ICMSF methods studies. XIII. An international comparative study of the MPN procedure and the Anderson–Baird-Parker direct plating method for the enumeration of *Escherichia coli* biotype I in raw meats

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The most probable number (MPN) and a direct membrane-plating (DP) method were compared for enumeration of *Escherichia coli* biotype I in raw meats by 11 laboratories. The DP method yielded higher counts of *E. coli* than the MPN method for frozen samples but neither method consistently gave higher counts for non-frozen samples. The DP method was less variable and gave a higher rate of detection of low numbers of *E. coli* in frozen samples. Despite the inability of the DP method to enumerate *E. coli* biotype II and intermediate types, which comprise only 3–5% of the *Escherichia* strains (Ewing 1972), the method is preferable to the MPN method for enumerating *E. coli* in raw meats because of its lower variability, better recovery from frozen samples, rapidity, decreased requirement for media, and decreased costs for analysts' time.

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Onze laboratoires ont comparé la méthode du nombre le plus probable (MPN) et la méthode de l'ensemencement direct sur membrane (DP) pour la numération d'*Escherichia coli*, biotype I, dans les viandes crues. A partir d'échantillons congelés, la méthode DP donne des valeurs d'*E. coli* plus élevées que la méthode MPN, mais ni l'une ni l'autre ne donnent des décomptes régulièrement plus élevés à partir d'échantillons non-congelés. La méthode DP est moins variable et serait plus sensible, permettant de détecter à un taux plus élevé les faibles quantités d'*E. coli* dans les échantillons congelés. Malgré son incapacité à faire le décompte d'*E. coli* du biotype II ou de types intermédiaires, ce qui représente 3 à 5% des souches d'*Escherichia* (Ewing 1972), la méthode DP est préférable à la méthode MPN à cause de sa plus faible variabilité, son taux supérieur de récupération à partir d'échantillons congelés, sa rapidité, sa consommation réduite de milieux et à cause d'une réduction des coûts de temps de manipulation.

[Traduit par le journal]

Introduction

The International Commission on Microbiological Specifications for Foods (ICMSF) recommends two most probable number (MPN) methods for the enumeration of fecal coliforms and *E. coli* in foods (ICMSF 1978). In one of the methods used extensively in North America, presumptive coliforms are enumerated in lauryl sulfate tryptose broth at 35–37°C and fecal coliforms in EC broth at 44.5 ± 0.2°C (American Public Health Association 1970). The other, a European method, uses MacConkey broth at 35–37°C for enumeration of coliforms and brilliant green lactose broth at 44 ± 0.1°C for fecal coliforms (Guinée and Mossel 1963). Both methods take 3–4 days and if the fecal coliform organisms are to be identified, an additional 5–8 days would be required to conduct IMViC tests on the isolates (ICMSF 1978). The present concern about the enumeration of fecal coliforms, or more specifically *Escherichia coli* in foods, is the length of time and the amount of media and analysts' time required to complete the analyses. This concern has prompted the development of a number of rapid methods among which are direct plating on modified MacConkey agar (Hefferman and Cabelli 1967), and direct plating onto membranes overlying tryptone bile agar (Anderson and Baird-Parker 1975). Andrews and Presnell (1972) have also proposed a shortened MPN method known as the A1 test, which has recently been modified to include a resuscitation step (Hunt and Springer 1978; Andrews *et al.* 1979). Common to these methods, except the modified A1 test, is direct incubation at an elevated temperature of 44.0–44.5°C.

After review of the rapid methods for enumerating fecal coliforms and *E. coli* (the modified A1 test was not reviewed since it was only recently developed), the ICMSF selected as most promising the direct plating method of Anderson and Baird-Parker (1975) which is a modification of the method of Delaney *et al.* (1962). The method yields results in 24–30 h, eliminates the use of costly multiple tubes of media, and reduces the requirement for analysts' time. The same is applicable to plating on modified MacConkey agar (Hefferman and Cabelli 1967), but this method has been shown to yield 10% lower counts than the North American MPN procedure (Varga *et al.* 1977).

In this paper we present the results of an ICMSF sponsored study in which 11 laboratories participated to compare the direct plating method of Anderson and Baird-Parker (1975) and the North American MPN method (American Public Health Association 1970) for enumerating *E. coli* in raw meats.

Materials and Methods

Samples

Raw red meats, poultry and pork, and washings and surface swabs of carcasses thereof were examined. All samples, frozen or non-frozen, were obtained in the country of the participating laboratory and were naturally contaminated.

Media and Membranes

All media were supplied gratis by Difco Laboratories Ltd., and cellulose acetate membranes were generously donated by Oxoid Ltd. Each participant received media and membranes from the same lot.

Enumeration Procedure

Two analysts in each laboratory simultaneously but independently carried out the analyses. To avoid sampling errors, solids were weighed and homogenized in 4 parts (v/w) of 0.1% peptone water by one analyst, and the homogenate (1:5) was divided between the two analysts. Samples were examined by the direct plating (DP) and most probable number (MPN) methods for enumerating *E. coli* biotype I. Biotypes of *E. coli* are defined in *Microorganisms in foods* (ICMSF 1978).

(a) DP Method

The method of Anderson and Baird-Parker (1975) was used. Cellulose-acetate membranes (85-mm diameter, 450-nm pore size, Oxoid Ltd.) were placed on predried surfaces of tryptone bile agar (TBA) contained in 90-mm Petri dishes; the membranes were gently flattened against the agar with a sterile glass spreader so that complete contact was made with the surface of the medium. Duplicate 1.0-mL volumes of the 1:5 and 1:10

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dilutions of solid samples were evenly spread on the membranes, taking care that the inocula did not spill over the edges. When suspensions contained less than 10% meat, 0.5-mL volumes were plated to avoid spillage over the edges of the membranes. After absorption of the inocula (usually 20–30 min), the plates were incubated in the upright position in stacks of not more than three in a 44.5 ± 0.5°C water-jacketed or similarly accurate incubator for 24 h. It is important that this temperature range be maintained throughout the incubation period.

A resuscitation step was included for the plating of frozen samples. The appropriate inocula were first spread on the surface of membranes overlying tryptic soy agar (TSA), and the plates incubated at 35–37°C in the upright position for 4 h. The membranes were then aseptically transferred from the TSA to TBA plates and the latter were incubated for 24 h at 44.5 ± 0.5°C as above. This modification has been reported to yield higher counts of *E. coli* in samples which have been subjected to conditions likely to cause stress in the organisms (Baird-Parker, personal communication; Rayman and Aris, unpublished results).

After incubation at 44.5°C, the membranes were lifted off the TBA, placed in clean covers of the Petri dishes, and stained for 10–15 min with 2.0 mL of 5% *p*-dimethylaminobenzaldehyde reagent (Anderson and Baird-Parker 1975). The membranes were then removed from the covers and dried under a low-pressure UV lamp or in sunlight (window glass does not interfere). The pink indole-positive colonies were enumerated as *E. coli*.

(b) MPN Method

The North American procedure for determination of coliform organisms of fecal origin (American Public Health Association 1970) was used. Since a maximum of 0.4 g of meat could be inoculated over two plates, the lower limit of detection by the DP method was 2.5 cells of *E. coli* biotype I per gram. To obtain comparable results, the lowest dilution used in the three-tube MPN method was 1:10 which yielded a minimum of three cells of *E. coli* per gram. Frozen and non-frozen samples were treated identically. One millilitre of decimal dilutions was transferred to each of three separate tubes containing inverted fermentation vials and 10 mL of lauryl sulfate tryptose broth (LST). These were incubated at 35–37°C and examined for gas production at 24 and 48 h. From gas-positive LST tubes, transfers were made to tubes containing inverted fermentation vials and 10 mL of EC broth; the tubes were incubated at 44.5 ± 0.5°C for 24–48 h. Aliquots from the EC tubes showing gas production were then streaked onto plates of Levine eosin methylene blue agar and after incubation at 35–37°C for 24 h, two typical colonies were picked and purified by streaking onto plates of nutrient agar. IMViC tests (ICMSF 1978) were run on the purified isolates. Only those colonies yielding ++−− IMViC reactions were used in determining the MPN of *E. coli*. The entire procedure took 9–11 days.

Statistical Methods

All statistical tests were chosen to accommodate the lack of independence of the four observations from each food sample, and the non-normality apparent in the data. For some procedures, it was necessary to edit the data for technical purposes. All such edits were kept to a minimum and were applied consistently over laboratories. These edits account for apparent sample-size discrepancies.

For each analyst, the Wilcoxin signed rank test (Hollander and Wolfe 1973) was used to test whether either method yielded consistently higher *E. coli* counts.

For each laboratory and method, variability in the data not attributable to variation in contamination levels from food sample to food sample, nor to consistent analyst differences, was calculated from $\log_{10}$ *E. coli* counts and presented as an estimate of method variability. To test for differences between method variability within each laboratory, an approximate significance test was performed using the "jackknife" technique (Miller 1974) on the test statistic described by Snedecor and Cochran (1973).

An alternative measure of method variability was obtained by modifying the above procedure to include analyst differences as a source of "method" variation. Thus if consistent within-laboratory analyst differences were present, they would inflate this measure of variability accordingly.

Results and Discussion

Comparison of E. coli Counts by the MPN and DP Methods

A comparison of the two methods for enumerating *E. coli* is shown in Table 1. The MPN method yielded counts of *E. coli* lower than the DP method in a significant number of frozen samples examined in laboratories 3 and 5. This was also true in laboratories 7 and 10, but for one analyst only. In laboratories analyzing more than 10 contaminated frozen samples, all but laboratory 1 reported more samples yielding higher *E. coli* counts by the DP method, though not all differences in counts by the two methods were significant.

A different pattern was seen for non-frozen samples. In laboratory 8, the MPN method yielded counts of *E. coli* higher than the DP method in a significant number of samples examined, and in laboratory 4, similar results were obtained by one analyst. Apart from these observations, neither method was consistently favoured over the other for maximal enumeration of *E. coli*.

In both frozen and non-frozen samples, differences in *E. coli* counts by the two methods were small, usually less than a factor of 10. In 62.6% of the frozen samples, *E. coli* counts by the two methods were within 0.5 $\log_{10}$ cycle of each other and in 83.9% of these samples, the counts differed by no more than 1 $\log_{10}$ cycle (Fig. 1A). Similar results were obtained with non-frozen samples where 73.3 and 95.4% of the samples yielded counts by the two methods which differed by no more than 0.5 and 1 $\log_{10}$ cycle, respectively (Fig. 1B). For frozen samples, the skewing of the histogram to the left of zero confirmed the previous observation that in the majority of these samples, the DP method yielded higher counts of *E. coli*. In these samples, it is likely that the cells were damaged and the resuscitation step included in the DP method was responsible for the higher counts.

Variability in the MPN and DP Methods for Enumerating E. coli

The standard deviations of $\log_{10}$ *E. coli* counts for the two methods are listed in Table 2. For frozen foods, the DP method yielded counts which were significantly less variable than those of the MPN

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TABLE 1. Comparison of $\log_{10}$ *E. coli* counts yielded by the MPN and DP methods

Laboratory	Analyst 1				Analyst 2			
	MPN > DP[a]	MPN < DP	Ties	No. samples	MPN > DP	MPN < DP	Ties	No. samples
				Frozen samples				
1	9	3	0	12	7	5	0	12
2	1	0	0	1	0	1	0	1
3	6	19	2	27**	10	16	0	26*
4	1	0	0	1	2	1	0	3
5	5	42	0	47**	0	39	0	39**
6	12	15	0	27	9	16	0	25
7	3	11	0	14*	4	8	0	12
9	3	5	1	9	5	3	0	8
10	14	33	0	47*	—[b]	—	—	—
11	5	12	1	18	—	—	—	—
				Non-frozen samples				
1	16	21	1	38	20	19	0	39
2	28	21	0	49	17	27	2	46
3	1	4	0	5	1	2	0	3
4	20	4	1	25**	16	15	0	31
7	10	10	1	21	8	14	0	22
8	16	4	0	20**	15	10	0	25*
9	20	14	1	35	19	15	0	34

[a]MPN = most probable number method; DP = direct-plating method.
[b]Only one analyst performed tests in laboratories 10 and 11.
*Significantly different at 0.10 level.
**Significantly different at 0.05 level.

method in laboratories 3, 5, and 7. Similar results were obtained for non-frozen foods analyzed in laboratories 1, 2, 4, and 9. Overall, the DP method was less variable than the MPN method except in laboratory 6, though not all differences were significant. The variability of the methods could not be compared in laboratories 10 and 11 because only one analyst performed the tests in these laboratories. Standard deviations were virtually unchanged when the variability due to consistent differences between analysts was included in the above calculations, indicating close agreement between analysts within laboratories (estimates not reported).

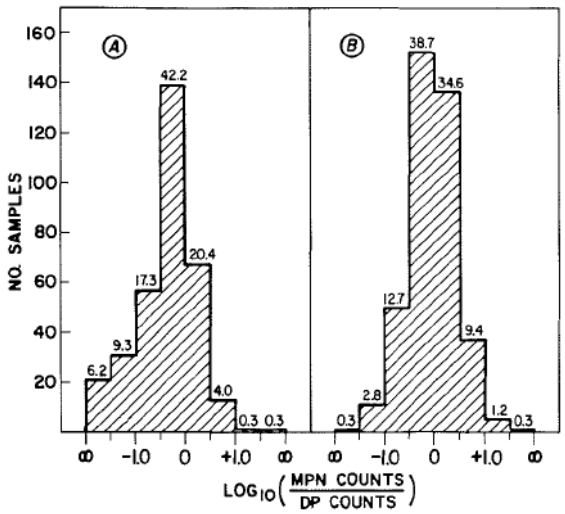

FIG. 1. Frequency distribution of $\log_{10}$ *E. coli* counts obtained by the MPN and DP methods. (A) Frozen samples; (B) non-frozen samples. Numbers at the top of the histograms represent percentages.

IMViC Tests

Only isolates from the MPN method were subjected to IMViC tests. As shown in Table 3, 96.6% of the indole-positive isolates gave IMViC reactions characteristic of *E. coli* biotype I. These figures compare favourably with those of Anderson and Baird-Parker (1975), who reported that 95.1% of 555 indole-positive colonies growing on membranes overlying TBA were *E. coli* biotype I. We did not run confirmatory tests on the indole-positive colonies growing on membranes because they are not a part of the DP method; the method assumes that production of indole at 44.5 ± 0.5°C on TBA is sufficient to identify the colonies as *E. coli*. Based on our results and those of Anderson and Baird-Parker (1975), this assumption is not entirely correct in that 3–5% of the indole-positive isolates were not *E. coli* biotype I. However, the

National Beef Ex. 1010

TABLE 2. Estimated standard deviations of $\log_{10}$ *E. coli* counts yielded by the MPN and DP methods

Laboratory	No. samples	Standard deviation MPN[a]	Standard deviation DP[a]	P^b
		Frozen samples		
1	12	0.173	0.114	0.117
2	1	—	—	—
3	22	0.310	0.173	0.009***
4	1	—	—	—
5	37	0.379	0.263	0.062*
6	23	0.221	0.288	0.36
7	11	0.446	0.170	0.003***
9	8	0.333	0.195	0.428
		Non-frozen samples		
1	38	0.448	0.122	<0.0001***
2	46	0.332	0.187	0.015**
3	3	—	—	—
4	23	0.562	0.192	0.001***
7	18	0.329	0.229	0.381
8	19	0.439	0.243	0.234
9	34	0.472	0.164	0.0001***

[a]See footnote to Table 1.
[b]Observed level of significance of the test of the hypothesis that standard deviations of the two methods are equal.
*Significantly different at 0.10 level.
**Significantly different at 0.05 level.
***Significantly different at 0.01 level.

TABLE 3. Biotyping of indole-positive organisms isolated by the MPN method

Laboratory	No. indole-positive isolates	% *E. coli* biotype I	% not *E. coli* biotype I
1	645	99.1	0.9
2	560	99.6	0.4
3	519	97.7	2.3
4	349	96.0	4.0
5	260	79.6	20.4
6	163	100.0	0
7	—[a]	—	—
8	207	99.5	0.5
9	449	94.7	5.3
10	360	99.2	0.8
11	75	96.0	4.0
Overall	3487	96.6	3.4

[a]IMViC reactions for isolates in this laboratory not reported.

time-saving advantage of the DP method greatly outweighs the disadvantage of including a small number of non-biotype I *E. coli* in the final count. A more serious disadvantage inherent in the method is its inability to enumerate *E. coli* incapable of producing indole. Fortunately, only 1% of all *E. coli* strains fall into this category (Ewing 1972). On the other hand, the DP method enumerates anaerogenic and late lactose-fermenting strains of *E. coli* biotype I which would be missed by the MPN method; according to Ewing (1972), these organisms comprise as much as 10% of *Escherichia* strains.

Detection of E. coli Biotype I in Samples

Although both methods are quantitative, we calculated the number of samples found to be positive for *E. coli* biotype I by either method (Table 4). Of 868 positive samples, the MPN method detected 797 and the DP method 823. Both methods detected *E. coli* biotype I in 752 samples and either method was capable of enumerating low numbers. The organism was also detected in 45 samples by the MPN but not the DP method; of the 45 samples, 20 were frozen and 25 non-frozen, and only 3 samples contained greater than 50 *E. coli* per gram. Conversely, 71 samples yielded *E. coli* biotype I by the DP method alone, and 49 of these were frozen; only 6 of the 71 had counts exceeding 50 *E. coli* per gram. These results indicate that both methods, as used in this study, were capable of detecting *E. coli* biotype I when low numbers are present, but the DP method gave a higher rate of detection of low numbers in frozen samples.

Specific Comments on DP Method

Some useful observations were made on the DP method. Analysts were initially instructed to dry the stained membranes under a low-pressure UV lamp, in direct or in indirect sunlight. In one laboratory good differentiation of colonies on membranes dried under fluorescent lights was reported, while in three laboratories indirect sunlight was inadequate for developing the pink color of indole-positive colonies; it appears that best results were obtained when membranes were dried in direct sunlight or under a UV lamp. Some laboratories

TABLE 4. Number of samples found to contain *E. coli* biotype I by the MPN and DP methods

Method[a]	No. positive samples	No. positive samples containing *E. coli* biotype I per gram: >50	10–50	<10
MPN	797	406	203	188
DP	823	499	221	103
Both MPN and DP	1504[b]	896[c]	393[d]	215[e]
MPN only	45	3	6	36
DP only	71	6	25	40

[a]See footnote to Table 1; minimum number of *E. coli* biotype I detected by MPN method was 3.0 per gram, by DP method 2.5 per gram.
[b]Comprising 752 samples by each method.
[c]Comprising 403 and 493 samples by the MPN and DP methods, respectively.
[d]Comprising 197 and 196 samples by the MPN and DP methods, respectively.
[e]Comprising 152 and 63 samples by the MPN and DP methods, respectively.

National Beef Ex. 1010

also reported that optimal differentiation was obtained after 10–30 min of drying; after this time, the color of indole-positive colonies faded. Another minor problem encountered was the extension of the working day if the resuscitation step was required. To overcome this, one laboratory found that they could refrigerate the plates with membranes overnight and stain them the following day without loss in differentiation. This step would be useful in laboratories with fixed working hours. On the whole, no major adverse comments were made on the DP method which appears to be superior to the MPN method because of its lower variability, better recovery from frozen samples, rapidity, and decreased requirement for media and analysts' time.

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National Beef Ex. 1010

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National Beef Ex. 1010