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THE QUANTITATIVE DETERMINATION OF ENTEROBACTERIACEAE IN PHARMACEUTICAL PREPARATIONS

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SUMMARY

In the Dutch Pharmacopoeia a method is given for the quantitative determination of Enterobacteriaceae. The method includes a resuscitation step. The necessity for this resuscitation is confirmed. The results indicate that growth during resuscitation may dramatically influence the result of the examination and alternative methods without this disadvantage are suggested.

INTRODUCTION

In the latest edition of the Dutch Pharmacopoeia (1978) a most probable number (M.P.N.) technique is suggested for the quantitative determination of Enterobacteriaceae in pharmaceutical preparations. The method consists of three consecutive steps, viz. (i) a resuscitation treatment lasting 5 h at 37°C in Lactose Broth (LB); (ii) a selective enrichment for 24–48 h at 37°C in Enterobacteria Enrichment Broth (EE) and (iii) a differential plating procedure on Violet Red Bile Glucose Agar (VRBG). The reliability of the method depends largely upon the resuscitation treatment involved. Criticism can be made on two points. Firstly, the sample is mixed with LB and incubated for 5 h at 37°C, although in the draft monograph 2–5 h is stated (Berg et al., 1976). At the end of this period dilutions are made in EE. Consequently if growth of Enterobacteriaceae has occurred during the resuscitation period, the observed M.P.N. will only be remotely correlated to the actual number of Enterobacteriaceae present in the preparation. Therefore, a better approach would be to make the dilutions before the resuscitation treatment, and then any subsequent proliferation of Enterobacteriaceae will not affect the final result. Secondly, in M.P.N. procedures the nutrients in the resuscitation medium may be of importance, particularly in higher dilutions. The use of buffered Tryptone Soya Peptone Broth (TSB) has been recommended (Clark and Ordal, 1969; Dabbah and Moats, 1969; Mossel and Ratto, 1970; Warseck et al., 1973). Ray and Speck (1973)

demonstrated that TSB supplemented with yeast extract is superior to LB.

In the present study four different resuscitation procedures were compared.

METHODS AND MATERIALS

Method 1 (Dutch Pharmacopoeia method)

A 10 g sample was weighed out in 100 ml prewarmed (37°C) Lactose Broth (Merck), homogenized and incubated for 5 h at 37°C. Amounts corresponding to 0.1 g, 0.01 g, etc. were transferred to 10 ml Enterobacteria Enrichment Broth (Merck) and incubated for 24 h at 37°C. Five tubes of EE were used for each level of dilution. From each tube of EE subsequent to incubation a loopfull was streaked on to Violet Red Bile Glucose Agar (Merck) and incubated for 24 h at 37°C.

Method 2

Different amounts of sample, 1 g, 0.1 g, 0.01 g, etc., were weighed out into tubes containing 10 ml prewarmed (37°C) LB and homogenized. From each tube 5 portions of 1 ml were pipetted into 5 tubes, and incubated at 37°C for 5 h. Thereupon 9 ml EE (10/9 strength) were added, incubated at 37°C for 24 h and treated as described under Method 1.

Method 3

This procedure was identical to that described under Method 2, except that LB was replaced by buffered Tryptone Soya Peptone Broth (Caso Bouillon, Merck) supplemented with 0.3% w/v yeast extract (Bio-Merieux).

Method 4

Different amounts of samples were weighed out directly into EE (5 tubes for each amount), incubated for 24 h at 37°C and then treated as described under Method 1.

Characteristic colonies on VRBG were Gram-stained and tested for their oxidase reaction; Gram-negative, oxidase-negative bacteria were presumptively identified as Enterobacteriaceae. Confirmation was obtained by testing for anaerobic glucose dissimilation. Computation of the M.P.N. was carried out using the tables given by Taylor (1962). Using the formula given by Cochran (1950), it was assumed that the results obtained from two different methods were significantly different ($P < 0.05$) when their ratio was more than 5, or less than 0.2.

Ecograms were obtained in the following way. Ten gram samples were suspended in both LB abd TSYB (100 ml in each case) at temperatures of 20 and 37°C and incubated. Immediately after mixing and after specific time intervals, viable counts were made on TSA, VRBG with overlay of about 3 mn to produce anaerobic conditions, phenylethanol agar (PEA), and crystal violet agar (CVA). TSA, PEA and CVA plates were incubated for 48 h at 30°C, VRBG plates were incubated at 37°C for 24 h.

Media

TSYB: 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, water 1000 ml. Sterilization time 20 min at 120°C.

LB: beef extract 3.0, peptone from gelatin 5.0, lactose 5.0, distilled water 1000 ml. Sterilization time, 20 min at 120°C.

VRBG: peptone 7.0, yeast extract 3.0, glucose 10.0, sodium chloride 5.0, bile salts 1.5, neutral red 0.03, crystal violet 0.002, agar 13, distilled water 1000 ml. Sterilization time, 30 min in boiling water bath.

EE: peptone 10.0, glucose 5.0, ox bile 20.0, brilliant green 0.015, disodium hydrogen phosphate 8.0, potassium dihydrogen phosphate 2.0, distilled water 1000 ml. Sterilization time, 15 min in boiling water bath.

TSA: peptone from casein 17.0, peptone from soya meal 3.0, glucose 2.5, sodium chloride 5.0, dipotassium hydrogen phosphate 2.5, distilled water 1000 ml. Sterilization time, 20 min at 120°C.

CVA: as TSA. After sterilization, 2 mg crystal violet are added to 1000 ml medium.

PEA: as TSA. Before sterilization, 3.5 g of 2-phenyl ethanol are added to 1000 ml medium.

RESULTS AND DISCUSSION

The purpose of the present investigation was not to survey the microbiological quality of products available on the Dutch market, but to compare the results obtained by modification of the Pharmacapoeial test for Enterobacteriaceae. A wide range of samples were obtained from randomly chosen sources, including frequently opened and refilled containers present in our laboratory. Hence, no conclusions regarding the microbiological quality of the products should be made.

In Table I the results obtained by the various methods are summarized.

The following samples were found to contain less than 1.8/g Enterobacteriaceae by all 4 methods, which is the lower limit of detection: wheat flour, gummi arabicum, thyroid powder, bentonite (3 samples), and veegum (3 samples).

TABLE I

COMPARISON BETWEEN 4 DIFFERENT METHODS FOR THE ESTIMATION OF THE NUMBER OF ENTEROBACTERIACEAE

Preparation	M.P.N. counts, expressed per gram			
Method	1	2	3	4
(1) Canal water	3.3×10^5	1.4×10^3	1.4×10^3	1.7×10^2
(2) Pancreatic powder (a)	2.4×10^3	4.5	13	4.5
(3) Pancreatic powder (b)	1.3×10^3	<1.8	<1.8	<1.8
(4) Croquette (deep frozen)	$>2.4 \times 10^5$	4.9×10^3	7.9×10^3	2.4×10^3
(5) Minced meat	1.6×10^7	9.2×10^3	2.4×10^4	2.4×10^4
(6) Liquiritiae radix [a]	$<2.4 \times 10^7$	2.4×10^4	2.4×10^5	3.3×10^2
(7) Tragaranth	7.9×10^3	3.3×10^2	4.9×10^3	14
(8) Pulvis gummosis [b]	4.8×10^4	66	4.8×10^3	98

[a] Liquorice.

[b] A mixture of equal parts of tragaranth, acacia and saccharose.

With specimen 1 Method 4 (without resuscitation) yielded lower results than Method 2 (resuscitation in LB) and Method 3 (resuscitation in TSYB), the latter two giving identical results. Hence, in this case TSYB and LB seem to be equally suitable for the restoration of the impaired cells. With Method 1 (resuscitation in LB of undiluted water) the highest number of Enterobacteriaceae were recovered. The differences can be explained by assuming that either (i) multiplication has occurred during resuscitation, or that (ii) the other two resuscitation treatments are inadequate.

In preparations 2, 3, 4 and 5, apparently no sublethally injured organisms are present, or their presence cannot be revealed with the chosen selective medium. Since the results obtained with Methods 2 and 3 (including a resuscitation step) and Method 4 (without resuscitation) were not significantly different. The results obtained with Method I were much higher than with the other three methods. Hence, the occurrence of growth seems to be the most likely explanation, otherwise the complete failure of both other resuscitation steps has to be admitted. A large fraction of the Enterobacteriaceae present in liquiritiae radix, tragacanth and pulvis gummosus (preparations 6, 7 and 8) has suffered from sublethal injury. When applying resuscitation in TSYB (Method 3), more Enterobacteriaceae were recovered than without resuscitation (Method 4). When using LB as a resuscitation medium (Method 2), intermediate results were obtained, demonstrating that LB is less suitable as a resuscitation medium in this instance.

Although TSYB was found to be superior to LB as a resuscitation medium, generally higher numbers of Enterobacteriaceae were recovered with the original pharmacopoeia method than with TSYB as resuscitation medium. In addition to the composition of the medium, time and temperature will also affect the results. Mossel and Ratto (1970) showed that 2 h resucitation was adequate for the recovery of Enterobacteriaceae in foods, whereas van Schothorst and van Leusden (1972, 1975) demonstrated that more than 6 h were required for the restorement of severely injured Salmonellae. Therefore, the influence of time and temperature on the numbers of Enterobacteriaceae recovered from pancreatic powder and liquiritiae radix was studied. Results are shown in Table 2.

From these results it may be concluded that 37°C is the optimum temperature for resuscitation. Prolonged resuscitation may even decrease the apparent number of Enterobacteriaceae, most probably due to overcrowding of the selective medium by other bacteria (Mossel and Ratto, 1970). These results once more support the hypothesis that the exceptionally high numbers observed by using Method I are a result of growth and multiplication.

The results are supported further by the ecogram made for liquiritiae radix. The following taxonomic groups were selectively enumerated: total flora, Gram-negative bacteria, Gram-positive bacteria and Enterobacteriaceae. Total flora was estimated on TSA, Gram-negative bacteria were enumerated on Crystal Violet Agar (CVA) (van Doorne, 1977; Mossel et al., 1977), Gram-positive bacteria were counted on Phenyl Ethanol Agar (PEA) (van Doorne, 1977; Mossel et al., 1977) and Enterobacteriaceae on VRBG.

In Fig. 1a–d the apparent number of the various types of organisms in liquiritiae radix, obtained with the 4 resuscitation treatments are plotted against time of incubation.

When the specimen is incubated in either TSYB or LB at 20°C, the total count remains constant for at least 6 h (Fig. 1a). When 37°C is used as resuscitation temperature, a

TABLE 2

INFLUENCE OF RESUSCITATION TIME AND TEMPERATURE ON NUMBERS OF ENTEROBACTERIACEAE RECOVERED FROM TWO PREPARATIONS

Procedure	Pancreatic powder	Liquiritiae radix
Method 1	2.4×10^3	2.4×10^7
Method 2	18	2.4×10^4
Method 3 resuscitation 5 h at 37°C	13	2.4×10^5
Method 3 resuscitation 5 h at 20°C	<1.8	2.4×10^4
Method 3 resuscitation 5 h at 30°C	–	2.4×10^4
Method 3 resuscitation 22 h at 37°C	8	7.9×10^3

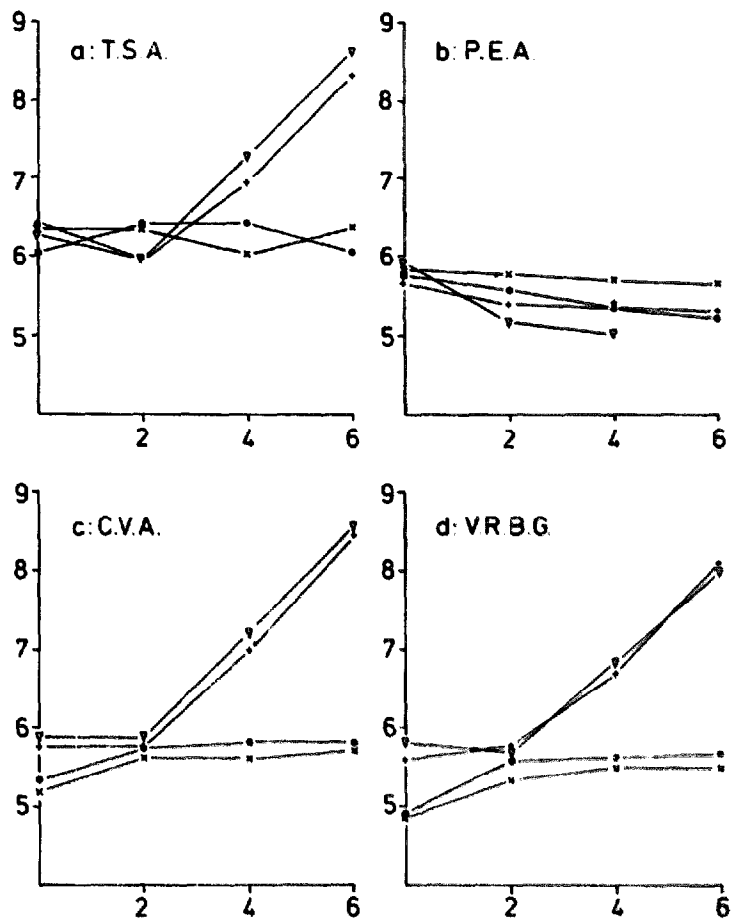

Fig. 1. Ecogram for liquiritiae radix. Logarithms of viable counts are plotted against time in hours. ×, resuscitation in TSYB at 20°C; •, resuscitation in LB at 20°C; +, resuscitation in TSYB at 37°C; ▽, resuscitation in LB at 37°C.

decrease in the total count during the first 2 h is noted. This decrease is fully accounted for by the decrease observed on PEA (Fig. 1b). Consequently, some of the Gram-positive flora of this preparation seem to die off under these conditions. After 2 h at 37°C growth is resumed both in TSYB and LB (Fig. 1a). Fig. 1c shows that an increase in the number of Gram-negative bacteria is observed when the specimen is incubated at 20°C in TSYB and LB. After 2 h a level is reached which remains constant for at least another 4 h. This type of curve is characteristic for resuscitation followed by a normal lag phase. At 37°C the number of bacteria recovered immediately after mixing are higher than at 20°C (Fig. 1c). After 2 h at 37°C the Gram-negative bacteria resume growth. These results suggest that resuscitation at 37°C may be almost instantaneous, whereas at 20°C longer periods are needed.

The results obtained with VRBG (Fig. 1d) are qualitatively and quantitatively the same as with CVA, suggesting that in this preparation the Gram-negative flora consists entirely of Enterobacteriaceae. Results similar to these were obtained for pulvis gummosis; no decrease in Gram-positive bacteria was observed, and growth of Gram-negative bacteria was resumed only after 4 h. These results demonstrate clearly that under conditions specified in the Dutch Pharmacopoeia for resuscitation, growth of micro-organisms may take place, which will dramatically influence the result of the examination.

CONCLUSIONS

For the determination of numbers of Enterobacteriaceae, a method involving resuscitation of previously diluted samples in TSYB is to be preferred to the pharmacopoeial method for the following reasons:

(a) TSYB is widely recognized as a most effective resuscitation medium;

(b) Resuscitation time is not too critical, because if growth of Enterobacteriaceae occurs, this does not influence the result of this estimation.

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