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A rapid and simple method for the detection and enumeration of *Escherichia coli* in cleansed shellfish

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SUMMARY

A multiple-tube technique based on peptone water incubated at 44 °C for 24 h followed by detection of indole was found to be sensitive and specific for the detection of *Escherichia coli* in oysters and mussels. The method has the advantage of providing rapid results and is both less expensive and less time-consuming than other MPN techniques.

INTRODUCTION

Molluscan shellfish are often cultivated in estuarine and coastal waters polluted with sewage. Given their ability to concentrate bacterial and viral pathogens present in the water (Hoff & Becker, 1968; Canzonier, 1971) shellfish consumption is potentially hazardous and has featured in a large number of food poisoning outbreaks (Ayres, 1979; Appleton, Palmer & Gilbert, 1981; Hobbs, 1983). In an attempt to safeguard the public health, many countries set microbiological guidelines for products of this type and the water in which they are cultivated (Ayres, 1979).

At the present time in the UK, public health guidelines are based on the number of *Escherichia coli* per unit volume of shellfish flesh or growing water. Although the specificity of this faecal bacteria indicator has been questioned (Hood, Ness & Blake, 1983), it has the advantage of being relatively easy to detect. In the United Kingdom *E. coli* in shellfish are still mainly enumerated using the technique of Clegg & Sherwood (1947). This involves the addition of a dilution of macerated shellfish tissue to molten agar containing bile salts, followed by incubation at 44 °C for 18 h, either as a pour plate or roll tube.

A combination of nutrient depletion, osmotic and low-temperature stress encountered in marine environments is likely to cause sublethal injury to many of the faecal bacteria present in water and shellfish tissue (Cooke & Lamb, 1983). A common manifestation of this phenomenon is an inability for these organisms to grow in the presence of selective agents, such as bile salts. Thus a proportion of the *E. coli* added to the molten agar will be unable to form colonies and will remain undetected (Maxcy, 1970; Speck, Ray & Read, 1975; Draughon & Nelson, 1981).

In recognition of this problem, both the European Economic Community and the United States (West, 1984) have stipulated that the sanitary quality of shellfish should be determined by MPN techniques. These use media and incubation

procedures that are less toxic to injured cells and are likely to be more sensitive than the more traditional methods. They have the disadvantage, however, of not providing results often until up to 72 h after the receipt of the samples and are thus less useful as a screening procedure.

In an attempt to overcome this problem, rapid MPN techniques for shellfish bacteriology have been developed (Andrews & Presnell, 1972; Hunt & Springer, 1978; Hastback, 1981). As these use comparatively expensive media and have been shown by some workers to be less sensitive than standard MPN techniques (Hunt & Springer, 1978; Rowse, 1981; Yoovidhya & Fleet, 1981) they are unlikely to gain wide acceptance at the present time.

This paper reports a simple and rapid method for detecting and enumerating *E. coli* in molluscan shellfish and compares its efficacy with standard methods.

MATERIALS AND METHODS

Shellfish

Oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) cultivated in the rivers Exe, Taw and Teign and submitted to the laboratory by local authorities were used in the study. Most of the 200 samples, each of which comprised 5–10 oysters or mussels, were subjected to controlled self-purification in an approved plant for up to 5 days, but a small proportion were tested without prior cleansing. The shellfish were transported to the laboratory in insulated containers and stored at 4 °C until testing, which was usually within 1 h.

The surfaces of the shells were scrubbed with a nail brush under running water to remove mud and weed. They were opened using a sterile shucking knife and the contents transferred to a sterile measuring cylinder. The flesh was then homogenized with two volumes of $\frac{1}{4}$ strength Ringer solution in a blender (MSE Atomix) at full speed for 3 min. The resultant macerate was either used at that dilution or further diluted with $\frac{1}{4}$ strength ringer solution to achieve a final dilution of 1 : 10. Microbiological examinations, as described below, were carried out immediately.

Enumeration of total coliforms and Escherichia coli

In the first 50 samples, *E. coli* were enumerated using MacConkey agar (Oxoid No. 3) pour plates (Thomas & Jones, 1971) in parallel with a two-stage 5-tube MPN technique, using Formate Lactose Glutamate Medium (FLG) as the initial medium. With the former method, which has a detection limit of 300 *E. coli*/100 ml, one plate was prepared per sample using 1 ml of the 1:3 dilution. For the MPN method, this dilution was further diluted to 1 : 10. Five-tube dilutions ranging from 10 ml to 0·001 ml of this were then ioculated into and cultured in FLG broth in the manner recommended for water (DHSS, 1982). Tubes showing acid and gas after 48 h at $37 \pm 0{\cdot}2$ °C were considered to be positive for total coliforms. The MPN per 100 ml of flesh was obtained by multiplying the table value by the appropriate correction factor. This was used subsequently to calculate the total coliform/*E. coli* ratio.

Tubes positive at 37 °C were subcultured in parallel into Brilliant Green Bile Broth (Oxoid CM 31) and peptone water (Oxoid L 37). These were incubated at

44±0·2 °C for 18–24 h. The production of gas in Brilliant Green broth and of indole in peptone water was taken to be indicative of the presence of *E. coli* type I. The MPN *E. coli* was calculated in the same way as for total coliforms.

The remaining 150 samples were examined for *E. coli* using the above MPN method in parallel with a technique based solely on peptone water. This also used a 1 : 10 dilution of shellfish tissue and involved the addition of 5 × 10 ml to 5 × 10 ml of double-strength peptone water and 5 × 1 ml, 5 × 0·1 ml and 5 × 0·01 ml to tubes containing 5 ml of single-strength medium. The tubes were incubated at 44±0·2 °C for up to 48 h and then tested for indole. A positive result was taken to indicate the presence of *E. coli* type I and was used to determine the MPN *E. coli* per 100 ml flesh in the manner described above.

The microbial flora of indole-positive tubes from 50 samples was determined by performing total counts aerobically and anaerobically, using the technique of Miles & Misra (1938), on an agar comprising 5% sterilized, macerated oyster flesh and peptone water. All plates were incubated at 44±0·2 °C for 24 h. Colonies were subcultured into peptone water and tested for their ability to produce indole at 44 °C. Those found to be positive were identified (Cowan & Steele, 1974).

To determine the selective effect of incubation at 44 °C total bacterial counts of diluted, macerated oyster and mussel flesh were also performed as above, with the difference that replicate plates were incubated at 30 and 37 °C in addition to those at 44 °C.

Statistical analysis of the data

The differences between the two MPN methods were compared using McNemar's test for discrepant pairs.

RESULTS

The enumeration of E. coli *in shellfish using MacConkey agar or Formate Lactose Glutamate broth*

An MPN method using Formate Lactose Glutamate (FLG) at 37 °C/48 h followed by confirmation of positive tubes was found to be more sensitive than MacConkey agar pour plates incubated at 44 °C/18 h. Ten of 50 samples examined in parallel were shown to contain *E. coli* by the pour-plate method and 40 by the MPN method ($P < 0{\cdot}001$). In more heavily contaminated samples where *E. coli* was detectable by both techniques, the pour-plate method gave a mean level of 750±80 per 100 ml of flesh, while with the MPN technique the mean figure was 1480±70.

A comparison between Formate Lactose Glutamate broth and peptone water for the detection and enumeration of E. coli *in shellfish*

In 50 samples of cleansed oysters the mean number of total coliforms was found to be 3410 per 100 ml of flesh, while the mean number of *E. coli* was 29. This meant that during routine examination for *E. coli* using FLG broth a large number of positive tubes at 37 °C were unnecessarily confirmed and suggested the need for a direct technique for this organism.

The substitution of peptone water for FLG broth, with indole production being measured after 24 h at 44 °C, was examined as a possible method for the detection

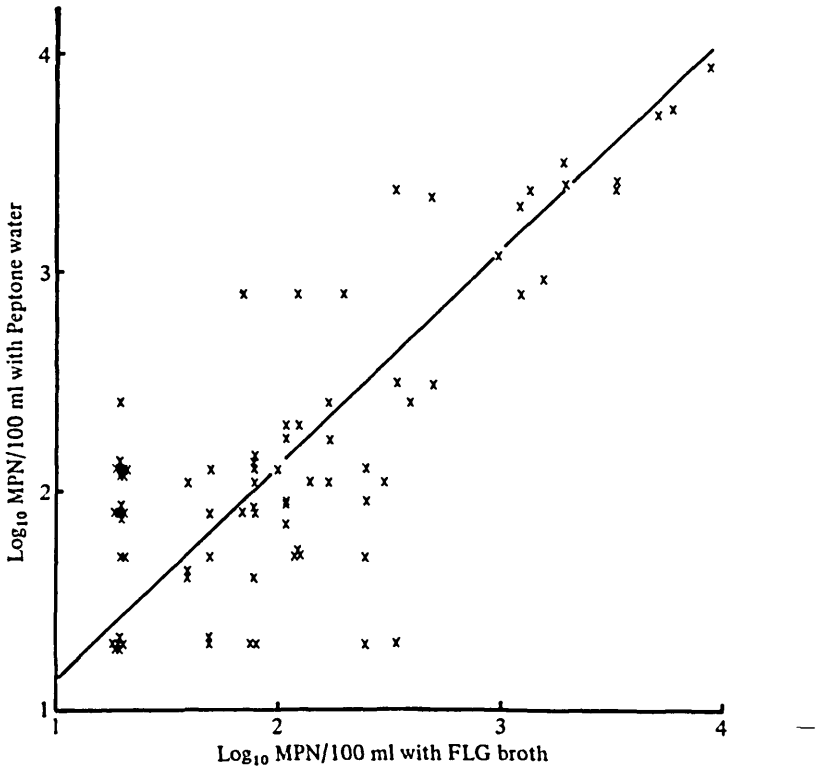

Fig. 1. The correlation between Formate Lactose Glutamate broth and peptone water for the enumeration of *Escherichia coli* in shellfish. Line = regression line ($r = 0{\cdot}949$; $\log_{10}$ MPN tryptone $= 0{\cdot}169 + 0{\cdot}966 \times \log_{10}$ MPN glutamate).

and enumeration of *E. coli* in oysters and mussels. In 150 samples tested in parallel, the two techniques gave the same MPN *E. coli*/100 ml in 37 samples. With 75 of the remaining 113 samples the MPN obtained with peptone water exceeded that found with the other method. These results indicated that the two methods were detecting different levels of contamination ($P = 0{\cdot}001$). Further analysis showed that while there was a degree of correlation between the methods, with a slight bias towards peptone water, where positive results were obtained with both techniques (Fig. 1), this method was significantly better for the detection of *E. coli* (Tables 1 and 2), especially in samples with low levels of contamination, i.e. this method was more successful at detecting small numbers of *E. coli*. In 78 samples where *E. coli* was detected by both methods there was no difference in the efficacy of the two techniques. Thus the same result was obtained in 15 samples, in 35 others peptone water was more sensitive and in the remaining 28 FLG broth gave higher MPN values ($P = 0{\cdot}4$).

Microflora of shellfish and indole-positive tubes

All indole-positive organisms from 50 samples were subsequently indentified as *E. coli*, indicating that for shellfish from temperature waters false positives would not appear to be a problem.

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Table 1. *Detection of* Escherichia coli *using FLG broth or peptone water*

FLG broth: no. of samples where *E. coli* was:	Peptone water: no. of samples where *E. coli* was: Absent	Present	Total
Absent	22	40	62
Present	10	78	88
	32	118	150

$\chi^2 = 17{\cdot}4$, $P = < 0{\cdot}0001$.

Table 2. *The enumeration of* E. coli *in uncleansed shellfish using peptone water or FLG broth*

	MPN *E. coli*/100 ml flesh in FLG	Tryptone
Mean ± S.E.	4380 ± 1100	3760 ± 1180
Range	700–16000	500–16000

$P > 0{\cdot}05$.

Many organisms that are found in shellfish and estuarine waters are capable of indole production at temperatures lower than 44 °C (Cowan & Steele, 1974). However, this is unlikely to produce false positives, as a mixture of 10 ml tryptone water at room temperature and 10 ml of shellfish macerate at 4 °C was found to reach 44 °C within 4 min when placed in a water bath set at that temperature. The elevated incubation temperature was also found to have a significant selective effect, as only 6·4 % of the microbial population of shellfish tissue that was capable of growth on peptone/shellfish agar at 30 °C grew at 44 °C. The microflora of positive tubes and shellfish tissue at 44 °C was dominated by *E. coli* and Bacillus spp., although in some uncleansed samples high numbers of *Clostridium perfringens* and *Clostridium sporogenes* were present.

Enumeration of E. coli *in uncleansed samples of oysters and mussels*

Fourteen samples of shellfish were tested without prior cleansing. Although there was no significant difference in the mean MPN values obtained with the two methods (Table 2), FLG broth gave the higher value in 9 of the 14 samples. The differences were generally quite small and were due to the presence of indole-degrading clostridia, such as *Cl. sporogenes* and *Cl. perfringens*, which caused some false negative tubes with the peptone water method.

DISCUSSION

The detection and enumeration of *E. coli* in foodstuffs has relied on its ability to produce acid and gas from lactose and indole from tryptophan at elevated temperatures. The ability to do the former, however, is easily lost (Meadows,

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Anderson & Patel, 1980; Meadows *et al.* 1980; Bueschkens & Stiles, 1984) or may go undetected because of the presence of food particles in the medium (Varga & Doucet, 1984). For these reasons indole production is considered to be a more reliable indicator of the organism's presence (Anderson & Baird-Parker, 1975; Holbrook, Anderson & Baird-Parker, 1980) and Delany, McCarthey & Grasso (1962) proposed a rapid test for the detection of *E. coli* in water based on this test only. Indole detection alone has not been used for the enumeration of *E. coli* in foods, although the plate technique of Anderson & Baird-Parker (1975) used this attribute in combination with bile tolerance. However, this method has been shown to be less sensitive than MPN methods (Motes, McPherson & De Paola, 1984).

The data presented in this paper show that the measurement of indole production, after 24 h at 44 °C, is a simple and inexpensive way of enumerating *E. coli* in purified molluscan shellfish. While the reliance on a single characteristic as a means of identification could be criticized, it did not appear to lead to inaccuracies in this study. The higher rate of detection of low numbers of *E. coli* in depurated shellfish may be because sublethal injury to some of the organisms present in the tissue caused them to lose the ability to ferment lactose.

The results obtained with the samples tested straight from the estuary (Table 2) suggest that the technique may not be suitable for shellfish taken from beds known to be subject to chronic high levels of faecal pollution. False negatives or 'skipping', which sometimes occurred with FLG broth, only happened with samples containing high numbers of indole-degrading clostridia. These bacteria grew well in tubes inoculated with 10 ml of macerate, and because the uncleansed shellfish generally contained high levels of *E. coli*, it was possible to reduce the effect of the clostridia by using the 1, 0·1 and 0·01 ml dilutions to determine the MPN values. Indole degradation was not a problem in samples that had been improperly cleansed, i.e. those that still contained high numbers of *E. coli*. It would be prudent, at this stage, to confine the peptone water technique to shellfish that have received complete or partial purification. The method has a value as a rapid screening procedure and would enable the regulatory authorities to delay the sale and distribution of shellfish until they have been shown to be free of significant levels of contamination. The enumeration of *E. coli* in freshly harvested shellfish is not urgent and could be carried out some days prior to possible cleansing, as the degree of contamination is unlikely to change to any great extent within that time.

As with any study proposing a new technique, the data presented in this paper are open to criticism, particularly on the grounds that we are redefining *E. coli*. We recognize that this may cause genuine concern, but believe that every effort has been made to demonstrate that the organisms producing indole in the positive tubes are, in fact, *E. coli*. While there must be a number of indole-positive bacteria that are incapable of growth on an agar containing a mixture of shellfish flesh and peptone water, which, therefore, would have been missed in the examination of positive tubes, these are very unlikely to have proliferated under standard test conditions. For a screening test to have the widest possible application it must be simple to use, inexpensive and provide rapid answers. The peptone water method would appear to fulfil these criteria, and while false positive results were not a

problem in this study, it would be of interest to see the technique assessed using shellfish cultivated in different waters.

Maddern, Buller & McDowell (1986) have suggested that *Cl. perfringens* has a value as an indicator of faecal contamination in shellfish. In common with the results presented in this paper, they found high numbers of the organism in freshly harvested oysters and mussels, with most being removed by a relatively short period of cleansing.

The current guidelines for shellfish of 230–300 *E. coli* per 100 ml of flesh would seem, from the data presented in this paper, to be unrealistically high. Should there be a move to make them more stringent, sensitive techniques will be required, and the peptone water method would be worthy of consideration. Whatever techniques are finally adopted, it is surely time to abandon the roll tube or pour plate using Violet Red Bile agar or MacConkey agar. As with previous studies (West, 1984), we have found such techniques to be less sensitive than an MPN method.

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