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The Examination of Foods for Enterobacteriaceae using a Test of the Type Generally Adopted for the Detection of Salmonellae

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SUMMARY. In countries that have recently increased their bacteriological inspection of foods, many products have shown considerable improvement in microbiological quality; simultaneously, however, discrepancies between salmonella and coli-aerogenes tests, especially on dehydrated and frozen foods, have tended to become more frequent. These discrepancies have been eliminated by applying the following measures: incorporating glucose in culture media so as to reveal all Enterobacteriaceae; placing reliance on growth rather than gas formation so as to avoid missing anaerogenic organisms, and, especially, examining quantities of foods commensurate with those used in salmonella tests.

For this purpose a procedure is recommended in which 10 g of well homogenized food are enriched in 100 ml of buffered brilliant green-bile-glucose broth, with no attention being paid to gas formation; the enrichment cultures are then streaked on to MacConkey's glucose agar. Single colonies so obtained are tested for fermentative attack on glucose and may be further examined for other characteristics. The same enrichment fluid can be used for the so-called 'nonselective pre-enrichment' of samples of food containing salmonellae impaired by periods spent in conditions of low water activity, low pH, etc.

IT HAS BEEN frequently noted that various foods, especially dehydrated and frozen ones, which appeared to be contaminated with salmonellae nevertheless gave a negative standard coli-aerogenes test (Sutton & MacFarlane, 1947; Emmenegger, 1959; Silverstolpe *et al.*, 1961; van der Schaaf, van Zijl & Hagens, 1962; Thatcher & Montford, 1962). For such a discrepancy, which tends to impair the reputation of the coli-aerogenes test, four reasons may be suggested. First, the product may indeed contain salmonellae only, as might be expected with products made from whole duck eggs (Hobbs, 1955) where the yolk may contain a pure culture of salmonellae arising from an intravital infection. Second, the commodities under investigation may contain only anaerogenic though lactose positive Enterobacteriaceae, which would clearly be missed in the usual type of test involving the detection of gas (Mossel, Bechet & Lambion, 1959; Lindberg, 1960; Schönherr, 1961). Third, the Enterobacteriaceae accompanying the salmonellae may be lactose negative, as are the salmonellae themselves (Mossel, 1957).

The three deficiencies of currently used coli-aerogenes tests mentioned above can be remedied by incorporating glucose in the medium, which also makes the tests efficient for salmonellae, *Proteus* spp. and other lactose negative Enterobacteriaceae (Mossel, Mengerink & Scholts, 1962); and by replacing gasometric techniques by the

use of solid media which also reveal the occurrence of anaerogenic strains (Mossel *et al.*, 1959). Table 1 shows that such measures are in fact effective; the logarithmic average counts of certain groups of Enterobacteriaceae, confirmed along the lines given by Mossel *et al.* (1962), in over 100 samples of various foods increased from 2·1 to 3·5 following the successive steps, mentioned above, introduced to increase the recovery of Enterobacteriaceae.

TABLE 1

A comparison of the results obtained by four different methods for the enumeration of fermentative Gram-negative rod shaped bacteria in foods

(From unpublished data of Mossel & Dorrestijn)

Food	No. samples examined	Log_{10} (average confirmed count/ml) using			
		Brilliant green-bile broth, with glucose		Violet red-bile agar, with glucose	
		Omitted	Added	Omitted	Added
Fruit ice cream	7	1·8	1·8	2·2	2·0
Vanilla ice cream	28	1·1	1·8	2·1	2·1
Meat salad	49	2·9	3·1	4·3	4·5
Egg salad	10	2·1	2·9	4·4	4·5
Fish and vegetable salads	10	2·5	3·3	4·0	4·3
Total	104				
Mean counts		2·1	2·6	3·4	3·5

The fourth possible reason for the discrepancy between salmonella and coli-aerogenes tests is the lack of commensurability. In the former test 20–25 g of substrate are usually examined, whereas in the latter the sample examined is only 0·01–1·0 g. This difference in sensitivity of the two types of tests is the more important because we have noticed, like Seidel & Schulz (1962), that the overall bacteriological quality of some food products has improved considerably during the last decade, probably because increasing numbers of countries have established bacteriological requirements for imported and locally manufactured foods.

We have therefore developed a procedure with the required sensitivity, which at the same time is based on the other principles mentioned for establishing a test in which all Enterobacteriaceae are detected.

Methods

Principles

Samples of 10 g are taken from the material after thorough homogenization. These are first enriched in a fluid medium containing glucose as the carbon source and brilliant green and bile salts to inhibit virtually all bacteria other than Enterobacteriaceae. When using this technique we occasionally noticed that no subcultures

could be obtained from apparently positive enrichment cultures. As in such instances the pH of the enrichment fluid had dropped to rather low values (*c.* 4·5) we supposed that autosterilization of the enrichment culture had occurred (Perry & Hajna, 1933; Smith, 1959). Hence, we thereafter buffered the fluid at pH *c.* 7·2. No further erratic results were obtained after this improvement had been made; the final pH was always $\geqslant$5·4 with an average of 6·1 in 36 assays.

Gas formation is irrelevant in this test, as it is with enrichment cultures made from foods with the aim of detecting salmonellae. As in the latter tests, all primary enrichment cultures are streaked on to a solid medium, generally a glucose-containing variety of MacConkey's crystal violet-neutral red-bile agar (Mossel *et al.*, 1962). However, when specific organisms, rather than Enterobacteriaceae in general, are sought by this test, subcultures should be made on either brilliant green-phenol red-agar, deoxycholate-citrate agar, MacConkey's lactose agar, or on similar media used for the detection of lactose negative organisms.

Positive subcultures on MacConkey's glucose agar hardly need more confirmation than deep stabbing into freshly steamed tubes of glucose-bromocresol purple agar (Mossel & Martin, 1961). When the attack on glucose is fermentative the only organisms that might still be mistaken for Enterobacteriaceae are *Aeromonas* spp. If ecological considerations make it necessary that this should be precluded (Mossel & Krol, 1962), an oxidase test according to Kovacs (1956) will have to be made (Steel, 1961).

Where closer identification of isolated Enterobacteriaceae is required the usual set of tests must be carried out; such tests are IMVEC-reactions—in which E stands for a suitable Eijkman test (Mushin & Ashburner, 1962; Guinee & Mossel, 1963)—mode of growth in Kligler's iron agar, attack on urea, tolerance of KCN (Buttiaux, Moriamez & Papavassiliou, 1956), and a lysine decarboxylase test, preferably the very useful modification suggested by Taylor (1961).

Standard procedure

Enrichment. The following composition and preparation of the enrichment medium was that finally adopted: peptone, 10 g; glucose, 5 g; $Na_2HPO_4.2H_2O$, 8 g; KH_2PO_4, 2 g; dehydrated ox gall, 20 g; solution of 1·35 g/l of brilliant green sterilized by boiling, 10 ml; distilled water, 1,000 ml; pH 7·2.

This fluid was distributed in 100 ml quantities in 250 ml conical flasks and sterilized for exactly 15 min at 121° after a heating up time of not more than 10 min; cooling after sterilization was done rapidly with running tap water. In this way the condensation reactions between brilliant green and certain other components of the medium were limited and hence the inhibitory power of the fluid would be satisfactorily standardized.

A portion of 10$\pm$1 g of the product under examination was added to each flask. For this purpose we used a spoon made of stainless steel, somewhat less wide than the opening of the flask and with a handle *c.* 15 cm long, so as to be able to introduce the samples into the flasks without any spilling. The mixture was shaken well, e.g. three successive times for half a minute, alternately in clockwise and counter-clockwise directions.

Incubation was for 20–24 h at 37°, with the shaking of the flasks being repeated after incubation for about 3 h.

Confirmation. A loopful from the enrichment cultures was streaked on to an agar medium of the following composition: dehydrated violet red-bile agar (B.B.L., Difco or Oxoid), 41·5 g; glucose, 10 g; distilled water, 1,000 ml; pH 7·2. After steaming to dissolve the ingredients, plates of the medium were poured and stored in the refrigerator until required.

The inoculated plates were incubated for 20–24 h at 37°. Every colony which showed a purple halo on a purple background was considered as presumptive evidence of the occurrence of Enterobacteriaceae.

Identification. Isolated colonies of this type were stabbed to the bottom of freshly steamed tubes containing agar of the following composition: trypticase (B.B.L.) or tryptone (Difco), 10 g; dehydrated yeast extract (B.B.L., Difco or Oxoid), 1·5 g; glucose, 10 g; NaCl, 5 g; bromocresol purple, 15 mg; agar, 15 g; distilled water, 1,000 ml; pH 7·0. Cultures were incubated overnight at 30°. A distinct colour change to yellow throughout the tube indicated the fermentative attack on glucose characteristic of Enterobacteriaceae and *Aeromonas* spp.

Further identification reactions can be carried out as required. Also, when examining certain foods (e.g. frozen raw meats and poultry) in which one might expect to find a large number of psychrotrophic Enterobacteriaceae (Eddy & Kitchell, 1959; Mossel & Zwart, 1960) proper significance should be placed on the presence of such organisms and hence separation of these from regular mesophilic types should be attempted by carrying out the growth test at 43±0·1° (Mossel & Zwart, 1960).

Results

Reliability of the procedure

The first trial was the examination of a representative selection of products in which salmonellae had been detected. If, for a significant proportion of these, the outcome of the new test was negative—even for reasons not understood *a priori*—the new test would clearly be of little value.

A total of 51 samples of egg products, fish meal and meat and bone meal was available for this examination. The following serotypes were detected in these samples: *Salmonella anatum*, *Salm. bareilly*, *Salm. bredeney*, *Salm. derby*, *Salm. gaminara*, *Salm. give*, *Salm. infantis*, *Salm. livingstone*, *Salm. manchester*, *Salm. montevideo*, *Salm. newington*, *Salm. newport*, *Salm. tennessee*, *Salm. typhi-murium*, *Salm. virchow* and *Salm. worthington*. All these samples gave a clear cut positive result when the new test was applied to them.

Incidentally we had the opportunity of examining one of these samples, from a consignment of fish flour, a second time after an interval of more than one month. The results of the first parallel tests (salmonellae and Enterobacteriaceae) were positive, the same tests performed with the sample after it had been stored for some time being negative. Probably under the influence of adverse conditions during storage (Mossel, 1963) both salmonellae and other Enterobacteriaceae had been reduced to levels at which they were not detected by the tests.

Suitability of the test

The next aspect to be investigated was whether products manufactured according to good practice, which ought to satisfy the new test, really did so. Clearly, if this test represented too stringent a requirement for such foods, it would be of little use. About 560 samples of various products were examined for this purpose. They were deliberately chosen from three groups providing ecologically different situations (Mossel & Krol, 1963).

TABLE 2

The occurrence of Enterobacteriaceae in 10 g samples of foods with an unfavourable epidemiological record with respect to salmonellae

Product	No. samples examined	Incidence of Enterobacteriaceae (% of samples)
Dehydrated egg products	103	80
Coconut flour	22	95
Animal feeds (fish meal, blood meal, cottonseed flour)	44	84
Total	169	
Mean		86

The first group was of dehydrated products which generally present epidemiological problems as they are either not sufficiently heated in the course of their manufacture or are frequently recontaminated following a suitable heat treatment. This group comprised dehydrated egg products and coconut flour as well as powdered animal feedstuffs like fish meal, blood meal, cottonseed flour, etc. The results of these tests are given in Table 2. The proportion of positive results was very high in this group, varying from *c.* 80–95% in the coconut and egg products to 84% for the animal feeds.

The second group consisted of materials which during their manufacture are generally subjected to a terminal heat treatment and have, therefore, good bacteriological and epidemiological records. Convenience products like 'instant' milk powder and rice and 'one minute' types of rolled oats, precooked dehydrated infant foods, and cocoa come under this heading. Margarine also has generally a very good public health record (Mossel & Zwart, 1958) and therefore this product was examined too. These commodities only appeared to be infected with Enterobacteriaceae in 10 g samples to an extent of *c.* 15%, although the results obtained with the baby foods are disappointing as more than a third of the samples examined were found positive (Table 3).

Finally, a small group of samples was examined from a category that is bound to contain Enterobacteriaceae to a certain extent, because the product consists of, or contain, unheated vegetable material. However, these foods are either usually heated before consumption or used raw in very small quantities; therefore they do not present a major epidemiological problem, unlike the products of the first group.

TABLE 3

The occurrence of Enterobacteriaceae in 10 g samples of foods subjected to terminal heat treatment

Product	No. samples examined	Incidence of Enterobacteriaceae (% of samples)
Instant milk powder	17	12
Precooked dehydrated infant foods	75	35
Rolled oats ('one minute' type)	32	9
Precooked rice	16	12
Cocoa	29	10
Margarine	158	15
Total	327	
Mean		16

TABLE 4

The occurrence of Enterobacteriaceae in 10 g samples of foods generally subjected to culinary heat treatment before consumption

Product	No. samples examined	Incidence of Enterobacteriaceae (% of samples)
Nonprecooked infant foods	10	100
Pepper and nutmeg	14	100
Dry 'noninstantized' soups	40	83
Total	64	
Mean		94

Nonprecooked infant cereals, dehydrated 'noninstantized' soups and spices belong to this group. As appears from Table 4, these products are contaminated with Enterobacteriaceae to a somewhat higher extent (94 *versus* 86%) than the products of group 1.

Use of the new enrichment fluid for other purposes

We have also found the new enrichment fluid to be a most useful 'nonselective' (in the sense of being free from tetrathionate or selenite) pre-enrichment medium for weakened cells of salmonellae which need resuscitation before being exposed to one of the rather toxic inhibitors just mentioned. The new medium is more inhibitory, especially to aerobic sporeformers, than either Taylor's (1961) mannitol broth or North's (1961) lactose broth and hence it gives impaired cells of salmonellae a better chance to grow from small inocula. Except for certain mutants of salmonella serotypes which possess a most unusual sensitivity to triphenylmethane dyes (Smith, 1962; Mossel *et al.*, 1962), our medium has never shown any unfavourable effects on salmonellae weakened by exposure to low water activities, low pH and similar unfavourable conditions.

Because the medium will clearly allow the development of other Enterobacteriaceae, it is necessary to pay some attention to the way in which subcultures from the enrichment fluid are made. The ordinary streaking procedure will generally not allow easy differentiation of salmonellae from other Enterobacteriaceae; it will in fact be quite useless if the latter outnumber the former. The technique first suggested by Silliker & Taylor (1958) and found very satisfactory by Montford & Thatcher (1961)—*viz.* to make spread drop plates of suitable dilutions of the enrichment cultures on the current media—appeared very effective indeed in obtaining well isolated colonies of lactose positive and negative types. In fact, spreading 0·1 ml of the 10^{-4} and occasionally also the 10^{-5} dilutions of the cultures on brilliant green-phenol red agar and deoxycholate-citrate agar plates of 15 cm diameter, dried for *c.* 30 min at 55° before inoculation, was successful in all cases.

Discussion

It is striking that amongst the foods of group 2 (Table 3) which should *a priori* be free from Enterobacteriaceae, the percentage of positives in samples of 10 g is of the same order of magnitude as the frequency of other bacteriological defects detected in such goods, in particular the too large discrepancy between total counts and counts of spores of Bacillaceae (Mossel & Krugers Dagneaux, 1959). This suggests that the new test for Enterobacteriaceae may be useful in the examination of certain foods and, more specifically, that it is not too stringent to require that the outcome of this test should be negative.

From the point of view of technology the 'reward' to be gained by food manufacturing industries, when improving their products so as to yield entirely negative results in the new test, is of course that such products will—as we have demonstrated—also almost certainly be free from salmonellae. This should be most welcome to industrial laboratories for two reasons. First, the new test is much simpler than a direct search for salmonellae, especially when the identification reactions are only carried out periodically. Second, the new test lacks a most inconvenient aspect of salmonella assays, viz. the frequent erratic results due to heterogeneous distribution of these organisms in many dehydrated and frozen foods (Adam, 1956; Semple, Graham & Dutton, 1961; Lorenz, 1961) in combination with a statistically far from ideal mode of sampling of the consignments.

When required the sensitivity of this test can be further increased. Samples up to 100 g can easily be enriched if the amount of buffered glucose-brilliant green-bile fluid is increased to 1 l, distributed in 200 ml quantities in five conical flasks of 1 l capacity in order to permit the required shaking of the flasks described above. Still larger quantities of foods or feeds cannot readily be examined in a similar way because this would become too cumbersome. Where such an examination might be necessary, Anderson & Woodruff's (1961) percolation technique can be followed in principle. For extraction of the material the same fluid as normally applied for enrichment can then be used while it can subsequently be examined by membrane filtration and cultivation of the membrane on a suitable medium based on MacConkey's agar, as explained earlier.

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