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The preparation of simulated water samples for the purpose of bacteriological quality control

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SUMMARY

As part of the bacteriological quality control programme of the Public Health Laboratory Service, we were asked to investigate the possibility of providing simulated water samples for distribution to the laboratories. For this purpose it was necessary to find some means whereby suspensions of coliform organisms and *Escherichia coli* could be kept relatively stable in number at room temperature for a period of 7–10 days. This, it was finally found, was best achieved by adding selected strains of the organisms to improved formate lactose glutamate medium (Gray, 1964) without the lactose but with added boric acid to a final concentration of 1·8 %.

The procedures adopted in the successful quality control programme are described.

INTRODUCTION

In the preparation of simulated samples of polluted water for the purpose of the quality control programme, the main problem was to find some means of maintaining the stability of suspensions of coliform organisms and *Escherichia coli* at ambient temperature for periods up to 10 days.

It is well known that coliform organisms have a limited survival time in water. Variations occur even after 6 hr. storage, with significant decreases in numbers occurring more frequently than increases (Reports, 1952, 1953). It was clear that, if liquid suspensions were to be used, some form of preservative plus a suitable nutritive source would have to be found.

An alternative method, of using freeze-dried cultures on soluble paper disks which could be reconstituted in sterile water, was also considered. This investigation was kindly carried out by Dr S. P. Lapage and Mr J. P. Fisher of the National Collection of Type Cultures, Central Public Health Laboratory, Colindale. Their results showed that it was impossible to produce, with any degree of accuracy, disks yielding the relatively small numbers of coliform organisms (50–100) required for the investigation. In addition, Mr Fisher (personal communication) found that some cultures of *Esch. coli* after freeze-drying had lost their ability to ferment lactose at 44° C. when reconstituted in the manner described.

Two methods employing chemical agents for the preservation of bacteria have been described. Hoather (1952, 1957) suggested that the addition of sodium thiosulphate to water samples was effective in the short term in stabilizing the

coliform and *Esch. coli* content. He suggested that sodium thiosulphate at 100 mg./l. was the most effective concentration. The American Public Health Association (1955) also recommended this concentration to decrease the death rate of coliform organisms in water on storage.

Storage experiments were subsequently carried out using a selected strain of *Esch. coli* contained in an aqueous solution of sodium thiosulphate, 100 mg./l. Although a stable count was maintained for 24 hr. rapid multiplication occurred after this time. It would appear that, after an initial resting period, the sodium thiosulphate was being utilized for growth by this particular strain of *Esch. coli*.

A more hopeful approach to the problem was offered by the findings of Porter & Brodie (1969), who found that the content of coliform bacteria and other organisms in urine samples was maintained at a stable figure by the addition of boric acid to a final concentration of 1·8 % to the urine on collection. It was therefore decided to observe the effect of boric acid on the stability of selected strains of coliform organisms and *Esch. coli* in media containing various nutrients.

MATERIALS AND METHODS

Test organisms

Pilot storage experiments were carried out using various strains of coliform organisms which were isolated from water samples examined in this laboratory. Single strength improved formate lactose glutamate medium (Gray, 1964) with the lactose, thiamine and indicator omitted (I.F.L.G. LAC−) plus 1·8 % boric acid was the medium used. The results obtained showed that variation existed between individual strains regarding survival after prolonged storage. However, a strain of *Esch. coli* (E.C. 1) was found to have a reasonable storage survival time, and this strain was selected for further investigations.

Storage media

The following storage media were investigated.

(1) I.F.L.G. LAC− medium plus boric acid.

(2) Sodium thiosulphate 100 mg./l. in deionized water.

(3) Sodium thiosulphate 100 mg./l. in deionized water plus boric acid.

(4) Glutamic acid 5 g./l., di-potassium phosphate 3 g./l. in deionized water plus boric acid.

(5) Nutrient broth plus boric acid.

Also included were deionized water and deionized water plus boric acid. Boric acid was used in all instances at 1·8 % concentration. The pH of media 1, 2, 3 and 5 was adjusted to 6·8 before the addition of the boric acid. The media were sterilized by steaming (100° C.) for 1½ hr.

Storage suspensions

Three hundred ml. of each storage medium was inoculated with a volume of an overnight I.F.L.G. culture of E.C. 1 calculated to give a final concentration of approximately 100 organisms/ml.

These media were then stored in a dark cupboard at room temperature. At suitable intervals the stored suspensions were well mixed and 3 ml. of each was added to 300 ml. of sterile deionized water. The prepared samples were then examined by the multiple tube method (5×10 ml., 5×1 ml. and $5 \times 0{\cdot}1$ ml. vols.) (Report, 1969). One hundred ml. of each sample was also passed through a membrane filter and grown on enriched Teepol broth (Report, 1969). The results of the multiple-tube method were recorded after incubation for 24 and 48 hr. at 37° C. The membrane filtration results were recorded after 24 hr. incubation at 37° C.

RESULTS

The counts on storage obtained by the multiple-tube method are shown in Table 1. The counts obtained by membrane filtration were similar but are not shown. It was apparent that with strain E.C. 1 it was possible to maintain a stable viable count of minimal numbers for 10 days in three of the storage media examined (see significant counts in bold figures). As expected, death occurred fairly rapidly in the sample of deionized water and the addition of boric acid prolonged survival for only a few more days. Multiplication occurred in the sodium thiosulphate solution after the first day of storage, but surprisingly, the addition of boric acid converted this into a reasonable storage medium. The I.F.L.G. LAC− medium and nutrient broth plus boric acid also provided good survival rates, but the glutamic acid phosphate mixture failed to maintain adequate numbers of *Esch. coli* after 4 days' storage.

Of the three successful media it was decided to adopt the I.F.L.G. LAC− boric acid medium for further storage experiments. It was subsequently found that, with experience, accurate counts which remained stable up to 10 days could be consistently obtained. Using this medium for storage, a strain of *Klebsiella aerogenes* (K.A. 1) which gave survivals on storage similar to those of culture E.C. 1 was found. This organism fermented lactose at 37° C. within 24 hr. and failed to do so at 44° C.

With the possession of strains of *Esch. coli* and *Klebsiella aerogenes*, representative of the so-called faecal and non-faecal coliform types, which could be maintained in relatively small numbers for up to 10 days at room temperature, it was felt that the preparation and distribution of simulated polluted water samples for the purpose of quality control could now be attempted.

PILOT TRIALS

Although samples with predictable coliform counts could be readily prepared it was felt that a series of simulated samples should be sent to other laboratories to assess our results. Eight Public Health Laboratories, in various parts of the country, who examine water samples routinely, participated in the pilot programme.

Suspensions of E.C. 1, K.A. 1 and a mixture of both organisms in I.F.L.G. LAC− boric acid medium were distributed in 6 ml. vols. into $\frac{1}{4}$ oz. screw-capped bijou bottles. The final counts of each suspension were calculated to give a predicted count when 3 ml. of the suspension was added to 200 ml. of sterile deionized water.

Table 1. *The survival of* Escherichia coli *on storage in various media*

Storage medium	Number of days of storage													
	0	1	2	4	6	8	10	12	14	16	18	20	22	24
Deionized water	170	110	5	4	0	—	—	—	—	—	—	—	—	—
Deionized water with boric acid	350	130	110	80	2	0	—	—	—	—	—	—	—	—
Deionized water with sodium thiosulphate	350	550	$1{\cdot}8\times10^{4}$	2×10^{8}	$1{\cdot}1\times10^{7}$	$2{\cdot}5\times10^{7}$	$3{\cdot}5\times10^{7}$	$2{\cdot}5\times10^{7}$	$2{\cdot}5\times10^{7}$	9×10^{6}	$1{\cdot}6\times10^{6}$	ND	$1{\cdot}6\times10^{7}$	ND
Deionized water, sodium thiosulphate and boric acid	250	140	80	170	80	80	130	130	ND	5	0	—	—	—
I.F.L.G. LAC – medium and boric acid	110	110	250	140	50	225	250	50	80	17	8	2	0	—
Glutamic acid, di-potassium phosphate and boric acid	350	170	175	130	80	0	—	—	—	—	—	—	—	—
Nutrient broth and boric acid	170	130	350	250	110	110	250	50	50	35	ND	13	4	0

The counts are the most probable number per 100 ml., obtained by the multiple-tube method.

Table 2. *Pilot investigation of simulated water samples*

Laboratory	Sample A: *Esch. coli* and *K. aerogenes* mixed		Sample B: *K. aerogenes* only		Sample C: *Esch. coli* only	
	Tube	Membrane	Tube	Membrane	Tube	Membrane
Newport						
Stored 1	90/25*	38†	17/0	13	50/50	50
Stored 2	50/25	50	17/0	7	50/50	43
Stored 3	180+/18	52	14/0	4	50/50	58
Postal	50/35	52	20/0	10	50/50	63
Brighton	90/50	—	35/0	—	50/50	—
Colindale	35/17	—	20/0	—	90/90	—
Manchester	160/50	—	17/0	—	180+/180+	—
Southend	90/50	—	13/0	—	160/160	—
Sunderland	90/50	—	25/0	—	50/50	—
Truro	25/25	32/20	8/0	12/0	25/25	36/21
Worcester	50/14	—	35/0	—	90/90	—
Wakefield	50/50	—	25/0	—	50/50	—

* In all pairs of figures separated by an oblique stroke, the first is the MPN of coliform bacilli per 100 ml., and the second is the MPN of *Escherichia coli* per 100 ml.

† Single figures represent total counts only, per 100 ml.

It was found necessary to submit the samples in concentrated small volumes to avoid postal problems (three simulated samples could be dispatched by letter post in a small box). Each laboratory was asked to store the samples on receipt in a dark cupboard at room temperature and to reconstitute and examine on a selected day. The day chosen was usually 5 days after the samples had been dispatched.

The results of a typical trial investigation are shown in Table 2. With the three simulated samples all of the examining laboratories gave comparable counts. It became evident that provided the instructions given regarding the storage and reconstitution of the simulated samples were strictly adhered to, the distribution of simulated polluted water samples giving dependable coliform counts was possible.

Results of quality-control programmes

Three annual programmes have now been successfully carried out and a considerable amount of information has been gathered regarding the quality of the methods used for the examination of water from most of the examining laboratories in this country. The simulated water samples showed very little variation from the day of preparation to the day of examination, and no apparent difficulties were experienced by the examining laboratories with regard to the reconstitution of the concentrated samples. The simulated samples presented have ranged from polluted swimming-bath water, seepage water suspected of consisting of sewage and various mixtures simulating contaminated rural and chlorinated supplies. Table 3 shows the results obtained in this laboratory from samples examined from various programmes.

Table 3. *Results obtained from simulated polluted water samples before and after storage*

	Counts on					
	Day of preparation			Day of examination (9 days storage)		
	MPN per 100 ml.			MPN per 100 ml.		
Type of simulated sample	Coliform organisms	*Esch. coli*	Plate count per 100 ml.	Coliform organisms	*Esch. coli*	Plate count per 100 ml.
Swimming bath water (1974 programme)	160	25	268	90	50	131
				90	35	460
				35	13	215
				90	20	502
				160	13	520
Unchlorinated rural supply (1974 programme)	90	0		20	0	—
				35	0	—
				50	0	—
				90	0	—
				90	0	—
Seepage water (consisting of sewage?) (1975 programme)	900	50		550	35	—
				450	17	—
				250	17	—
				550	35	—
				900	11	—

(The five samples of each simulated specimen examined were selected at random.)

DISCUSSION

The method described has proved quite satisfactory in Great Britain and the various distributions have shown the counts to remain reasonably stable for 10 days. It would seem possible, therefore, that the method could be applied on a wider scale, e.g. within the E.E.C. countries, as the 10-day period would be sufficient for postal transport to the continent. In any case the programme has provided much valuable quality control information and will continue in this country.

Since this quality control programme has been operating we have been asked whether the method of preserving the suspensions could be used for the submission of actual samples of water in parts of the world where the transport of the sample to the laboratory takes several days. Unfortunately this is not so. The whole essence of the method is that the suspensions are transmitted in concentrated form and that before examination the simulated sample is very considerably diluted whereby the boric acid effect is eliminated. For remote parts of the world where laboratories are sparsely distributed, it would seem to us that membrane filtration at the sampling point with transmission of the membrane would offer more hope of overcoming transport difficulties.

APPENDIX

The preparation of simulated polluted water samples

Two strains of coliform bacteria *Esch. coli* (E.C. 1) and *Klebsiella aerogenes* (K.A. 1) were used for preparing the simulated samples. A strain of *Serratia*

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liquefaciens was also used for the simulation of a high bacterial plate count when polluted swimming bath water was simulated.

Procedure

One standard drop (0·02 ml.) of an overnight I.F.L.G. culture of each of E.C. 1 and K.A. 1 was added to 200 ml. of I.F.L.G. LAC− boric acid medium contained in a 20 oz. bottle. After mixing by vigorous shaking a surface colony count was performed using the method of Miles & Misra (1938). When the viable count had been ascertained a suitable volume was added to a 2 l. flask containing 750 ml. of I.F.L.G. LAC− boric acid medium to give the desired concentration of organisms or organism mixtures. As an additional safeguard a volume of the suspension was also added to two further flasks giving an estimated count of 25 % over and 25 % under the desired count.

After vigorous shaking a 3 ml. volume was removed from each flask and added to 200 ml. of sterile deionized water contained in a 20 oz. bottle. These samples were well mixed by shaking and were examined by the multiple tube and membrane filtration methods. The counts were assessed after incubation for 24 and 48 hr. and the flasks of suspensions giving the desired final counts were selected for the preparation of samples for dispatch.

The selected flasks of suspensions were well mixed by vigorous shaking (for at least 5 min.) and distributed aseptically into sterile $\frac{1}{4}$ oz. screw-capped bijou bottles, filling to the top of the bottle leaving the minimum of air space. These samples were then dispatched by post to the laboratories participating in the programme. The time taken from preparation of the samples to the day of dispatch was usually 3 days. Six days were allowed from the day of dispatch until the selected day for examination.

When the strain of *Serratia liquefaciens* was included to simulate a high bacterial plate count a slightly different procedure was adopted. An overnight culture of this organism was grown in I.F.L.G. medium containing 1 % glucose in place of the lactose and a 1 ml. volume was added to 200 ml. of I.F.L.G. LAC− boric acid medium for Miles & Misra colony counts.

Instruction to laboratories

The following instructions which accompanied each set of samples for examination were prepared in collaboration with Mr W. B. Fletcher, Epidemiological Research Laboratory, Central Public Health Laboratory, Colindale:

'*Three specimens are here enclosed. On receipt please store at room temperature in a dark cupboard and examine them on the date stated.*

'Preparation of Samples – It is important that the instructions given are meticulously carried out.

'1. HALF THE CONTENTS OF EACH BIJOU BOTTLE SHOULD BE DECANTED INTO A STERILE UNIVERSAL BOTTLE.

'2. THE BIJOU BOTTLE SHOULD BE THEN SHAKEN VIGOROUSLY AND MIXED WELL BY ASPIRATION, USING A PASTEUR PIPETTE. THE CONTENTS ARE THEN PIPETTED INTO THE UNIVERSAL BOTTLE.

'3. THE UNIVERSAL BOTTLE SHOULD THEN BE VIGOROUSLY SHAKEN FOR AT LEAST TWO MINUTES AND 3 ml OF THE CONTENTS ADDED TO 200 ml OF STERILE DISTILLED OR DEIONIZED WATER.

'This constitutes the simulated water sample which should be mixed and examined in the usual way.'

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