bataviae	O'Connor	4	2	2/2	2/2
			19	1/2	0/2
		4	5	2/2	0/2
			6	—	0/2
			7	—	0/2
			19	2/2	0/2
bataviae	Kuching	5	2	2/2	2/2
			19	1/2	0/2
		5	5	1/2	0/2
			6	—	0/2
			7	—	0/2
			19	1/1	0/2

With one exception, growth resulted from the addition of the contents of one capillary tube to the culture medium; in one case two tubes were necessary. Not every tube has resulted in growth, some of the tubes were improperly sealed and the content of others was contaminated, probably by faulty manipulation at the time of culturing the thawed material. A total of 129 attempts to culture the preserved leptospires were made of which 88 (68%) were successful. Eight of 20 cultures prepared from leptospires preserved for $2\frac{1}{4}$ years resulted in satis-

factory growth of density equivalent to the original culture after 10 days' incubation; seven others were seen to contain a few motile leptospires but these failed to multiply even in subculture.

During January 1965, eight additional cultures of four strains of leptospira (1 *icterohaemorrhagiae* and 3 *bataviae*) were preserved by freezing. The ages of these cultures ranged from 3 to 8 weeks. They were tested for survival and ability to multiply at periods of 2–19 months after freezing. The results are given in Table 2.

Cultures of leptospires that were 3, 4 and 5 weeks old when frozen were satisfactorily cultured 2 months later. None of them, however, could be cultured after periods of 5–19 months' storage although active organisms were seen in 17 out of 22 of the cultures.

In all, 177 cultures have been attempted. Ninety-six (54 %) were satisfactory and 81 (46 %) failed to produce growth.

DISCUSSION

The results presented indicate that the long-term viable preservation of *Leptospira* materials is possible by storing suspensions in glass capillary tubes at −79° C. In most cases the organisms survived and could be re-established in culture for periods of up to over 2 years. The possible practical applications of such a method of preservation are obvious. It should be possible to lay down standard materials available for reference over long periods—the concept of stabilates as discussed by Lumsden & Hardy (1965). For this, further studies are required: quantitative to define the proportions of the organisms which ultimately survive the processes of preservation, storage and retrieval from preservation: and qualitative to establish the concordance of the characteristics of the organisms before and after treatment.

The factors that determine survival require fuller study. Even in the cases in which attempts to recover leptospires in culture were unsuccessful, actively motile leptospires could often be observed by dark-ground microscopy. Some may even have reproduced to some extent before dying out as motile leptospires were observed in subculture. The factors which seem likely to influence success are, in our consideration: the age of the culture when preserved and the quality of the culture medium used.

The use of a chemically-defined medium and the possibility of additional growth factors to stimulate multiplication of the organisms that have been devitalized by the freezing process would no doubt improve the reliability of the procedure.

SUMMARY

1. A simple method for the long-term viable-preservation of samples of *Leptospira* suspensions is described. Quantities of about 25 μl., with the addition of 7·5 % glycerol, are introduced into capillary tubes, cooled slowly to −79° C. and stored at that temperature.

2. More than half the attempts were successful in re-establishing growth in culture after storage at −79° C. for periods up to 27 months.

3. The possibility that the method could be used for establishing 'banks' of standard *Leptospira* material for reference is discussed.

Mrs Jean Smith contributed greatly to the work by her painstaking carrying out of the preparation and examination of cultures.

REFERENCES

ANNEAR, D. I. (1956). Preservation of leptospirae by drying. *J. Path. Bact.* **72**, 322.

ANNEAR, D. I. (1958). Observations on the preservation by drying of leptospirae and some other bacteria. *Aust. J. exp. Biol. med. Sci.* **36**, 1.

BRYAN, H. S. (1957). Studies on leptospirosis in domestic animals. *Vet. Med.* **52**, 111.

CUNNINGHAM, M. P., LUMSDEN, W. H. R. & WEBBER, W. A. F. (1963). Preservation of viable trypanosomes in lymph tubes at low temperature. *Expl Parasit.* **14**, 280.

KIRSCHNER, L. (1958). Growth, purification and maintenance of *Leptospira* on solid media. *Proc. 6th int. Congr. trop. Med. Malar.* **4**, 434.

LUMSDEN, W. H. R. & HARDY, G. J. C. (1965). Nomenclature of living parasitic material. *Nature, Lond.* **205**, 1032.

LUMSDEN, W. H. R., ROBERTSON, D. H. H. & MCNEILLAGE, G. J. C. (1966). Isolation, cultivation, low temperature preservation and infectivity titration of *Trichomonas vaginalis*, Donné, 1837. *Br. J. vener. Dis.* **42**, 145.

LUMSDEN, W. H. R. & WEBB, C. J. (1961). Dry-ice cabinet. *Rep. E. Afr. Trypan. Res. Org.* 1960, p. 46.

POLGE, C. & SOLTYS, M. A. (1957). Preservation of trypanosomes in the frozen state. *Trans. R. Soc. trop. Med. Hyg.* **51**, 519.

TARASEVICH, M. N., BULK, B. F. & MUDROVA, P. L. (1963). A method of conservation of pathogenic leptospira organisms while preserving their virulence. *J. Hyg. Epidem. Microbiol. Immun.* **7**, 352.

WEINMAN, D. & MCALLISTER, J. (1947). Prolonged storage of human pathogenic protozoa with conservation of virulence. *Am. J. Hyg.* **45**, 102.

World Health Organization (1965). Classification of leptospires and recent advances in leptospirosis. *Bull. Wld Hlth Org.* **32**, 889.

WOLFF, J. W. (1960). Preservation of cultures of leptospires. *Pol. Acad. Sci. Sess. Ser.* **19**, 11.