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RESUSCITATION AND ENUMERATION OF *ESCHERICHIA COLI* AFTER FREEZE-DRYING

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SUMMARY

The suitability and precision of a recently suggested method for the resuscitation of Enterobacteriaceae is discussed. Experiments with freeze-dried cells of *Escherichia coli* ATCC 8739, showed that the recovery of the damaged cells was quantitative and that growth did not affect the result of the subsequent MPN count in Enterobacteriaceae Enrichment Broth. The method seems to be applicable for the examination of all kinds of products, and is possibly also suitable for the determination of other types of organisms.

INTRODUCTION

In the latest edition of the Dutch Pharmacopoeia a most probable number (MPN) technique is suggested for the quantitative determination of Enterobacteriaceae in pharmaceutical preparations. This method is the same as suggested by a working party of the F.I.P. (anonymous, 1976). In a previous paper (van Doorne and Claushuis, 1979) it was shown that this method may yield too high values because of growth during the resuscitation treatment, and an alternative method was proposed. It was suggested that a suitable number of dilutions of the sample are to be mixed with the resuscitation medium, and that after a sufficiently long resuscitation period a 10-fold amount of the selective medium is added. In this way growth does not influence the result of the estimation. Although the new method seemed to function well in practice, a more detailed study using a pure culture, seemed necessary to demonstrate that reliable results are obtained with the suggested mode of resuscitation. Freeze-drying was chosen as the method for the production of sublethally injured micro-organisms (Bousfield and MacKenzie, 1976; Ingram and Mackey, 1976). Freeze-drying was chosen because it allows the production of a large number of identical samples, which can be stored. Thus the influence of variations in culture conditions and pre-stress treatment of the organisms is minimized. In addition, the behaviour of micro-organisms during lyophilization and subsequent storage could be studied.

METHODS AND MATERIALS

Test organism. Escherichia coli ATCC 8739. The stock culture was kept on slants of Tryptone Soya Peptone Yeast Extract Agar (TSA).

Lyophilization. E. coli was cultured overnight at 37°C in Tryptone Soya Peptone Yeast Extract Broth (TSB), cells were centrifuged, washed with buffered saline, centrifuged and resuspended in buffered saline. This suspension contained about 10^9 cells/ml. Volumes of 0.2 ml were added to 1.8 ml of a sterile solution of saccharose (75 g/l). The vials were loosely capped, and the suspension was freeze-dried in a (Mini Fast 470 *) freeze-dryer. The vials were closed under vacuum, sealed and stored at room temperature.

When needed, a sufficient number of vials was opened under aseptic conditions and the contents were reconstituted by adding 2 ml sterile distilled water. The reconstituted contents were pooled and used as such for further experiments.

Resuscitation and enumeration procedures

Method A. The pooled sample was mixed 1 : 10 with either Lactose Broth (LB) or TSB tempered at 37°C and incubated at 37°C. At particular time intervals aliquots were drawn and MPN counts in Enterobacteriaceae Enrichment Broth (EEB) were performed in the following way. Dilutions were made in EEB. (At zero time the pooled sample itself was used.) From appropriate levels of dilution 5 aliquots of 1 ml were used to inoculate 5 tubes with 9 ml EEB. After 24 h of incubation at 37°C the tubes were inspected for growth and the MPN-value was obtained from the table given by Meynell and Meynell (1970).

Method B. Serial 10-fold dilutions of the reconstituted suspension were made in warm (37°C) LB or TSB. From each level of dilution 5 aliquots of 1 ml were transferred to 5 empty, sterile tubes and incubated at 37°C for 6–8 h. Thereafter, 9 ml of 10/9 strength of EEB were added to each tube, and all tubes were incubated for 24 h at 37°C. MPN was obtained from the table given by Meynell and Meynell. This method is identical to the one suggested by van Doorne and Claushuis (1980).

Viable counts of the pooled sample were made on TSA by normal spread-plate procedure, using buffered peptone saline as the diluent.

Media (composition in g/l)

Buffered saline: dihydrogen potassium phosphate 3.56; disodium monohydrogen phosphate 7.23; sodium chloride 4.3. Sterilization: 20 min at 120°C.

Buffered Peptone Saline: dihydrogen potassium phosphate 3.56; sodium monohydrogen phosphate 7.23; sodium chloride 4.3; oxoid bacteriological peption 1. Sterilization: 20 min at 120°C.

Lactose Broth (Merck): beef extract 3; peptone (from gelatine) 5; lactose 5. Sterilization: 20 min at 120°C.

Enterobacteriaceae Enrichment Broth (Merck): peptone 10; glucose 5; ox bile 20;

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* Edwards Alto Vuoto, Trezzano, Italy.

brilliant green 0.015; disodium hydrogen phosphate 8.0; potassium dihydrogen phosphate 2.0. Sterilization 15 min at 100°C.

Tryptone Soya Peptone (Caso-Bouillon, Merck; yeast extract, Bio-Mérieux). Broth: peptone from casein 17.0; peptone from soya meal 3.0; glucose 2.5; sodium chloride 5.0; dipotassium hydrogen phosphate 2.5; yeast extract 3. Sterilization 20 min at 120°C. Tryptone Soya Peptone Yeast Extract Agar (Caso-Bouillon, Merck with Agar): peptone from casein 17.0; peptone from soya meal 3.0; glucose 2.5; sodium chloride 5.0; dipotassium hydrogen phosphate 2.5; yeast extract 3.0; Agar (Difco) 15. Sterilization 20 min at 120°C.

RESULTS AND DISCUSSION

The precision of MPN counts depends largely on the number of tubes inoculated from each level of dilution. Cochran (1950) calculated the standard error in $\log_1$ MPN units for up to 10 tubes. His data for 10-fold serial dilutions are shown in Fig. 1. It can be seen, that when only one tube for each level of dilution is used, as suggested in the Dutch Pharmacopoeia (1978), the standard error is 0.58. An increase in the number of tubes causes initially a sharp decrease in the standard error. However, this increase in precision levels off, and the use of more than 5 tubes seems not to be warranted, because of the large amount of work and material involved. These calculated values for the standard error can only be used when the samples are taken from ideally homogeneous suspensions. a situation which will be approached by our simple aqueous solutions. A second condition is that one cell causes visible growth within the chosen incubation period. When using a selective medium, in which sublethally injured cells are by definition inhibited, the observed MPN will represent the uninjured and the fully recovered fraction of the population. In our studies, using 10-fold serial dilutions and 5 tubes for each level of dilution, the corresponding value for the standard error is 0.259 (Cochran, 1950; cf. Fig. 1). From this value it was calculated that two results are significantly different ($P < 0.05$) when their ratio is more than 5.24.

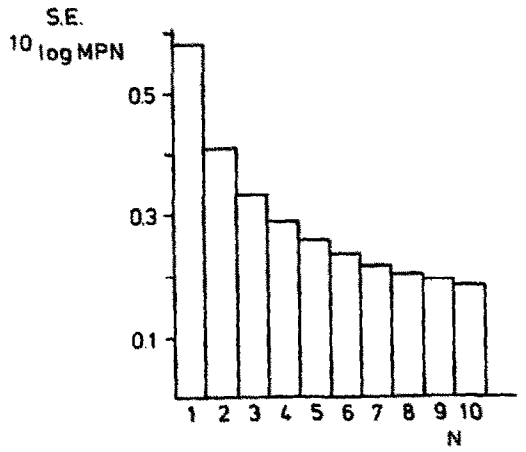

Fig. 1. Calculated standard error (S.E.) of MPN counts as function of number (N) of tubes for each level of serial 10-fold dilutions (after Cochran, 1950).

In the Dutch Pharmacopoeia LB is suggested as the resuscitation medium. It has been demonstrated that in some instances TSB is superior (Ray and Speck, 1973). The primary criterion on which preference for a certain medium should be based, is the time required to obtain complete recovery of all injured cells. Microbial antagonism between the Enterobacteriaceae and the associated part of the microbial flora may lead to erroneous results. Thus the length of the lag phase is another criterion for the selection of a suitable resuscitation medium. Using method A full stress–resuscitation curves were measured and a characteristic result is shown in Fig. 2. The point on the log N axis indicates the viable count of the suspension in peptone-saline before it was freeze-dried. The stress period, in Fig. 2 shown as the space between the vertical lines, is both the actual freeze-drying and the subsequent storage at room temperature. The right vertical line indicates the moment of resuscitation of the pellet, which is also the onset of resuscitation. The moment of complete resuscitation, t_r, cannot be determined with great precision, partly because of the large standard error of the method, and partly because only a limited number of individual measurements could be done. Interpolation of a curve drawn by hand is the best available way. The moment of resumption of growth, t_g, can be determined in an arbi-

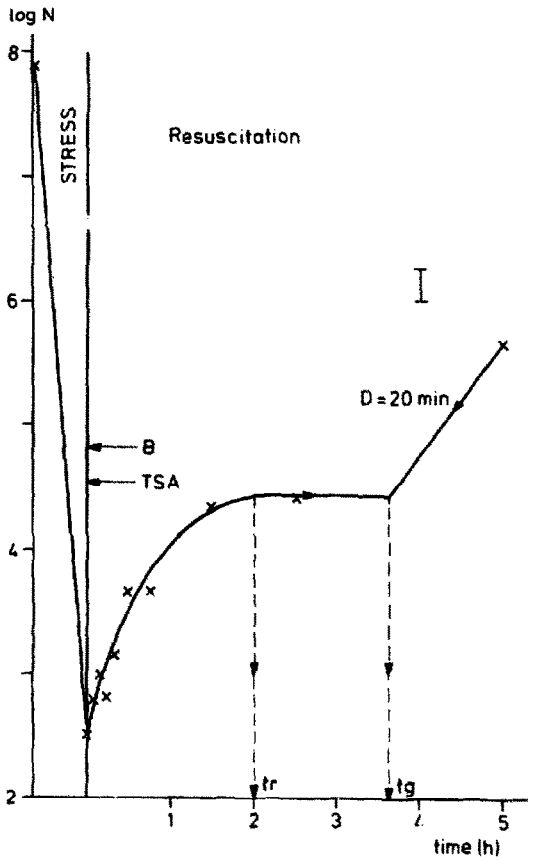

Fig. 2. Resuscitation of freeze-dried *E. coli* in Tryptone Soya Peptone Yeast Extract Broth at 37°C. Bar indicates calculated standard error of MPN counts. X: MPN counts observed with method A; arrow B: MPN count observed with method B; arrow TSA: viable count on TSA at zero time of resuscitation; t_r: complete resuscitation; t_g: resumption of growth.

trary way as shown in Fig. 2. After 5 h incubation the culture is growing. From this point a line is drawn with a slope corresponding to the generation time, D, of the bacteria (20 min); the intersection with the horizontal part of the resuscitation curve yields the moment of resumption of growth. In Fig. 2, the arrow marked B indicates the result of the MPN count in Enterobacteriaceae Enrichment Broth, using method B as the resuscitation procedure. The arrow marked TSA indicates the result of the plate count on TSA, immediately after reconstitution of the pellet.

The freeze-dried suspension was stored at room temperature for up to 98 days. At different times it was resuspended and t_r and t_g were measured by method A. The observed values for t_r and t_g in both LB and TSB are summarized in Table 1.

Under the chosen experimental conditions, LB and TSB seem to be equally suitable for the resuscitation of freeze-dried cells of *E. coli*. Resuscitation is generally complete within 3.5 h. Although under practical conditions even shorter periods appeared to be adequate (Mossel and Ratto, 1970; van Doorne and Claushuis, 1979) extremely long resuscitation periods have been noticed (Van Schothorst and Van Leusden, 1972; 1975).

Irrespective of the medium, a 4–5 h period before consumption of growth is observed (c.f. Table 1). However, shorter periods have been noticed earlier (van Doorne and Claushuis, 1979). Clearly t_r and t_g are largely determined by factors such as type of stress (e.g. heat, desiccation) and the conditions during the stress (e.g. time and temperature). The method suggested here offers the advantage that a sufficiently long resuscitation period can be used in all cases, without the disadvantage that in some cases the results is influenced by growth.

Fig. 3 summarizes the overall results of this study. The total count in the dry product decreases with increasing storage time. The factors influencing the death rate during freeze-drying and subsequent storage have been reviewed by Bousfield and MacKenzie

TABLE 1

TIME REQUIRED FROM COMPLETE RESUSCITATION AND FOR RESUMPTION OF GROWTH OF FREEZE-DRIED *E. COLI*

Storage (days)	Time (h)			
	Complete resuscitation (t_r)		Growth (t_g)	
	LB	TSB	LB	TSB
4	3	4	>4.5 *	>4.5
11	3.5	2.5	>4.5	– **
13	3.5	1.5	–	>4.5
32	1.5	3	5	4.5
81	1.5	–	4	–
83	–	2	–	3.5
98	–	–	–	–

* At the specified time no evidence for growth.
** – not done.

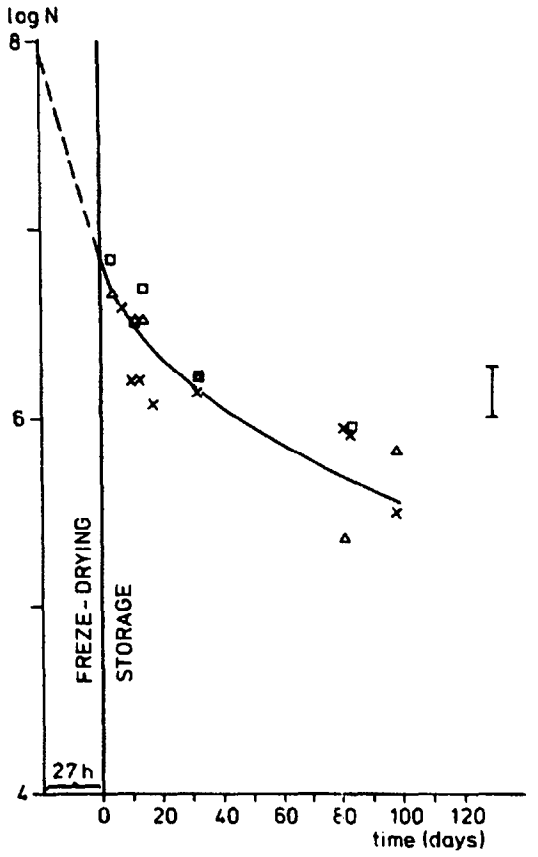

Fig. 3. Survival of freeze-dried *E. coli* at room temperature ×: plate count on TSA; □, MPN, resuscitation method B in Tryptone Soya Peptone Yeast Extract Broth at 37°C; △, MPN, resuscitation method B in Lactose Broth at 37°C; I, calculated standard error of MPN count.

(1976), and are beyond the scope of this paper. In Fig. 3 a comparison is made of the results obtained by the plate counts method and the MPN counts using resuscitation method B (c.f. methods and materials) with either LB or TSB. It can be seen that generally there is excellent agreement between the different methods. Only after 81 days of storage, resuscitation in LB seems to yield a lower result, but as this was not observed after 98 days of storage, experimental error rather than fundamental differences seems to be implicated.

Although it is extremely difficult, if not impossible, to prove that there are no exceptions, it is our belief that resuscitation of diluted samples is a versatile and reliable method, suitable for the quantitative determination of Enterobacteriaceae. The method is not confined to pharmaceutical preparations and raw materials and may also be used in the examination of, e.g., water and foodstuffs. Moreover, the method can also be used for the determination of other types of organisms, provided that a suitable, liquid selective medium is at hand.

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