Short-term storage of *Haemophilus influenzae*

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SYNOPSIS By a simple procedure, using a readily reproducible medium, most strains of *H. influenzae* can be stored for four weeks or more and can be sent through the post. Capsulate strains can be stored in this way without apparent change.

Haemophilus influenzae has a reputation for being difficult to maintain in culture. For example, Stokes (1962), reporting on short-term preservation of bacteria, suggested two days as the life expectation of haemophilus cultures on blood agar or chocolate agar, whereas all the other organisms listed except the pathogenic Neisseriae were given a much better prognosis. It is also generally believed that capsulate *H. influenzae* strains readily lose their capsules, and hence their type-specificity, in laboratory cultures (Cruickshank, 1960). These difficulties have discouraged full investigation of the role of this species, and of capsulate strains in particular, in human health and disease.

In our experience it is not in fact difficult to keep most strains of *H. influenzae* alive for weeks or months, using a medium and techniques well within the scope of any bacteriological department; and capsulate strains kept in this way have shown little tendency to lose their special characters.

The method as it is routinely used will be described first, and then some experiments showing its value and the consequences of varying it in certain particulars.

STORAGE PROCEDURE

MEDIUM This is a simple chocolate agar, made by adding 10% of oxalated horse blood to melted nutrient agar, heating the mixture in a water-bath at 75°C. until it is of a chocolate colour, and allowing 2 ml. amounts to solidify in a sloping position in $\frac{1}{4}$ -oz. screw-capped bottles. The following nutrient agar formula has proved satisfactory:—

Lab.-Lemco (Oxoid)	1%
Peptone (Oxoid)	1%
NaCl	0.5%
New Zealand agar (Oxoid)	1.2%

with pH adjusted to 7.5.

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USE OF MEDIUM A slope is heavily inoculated with a pure culture of the organism to be stored. The cap is firmly screwed on, and the culture is incubated at 37°C. for 18 to 24 hours. It is subsequently stored in a closed box at room temperature.

RESULTS

In the first three trials to be described (A, B, and C) the inocula for the chocolate agar slopes were taken from overnight plate cultures on Levinthal's agar, which was made according to the recommendations of Alexander (1958) apart from the substitution of oxalated for defibrinated horse blood. Capsulate strains of *H. influenzae* are recognizable on this medium by the iridescence and confluence of their colonies.

Survival of chocolate agar cultures after incubation and storage was tested by subculturing them on to oxalated-horse-blood agar plates across which a stock culture of *Staphylococcus aureus* was then streaked. Satellite growth in the vicinity of the staphylococcal streak after overnight incubation was taken to indicate survival of the *H. influenzae*. Strains which had been capsulate before storage were also examined for persistence of their ability to produce iridescent growth on Levinthal's agar.

MAINTENANCE OF CAPSULATE STRAINS Of the 20 capsulate strains used in trials A and B, 11 (eight of type b, two of type e, and one of type a) were locally isolated from apparently unconnected sources. The other nine (three of type c, two of type f, and one of each of the other four types) were kindly supplied by Dr. K. Zinnemann, Dr. H. Proom, and the National Collection of Type Cultures. All were typed by slide-agglutination with sera from Messrs. Burroughs Wellcome and Co. of London, and also by capsule-swelling with sera from Messrs. Hyland Laboratories of Los Angeles. Each strain gave a pure growth of

iridescent colonies on the Levinthal's agar plate from which it was inoculated on to the chocolate agar slopes.

Trial A Eighteen capsulate strains were used (those listed above except for one of the local type b strains and the N.C.T.C. type f). Three chocolate agar slope cultures were set up from each strain. Two slopes of each trio were subcultured weekly for four weeks; the third was not opened until the end of the fourth week. The results are given in Table I,

TABLE I
RESULTS OF TRIAL A

Bottles	Recovery of <i>H. influenzae</i> from Slopes Stored for											
	One Week			Two Weeks			Three Weeks			Four Weeks		
	C	NC	Nil	C	NC	Nil	C	NC	Nil	C	NC	Nil
1 + 2 3	18	0	0	17	1	0	15	1	2	8	0	10
	—	—	—	—	—	—	—	—	—	16	1	1

C = capsulate *H. influenzae* recovered

NC = only non-capsulate *H. influenzae* recovered

Nil = no *H. influenzae* recovered

— = not subcultured

and suggest that repeated opening of the bottles impaired the survival of the cultures. The only strain which failed to survive in the unopened bottle gave pure growths of iridescent colonies from the other two bottles after four weeks. Thus 17 of the 18 strains yielded iridescent colonies from at least one culture at the end of the trial, and these survivors gave specific slide-agglutination and capsule-swelling reactions. The strain which yielded only non-capsulate colonies from the third bottle after four weeks had given the same result from the other two bottles after two and three weeks but failed to grow at all from them after four weeks. One of the strains recorded in Table I as giving a growth of capsulate strains on all occasions in fact gave a mixture of iridescent and non-iridescent colonies from each of the first two bottles after two, three, and four weeks, but only iridescent colonies from the third bottle.

Trial B All 20 capsulate strains were stored on pairs of chocolate agar slopes which were not opened until the end of the fourth week. The strain which had given a mixture of iridescent and non-iridescent colonies in trial A did so again from both slopes. The strain which had ceased to be iridescent in trial A gave a growth of iridescent colonies from one bottle in trial B but failed to grow from the other. Each of the remaining 18 strains gave pure growths of iridescent colonies from both bottles. Thus 39 of the 40 cultures yielded growths of iridescent colonies after four weeks' storage. All

strains still gave appropriate type-specific capsule swelling reactions; their slide-agglutination reactions were not tested.

No growth was obtained from any of the 40 slopes when they were subcultured again after a further four weeks.

MAINTENANCE OF FRESHLY ISOLATED STRAINS All of the strains used in trials A and B were capsulate, and most of them had been isolated months or even years before the trials. The most useful application of short-term storage procedure is to the temporary maintenance of freshly isolated strains, capsulate and non-capsulate.

Trial C Fifty-seven strains of factor-V-dependent, non-haemolytic, Gram-negative bacilli were isolated from routine specimens in the Bacteriology Department of the Royal Victoria Infirmary during seven weeks of October, November, and December 1962. Twenty-five of these were tested for factor X dependence and were all found to be *H. influenzae*, but some of the others may have been *H. parainfluenzae*. So far as possible all strains were taken direct from the primary cultures (usually on staphylococcus-streaked blood agar) to Levinthal's agar and thence to the chocolate agar slopes, but purification and identification often required preliminary subcultures. Thus the strains underwent between two and five transfers before storage. Two of them failed to survive these procedures.

The remaining 55 strains were stored on pairs of chocolate agar slopes as follows:—

Series 1 Twenty-five strains (three of them capsulate) on the usual chocolate agar, which will be referred to as medium X.

Series 2 Twenty-five other strains (two of them capsulate) on a chocolate agar which will be referred to as medium Y. This was made from nutrient agar (Oxoid), and differed from medium X in containing only one-tenth of the amount of Lab-Lemco but an addition of yeast extract.

Series 3 Five other strains (all non-capsulate) both (a) on medium X and (b) on medium Y.

The inclusion of medium Y was at first accidental. In consequence, no attempt was made to ensure that series 1 and 2 were strictly comparable. But in series 3, cultures on the two media were set up simultaneously from the same strains and were incubated and stored together.

All slopes of all three series were subcultured once only, four weeks after inoculation. As shown in Table II, 27 out of 30 strains survived on medium X, but only nine out of 30 on medium Y. Furthermore, only scanty growths were recovered from some of the slopes of medium Y, whereas most of the subcultures from medium X grew profusely.

TABLE II

RESULTS OF TRIAL C

Series	No. of Strains Tested	Medium	Strains Recovered from			Survival Rates (%) of	
			Both Bottles	One Bottle	Neither Bottle	Strains	Individual Cultures
1	25(3) ¹	X	16(3)	6	3	88	76
2	25(2)	Y	3	3(1)	19(1)	24	18
3(a)	5	X	4	1	0	100	95
3(b)	5	Y	1	2	2	60	40

¹Figures in parentheses refer to capsulate strains. All such strains which survived gave pure growths of iridescent colonies on Levinthal's agar after recovery from the storage medium.

POSTAL TRANSMISSION OF STRAINS FROM OTHER LABORATORIES To exclude the possibility that unusually robust *haemophilus* strains prevail in Newcastle, and to confirm that organisms stored in this way can survive journeys by post, chocolate agar slopes (medium X) were sent to three other bacteriologists, who inoculated them with *haemophilus* strains which they had isolated, incubated them overnight, and returned them by post.

Dr. J. R. May, of the Institute of Diseases of the Chest, Brompton, London, sent 24 pairs of slopes, 12 of them inoculated with laboratory strains and 12 with fresh isolates. The inocula were taken from overnight cultures on agar containing yeast extract and haemin. All 48 slopes were subcultured between two and three weeks from their inoculation dates, and all yielded heavy growths of factor-V-dependent organisms. Three of the laboratory strains were identified as capsulate *H. influenzae*, of types b (two strains) and f. Dr. May subsequently confirmed that these were the only known capsulate strains included, and that the typing agreed with his own.

Dr. C. H. Jellard, of the Public Health Laboratory, Plymouth, sent 10 pairs of cultures of freshly isolated strains, taken from overnight cultures on blood agar or chocolate agar. All were subcultured between two and three weeks from their inoculation dates. One strain had grown well on both slopes but failed to grow on subcultures. The other 18 slopes all yielded heavy growths of factor-V-dependent organisms.

Dr. E. J. Stokes, of University College Hospital, London, sent pairs of cultures from five freshly isolated strains and single cultures from seven others, all taken from overnight cultures on oxalated-horse-blood agar. Most of these were subcultured as soon as they were received, the intervals between inoculation and subculture varying from two days to six weeks but mostly exceeding two weeks. Of the 17 slopes, three subcultured after 15, 17, and 28 days respectively failed to yield *haemophili*. Since there was a duplicate culture for one of these three which yielded growths 10 of the 12 strains survived.

No capsulate strains were known to have been included among those sent by Dr. Jellard and Dr. Stokes, and none was found among their subcultures.

One of Dr. Stokes' strains was markedly haemolytic when first isolated, but gave only feeble lysis when received in Newcastle and none at all after being returned to Dr. Stokes.

DISCUSSION

Freeze-drying is the safest and least troublesome method of storing *H. influenzae* cultures for long periods. The method described here should enable laboratories which have no freeze-drying facilities to maintain *H. influenzae* strains and to send them elsewhere for further investigation. It is in any case more convenient than freeze-drying for the temporary storage of strains that are awaiting identification or are in frequent use.

No thorough attempt has been made to find out which features of the method are essential for survival of *H. influenzae*. But some comments can be made.

MEDIUM FROM WHICH STRAINS ARE TAKEN Satisfactory results have been obtained with cultures taken from Levinthal's agar, from an agar containing yeast extract and haemin, from chocolate agar, and from blood agar made in two different laboratories, and no unsatisfactory media have yet been found.

STORAGE MEDIUM There are many ways of making 'chocolate agar'. The comparison of media X and Y in trial C shows that the composition of the storage medium is important. One variety of chocolate agar in routine use here, which is based on a locally made ox-heart infusion and supports far more profuse growth of *H. influenzae* than does medium X, is at least comparable with the latter for storage purposes; but it was not included in the trials because it would not be readily reproducible elsewhere.

STORAGE TEMPERATURE In a limited number of tests with only a few strains of *H. influenzae*, cultures stored at room temperature after incubation survived better than did those stored at 4°C. or 37°C. after incubation or those stored at room temperature without preliminary incubation.

OTHER STORAGE CONDITIONS The use of a cardboard box for holding the stored cultures has been a matter of convenience, but may have some other merit, such as the exclusion of light. Opening of the cultures shortens their survival. As a corollary of that, it is probably important that the caps of the bottles should fit well and be tightly screwed up.

RECOVERY OF STORED STRAINS *H. influenzae* usually grows well on the Levinthal's agar used in this work. However, strains stored on chocolate agar slopes often fail to grow if transferred directly to Levinthal's agar, although they grow well on it if allowed to grow first on staphylococcus-streaked blood agar. This may be due to the fact that the Levinthal's agar

is made largely from dehydrated materials and the blood agar from fresh ox-heart infusion. In any case, it is clear that failure to recover stored haemophilus strains may result from inadequacy of the medium used for subculture rather than from death of the stored organisms.

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