

A Rapid and Direct Plate Method for Enumerating *Escherichia coli* biotype 1 in Food

JUDITH M. ANDERSON AND A. C. BAIRD-PARKER

Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England

Received 20 January 1975 and accepted 16 June 1975

Direct enumeration of *Escherichia coli* biotype 1 in foods within 24 h has been achieved by a development of the method of Delaney, McCarthy & Grasso (1962) which is based on the production of indole at 44°. Indole positive *E. coli* growing at this temperature on a cellulose acetate membrane overlaying tryptone bile agar can be demonstrated. Characterization of 555 indole positive colonies from 843 samples of food showed that 95% were *E. coli* biotype 1 and a further 3.4% were 'faecal coliforms'. Anaerogenic and non-lactose fermenting *E. coli* are detected and the significance of these strains is discussed.

ESCHERICHIA COLI biotype 1 is used as an indicator of faecal contamination in food and water and has been implicated in cases of foodborne enteritis in children and adults (Anon, 1973*a, b*; Marier *et al.*, 1973). The presence of *E. coli* in foods is usually determined qualitatively by enrichment or quantitatively by Most Probable Number (MPN) counts (Geldreich, 1966; Thatcher & Clark, 1968; Mossel & Vega, 1973). The determination of 'Presumptive *E. coli*' as faecal coliforms by such techniques takes a minimum of 2 days and complete confirmation of an isolate as *E. coli* a further 3 days (Surkiewicz, 1966; Thatcher & Clark, 1968). The more rapid enrichment-serology technique is not yet suitable for the detection of *E. coli* in food (Cranston & Matthews, 1973).

Procedures based on MPN counts are laborious and, unless large numbers of replicates and at least 3 dilutions are set up, they are less accurate than direct counts (Cowell & Morisetti, 1969). When considering alternative methods of counting *E. coli* in foods it was decided to investigate the possible application of a direct count method. Equivocal results were obtained from a survey of selective agar media containing various differential substrates and incubation at 37°, 41° or 44° for 18-24 h. Thus even acid formation from lactose was questionable (Mossel & Vega, 1973) and the method of Delaney, McCarthy & Grasso (1962) was the only one which showed promise. This method, developed for detecting and enumerating *E. coli* in water samples, exploits the ability of *E. coli* to produce indole when growing at 44° on a cellulose acetate membrane placed on a bile medium in a Petri dish. This paper describes the development of this method and its application to the rapid and direct enumeration of *E. coli* in foods.

Materials and Methods

Media

Tryptone Bile Agar (TBA) was prepared as described by Delaney *et al.* (1962). It contains (% w/v): Tryptone (Difco), 2.0; Bile Salts No. 3 (Oxoid), 0.15; Agar, 1.5;

pH 7.2. The medium (100 ml) was dispensed in screw capped bottles and sterilized at 121° for 15 min. For use it was melted and 12–15 ml poured in Petri dishes (9 cm diam.). It may be stored in these dishes at room temperature or 4° for at least 5 days without affecting performance. Purple MacConkey broth, EC broth and IMViC test media for confirming strains as *E. coli* were prepared as described by Thatcher & Clark (1968).

Reagents

Standard reagents were used for all tests (Thatcher & Clark, 1968) except for indole detection in the Direct Plate Count technique. For this different indole reagents were tested. Five per cent *p*-dimethylaminobenzaldehyde in *N*-HCl described by Vracko & Sherris (1963) was found to be the reagent best suited for detecting indole produced by colonies on cellulose membranes.

Strains

Bile resistant and/or indole producing aerobic bacteria (Table 1) which are likely to occur in foods were tested for growth and indole formation by streaking broth cultures or spreading dilutions of these on membranes placed on TBA and incubated at 37°, 41.5° or 44° for 24 h.

TABLE 1
*Strains tested for growth and indole production on membranes on tryptone
bile agar*

Organism	No. of strains	Source
<i>Escherichia</i>	<i>coli</i> 85	NCTC, NCIB and Colworth (including enteropathogenic serotypes 025, 026, 044, 055, 078, 086, 0111, 0119, 0124, 0125, 0126, 0127 and 0128)
	<i>alkalescens</i> 1	NCTC
	<i>dispar</i> 2	NCTC
<i>Klebsiella</i>	62	NCIB, Colworth and indole positive strains from Dr W. Newsom, Papworth Hospital, Cambridge, England
<i>Enterobacter</i>	2	Colworth
<i>Citrobacter</i>	4	NCTC
<i>Levinea</i>	2	ATCC
<i>Edwardsiella</i>	3	NCTC
<i>Erwinia</i>	5	NCPPB
<i>Salmonella</i>	7	NCTC and Colworth
<i>Shigella</i>	14	NCTC
<i>Proteus</i>	9	NCIB and Colworth
<i>Providencia</i>	3	NCTC and Colworth
<i>Pseudomonas</i>	4	NCTC and Colworth
<i>Chromobacterium</i>	1	NCTC
<i>Flavobacterium</i>	1	Colworth
<i>Bacillus</i>	4	NCTC, ATCC, NCIB and Colworth
<i>Staphylococcus</i>	6	ATCC and Colworth
<i>Streptococcus</i>	5	Colworth

Foods tested

A large number of raw and cooked meat products were tested together with samples of food which had been found to contain *E. coli* by the Most Probable Number (MPN) method (see below).

Techniques for foods

Direct plate count on membranes on TBA. Cellulose acetate filter membranes (pore size 450 nm; 85 mm diam.—available as a special order from Oxoid Ltd, Southwark Bridge Road, London, SE1 9HF) for which there was no need to sterilize were placed on the well dried surface of TBA in Petri dishes. They were gently flattened with a sterilized spreader. Samples of food were diluted 1 in 5 or 1 in 10 with 0.1% (w/v) peptone water and homogenized using a 'Stomacher' Lab-Blender 400 (Sharpe & Jackson, 1972) (supplied by A. J. Seward, 3 Cavendish Road, Bury St. Edmunds, Suffolk IP33 3TE). Then, 0.5 or 1.0 ml of the homogenate was pipetted on to the membrane and spread completely over it with a sterile spreader. After the homogenate had soaked in, the Petri dishes, in piles of not more than 3 and with their lids uppermost, were incubated in a water jacketed incubator at 44° ($\pm 1^\circ$). After overnight incubation, 1–2 ml of the indole reagent (5% *p*-dimethylaminobenzaldehyde in N-HCl) of Vracko & Sherris (1963) were pipetted into each labelled lid. The membrane was then lifted with a pair of forceps from the TBA in the appropriate Petri dish and lowered on to the reagent. This soaked quickly into the membrane and indole-positive colonies 'stained' pink within 5 min. After excess reagent had been pipetted off, the number of pink colonies multiplied by the dilution factor gave the number of *E. coli*/g food. The 'stained' membrane may be 'fixed' by drying in sunlight or under a u.v. lamp (see below) and kept for reference.

Identification of isolates from indole-positive colonies on membranes on TBA. To estimate what proportion of pink 'staining' colonies on membranes were *E. coli*, more than 1000 colonies were examined from membranes inoculated with a range of food. Before the addition of the indole reagent a small portion of each colony was subcultured in nutrient broth. The position of each colony on the membrane was charted on graph paper so that it was possible to correlate pink 'stained' colonies and confirmatory tests for indole production.

The subcultures from colonies were tested with Kovac's reagent for indole production in Tryptone broth at 44° and positive strains tested for acid and gas formation in EC and MacConkey broth at 44°; all indole positive non-acid or non-acid and gas forming strains were tested in EC and MacConkey broth at 37°. All strains producing indole and pink 'staining' colonies were tested by the procedures of Thatcher & Clark (1968) for IMViC reactions.

Most Probable Number technique in Purple MacConkey Broth. Non-heat processed foods were tested for *E. coli* by direct MPN (3 tubes) counts in Purple MacConkey broth incubated at 44° for 18–24 h (Fishbein, 1973). Most Probable Number counts on samples of heat-treated foods were incubated at 37° followed by subculture from tubes showing acid and gas to fresh media which was incubated at 44° (Thatcher & Clark, 1968). In both cases, cultures producing gas at 44° were subcultured in tryptone

broth, incubated at 44° for 24–48 h and tested for indole with Kovac's reagent. These procedures were run in parallel with the direct counts on membranes on TBA.

Results

Growth of laboratory isolates on membranes on TBA

Most bile tolerant, indole-producing bacteria grew well and formed indole on membranes on TBA at 37° or 41.5° and thus could not be distinguished from *E. coli*. At 44°, however, good differentiation was achieved and all indole-producing bacteria other than strains of *E. coli* either failed to grow or grew insufficiently to form demonstrable amounts of indole during 24 h. This method has been used successfully to differentiate between indole positive strains of shigellae and anaerogenic *E. coli*. Although the success of the method is dependent on incubation at 44°, it does not require the technique of submerging Petri dishes sealed in cans (Thomas & Jones, 1971) or agar in pouches (Mossel & Vega, 1973) in a waterbath at 44°.

Indole detection on membranes on TBA at 44° and the membrane 'staining' technique

As none of the methods of indole detection are able to demonstrate indole produced by colonies of *E. coli* growing on the surface of agar, a membrane which enabled the transfer of colonies for indole 'staining' was used. The Vracko & Sherris (1963) reagent gave the most distinct reaction and reproducibility. The reagent was easy to prepare and did not deteriorate when kept for 3 months in a dark cupboard at room temperature. Except for 2 bile-sensitive laboratory strains, all strains of *E. coli* grew well on membranes on TBA incubated overnight at 44° and gave well-defined pink colonies when 'stained'. Non-indole producing colonies were a straw colour. Two laboratory strains of serotypes 055 and 0127, which were either weak or non-indole producers at 37° and 44° when tested by conventional methods, produced negative or only pale pink colonies on membranes on TBA. However, 3 strains of serotype 055 isolated recently from clinical material have produced normal indole positive pink colonies.

A range of possible colony transfer/support materials (e.g. Cellophane and filter paper) have been tested but only bacteriological grade cellulose acetate membranes were successful. Other materials were either less permeable or gave diffuse colonies with poor colour reactions. Membranes should either be observed for pink colonies within 30 min (they fade on drying) or dried under a low pressure fluorescent u.v. lamp with 'Woods' type filter (P. W. Allen & Co., 253 Liverpool Road, London, N.1) or left to dry in sunlight. When dried the intensity of the 'staining' reaction is improved and made permanent; such membranes may be stored for reference.

*Comparison of the enumeration of *E. coli* in food samples by the membrane on TBA method and by MPN counts*

Statistical comparison of results obtained by the two methods on 248 food samples are given in Table 2. Except for pork sample 2 there was no significant difference ($P=0.05$) between counts given by the two methods. The discrepancy obtained with pork sample 2 was caused by the presence of a high proportion (see Table 3) of anaerogenic

strains of *E. coli* (acid but no gas produced from lactose), thus they were not detected by the MPN method. Many other food samples were tested also but there were insufficient *E. coli* per sample for statistical analysis.

TABLE 2

Statistical analysis of counts of Escherichia coli isolated from foods on membranes on tryptone bile agar and Most Probable Number (MPN) method

Food	No. of samples	Best estimate*	95% Confidence limits	
			Lower	Upper
Pork { 1	60	1.1	0.60	1.89
2	30	3.41	2.33	4.98
Chicken	60	1.05	0.72	1.55
Fish	54	1.05	0.78	1.42
Meat (raw and comminuted)	44	0.98	0.77	1.25

* Best estimate = Mean of plate counts / Mean of MPN estimations.

TABLE 3

Identification of 555 indole positive colonies isolated from food on membranes on tryptone bile agar at 44°

Food	No. of samples	Indole positive isolates*	Classification by IMViC tests†			
			<i>Escherichia coli</i> biotype 1	<i>Escherichia</i> of faecal origin	<i>Enterobacter aerogenes</i> Intermediate coliforms and <i>Alcaligenes bronchisepticus</i>	Eijkman test acid only‡
Pork { 1	60	174	167 (96%)	3 (1.7%)	4 (2.3%)	6 (3%)
2	30	106	95 (90%)	11 (10%)		78 (74%)
Chicken	60	15	15 (100%)			2 (13%)
Fish and shellfish	126	130	129 (99.2%)		1 (0.8%)	6 (5%)
Meat (raw and comminuted)	566	130	122 (94%)	5 (4%)	3 (2%)	13 (10%)
Total	843	555	528 (95.1%)	19 (3.4%)	8 (1.5%)	105 (19%)
			547 (98.5%)			

* Membrane and conventional indole method gave identical results.

† Geldreich (1966), Thatcher & Clark (1968).

‡ All anaerogenic isolates identified with *E. coli* biotype 1 by IMViC tests.

Identification of colonies picked from membranes on TBA

To find if colonies identified presumptively with *E. coli* by their pink reaction on membranes were really *E. coli*, 1186 colonies from membranes inoculated with food samples were examined; 555 colonies gave a positive reaction and were characterized by classical procedures [acid and gas production from lactose in a bile medium at 44° (Eijkman Test), and IMViC tests (indole, methyl red, Voges-Proskauer and citrate)]. The results obtained are summarized in Table 3. Ninety-five per cent of the presumptive

E. coli on membranes on TBA were identified with *E. coli* biotype 1 (IMViC + + - -) and a further 3.4% as faecal coliforms on the basis of + + + - and + - - - IMViC reactions (Geldreich, 1966; Thatcher & Clark, 1968). Two indole positive (oxidase positive) isolates were identified tentatively with *Alcaligenes bronchisepticus* and, although these organisms are probably rare in foods, they would be identified with *E. coli* on membranes on TBA. When considering the validity of the MPN method for *E. coli* detection, it is important to note that 105 of the 555 strains isolated from the foods (Table 3) and identified presumptively with *E. coli* on membranes on TBA were still anaerogenic after 5 days' incubation at 37° and would therefore not have been detected by the MPN method. The proportion of anaerogenic *E. coli* isolated from the different foods varied between 3 and 74% (Table 3).

Discussion

There are divergent views on whether a presumptive test for faecal coliforms should detect *E. coli* biotype 1 (acid and gas from lactose and indole at 44°), or should be based solely on acid and gas formation from lactose at 44°-45.5°. The latter is the basis for the North American method which will detect not only *E. coli* but also irregular types II and VI (Thatcher & Clark, 1968) which may not be of faecal origin (Mackenzie, Taylor & Gilbert, 1948). The European workers approach has been to base presumptive faecal coliform recognition on both acid with gas and indole formation at 44° [the tests of Mackenzie *et al.* (1948)], which exclude the irregular types and will detect only *E. coli* biotype 1. This difference in approach is also reflected by direct plate count methods for *E. coli* detection which either have been based on acid formation from lactose in media containing bile at 44° (Thomas & Jones, 1971; Mossel & Vega, 1973) or on the method of Delaney *et al.* (1962) for detecting indole formation in such a medium also at 44°. For several reasons we believe that the latter approach has advantages for detecting *E. coli* in foods. Thus the use of lactose fermentation as an indicator test for coliforms will miss those strains that are unable to ferment lactose (Mossel, 1974). According to Ewing (1972), only c. 90% of *Escherichia* strains produce acid from lactose within 2 days, while 99% of all strains produce indole. Also some enteropathogenic *E. coli* are late or non-lactose fermenters (Arbuzova, 1970; Molchenov, 1970). Sakazaki, Tamura & Saito (1967) isolated 789 strains from children and adults with diarrhoea; 10 of the 18 enteropathogenic serotypes isolated included 393 strains (49%) which were late or non-lactose fermenters, only 50 strains (6%) were indole negative. The food poisoning outbreaks in the United States (Marier *et al.*, 1973), in which imported cheese was implicated, provide an example of the low reliance which can be placed on acid and gas formation from lactose for coliform and *E. coli* detection leading to misdiagnosis or failure to detect the causative agent of food poisoning. A further point in considering lactose fermentation tests is the possibility of anaerogenic strains; our direct method would indicate that these may, occasionally, be more common than typical aerogenic strains in a food. It would not be claimed that all *E. coli* strains form indole from tryptophan and indeed some laboratory strains of two serotypes (055 and 0127) were found to be either weak or non-indole producers. Recent isolates of serotype 055 produced indole. However, the evidence indicates that the formation of indole is more often a character of enterotoxigenic and non-enterotoxigenic strains of *E. coli* than is lactose fermentation.

The main disadvantage of the membrane on TBA method is that 'staining' with indole reagent kills the cells. This can be overcome without interference with the 'staining' reaction by making an 'imprint' of the membrane on MacConkey agar before 'staining'. After incubation of the MacConkey agar at 44° for 3 h, lactose positive and negative colonies can be differentiated easily. These colonies may be typed biochemically and serologically, but as a screening test for testing food samples this would not be necessary. The direct plate method is simple and quick to use; less media and subculturing are required than in traditional methods. Results are obtained within 24 h. Greater accuracy and less chance of missing atypical strains outweigh the disadvantages of missing very low numbers of *E. coli* and loss of viability when 'stained'.

References

- ANON (1973a). *Escherichia coli* Gastroenteritis. *Br. med. J.* **1**, 244.
- ANON (1973b). Nosocomial gastroenteritis, Arizona. *Morbidity Mortality* **22**, 225.
- ARBUZOVA, V. A. (1970). Biological characteristics of enteropathogenic *Bacillus coli* 044 isolated from the newborn. *Trudy Inst. Épidem. Mikrobiol. Sanit.* **36**, 287.
- COWELL, N. D. & MORISETTI, M. D. (1969). Microbiological techniques—some statistical aspects. *J. Sci. Fd Agric.* **20**, 573.
- CRANSTON, P. M. & MATTHEWS, D. E. (1973). Evaluation of an enrichment serology technique for *Escherichia coli* in frozen foods. *Aust. J. Dairy Technol.* **28**, 172.
- DELANEY, J. E., MCCARTHY, J. A. & GRASSO, R. J. (1962). Measurement of *E. coli* Type 1 by the membrane filter. *Wat. Sewage Wks* **109**, 289.
- EWING, W. H. (1972). *Differentiation of Enterobacteriaceae by Biochemical Reactions*. CDC, Atlanta: U.S. Dept. of Health, Education and Welfare.
- FISHBEIN, M. (1973). *Bacteriological Analytical Manual for Foods*. Washington, U.S.A.: Division of Microbiology, F.D.A.
- GELBREICH, E. E. (1966). *Sanitary Significance of Faecal Coliforms in the Environment*. Cincinnati: Water Pollution Control Research Series. Publication No. WP 20-3. U.S. Federal Water Pollution Control Administration.
- MACKENZIE, E. F. W., TAYLOR, E. W. & GILBERT, W. E. (1948). Recent experiences in the rapid identification of *Bacterium coli* Type 1. *J. gen. Microbiol.* **2**, 197.
- MARIER, R., WELLS, J. G., SWANSON, R. C., CALLAHAN, W. & MEHLMAN, I. J. (1973). An outbreak of enteropathogenic *Escherichia coli* foodborne disease traced to imported French cheese. *Lancet* **ii**, 1376.
- MOLCHENOV, L. F. (1970). Clinical/epidemiological characteristics of illness caused by *Escherichia coli* 0124. *VoennoMeditsinskii Zh.* **7**, 54.
- MOSSEL, D. A. A. (1974). Bacteriological safety of foods. *Lancet*, **i**, 173.
- MOSSEL, D. A. A. & VEGA, C. L. (1973). The direct enumeration of *Escherichia coli* in water using MacConkey's agar at 44° in plastic pouches. *Health Lab. Sci.* **10**, 303.
- SAKAZAKI, R., TAMURA, K. & SAITO, M. (1967). Enteropathogenic *Escherichia coli* associated with diarrhoea in children and adults. *Jap. J. med. Sci. Biol.* **20**, 387.
- SHARPE, A. N. & JACKSON, A. K. (1972). Stomaching: a new concept in bacteriological sample preparation. *Appl. Microbiol.* **24**, 175.
- SURKIEWICZ, B. F. (1966). Microbiological methods for examination of frozen and/or prepared foods. *J. Ass. off. analyt. Chem.* **49**, 276.
- THATCHER, F. S. & CLARK, D. S. (1968). *Micro-organisms in foods*. Vol. I. Their significance and methods of enumeration. Toronto: University of Toronto Press.
- THOMAS, K. L. & JONES, A. M. (1971). Comparison of methods of estimating the number of *Escherichia coli* in edible mussels and the relationship between the presence of salmonellae and *E. coli*. *J. appl. Bact.* **34**, 717.
- VRACKO, R. & SHERRIS, J. C. (1963). Indole-spot test in bacteriology. *Am. J. clin. Path.* **39**, 429.