J. Hyg., Camb. (1982), **89**, 185–190
Printed in Great Britain

A most probable number method for estimating small numbers of campylobacters in water

By F. J. BOLTON, P. M. HINCHLIFFE, D. COATES* and L. ROBERTSON

Public Health Laboratory, Preston Infirmary, Meadow Street, Preston, Lancashire PR1 6PS, U.K.

(*Received* 24 *April* 1982; *accepted* 17 *May* 1982)

SUMMARY

A most probable number (MPN) method capable of estimating as few as ten campylobacters per 100 ml of water is described. The method gave results close to those obtained by the viable count method of Miles, Misra & Irwin (1938) with graded suspensions of *Campylobacter jejuni*. The method was used to test raw water samples: counts were obtained ranging from 10 to 230 campylobacters per 100 ml for 11 of 49 coastal and estuary water samples, and from 10 to 36 campylobacters per 100 ml for 7 of 44 river samples. Campylobacters were isolated from an additional 24 of the 'negative' samples by testing 200 ml volumes by glass microfibre filtration and enrichment culture methods. The MPN method should prove to be a useful epidemiological tool particularly suited to the enumeration of campylobacters in particulate fluids.

INTRODUCTION

The epidemiology of human campylobacter infections remains to be fully elucidated, although some sources of infection are now well established such as unpasteurized milk (e.g. Robinson & Jones, 1981), improperly cooked chicken (Brouwer *et al.* 1979), and infected dogs (e.g. Blaser *et al.* 1978) and cats (e.g. Svedhem & Norkrans, 1980). There is now strong circumstantial evidence that water can be a vehicle of infection. Pearson *et al.* (1977) and Knill, Suckling & Pearson (1978) isolated campylobacters from many fresh-water and sea-water samples by membrane filtration, but only in the presence of *Escherichia coli* type 1 (H.M. Stationery Office, 1969), which suggested that the campylobacters were derived from faeces of animals, birds or man. Two major outbreaks of probable water-borne campylobacter enteritis have been reported. In Bennington, Vermont, an estimated 3000 of the town's population of 10000 developed campylobacter enteritis after consuming water from the town's main water supply which probably was inadequately chlorinated (Vogt *et al.* 1982). In Sweden about 2000 people developed enteritis after consuming unchlorinated main water that possibly had

* Requests for reprints to: Dr D. Coates, Public Health Laboratory, Preston Infirmary, Meadow Street, Preston, Lancashire PR1 6PS, U.K.

become mixed with contaminated river water (Mentzing, 1981). Evidence implicating the water was strong in both outbreaks but in neither were campylobacters isolated from the water. A sensitive method for the detection and enumeration of campylobacters in water would be of great value, and we now describe a simple method of estimation based on the Poisson distribution.

MATERIALS AND METHODS

Evaluation of the MPN method with graded suspensions of C. jejuni

Test organism

The test organism was *Campylobacter jejuni* NCTC 11168, a biotype 1 strain (Skirrow & Benjamin, 1980).

Preparation of suspensions

The test organism was cultured for 24 h at 42 °C on Columbia agar (Oxoid CM331) containing 5 % horse blood in a microaerobic atmosphere (about 6 % oxygen, 10 % carbon dioxide and 84 % nitrogen). Organisms from this culture were suspended in 0·1 % peptone water (Oxoid L37) to a density of about 10^8 colony-forming units (c.f.u.) per ml with a Perkin–Elmer Model 6/20 spectrophotometer set at a wavelength of 450 nm. Dilutions of 10^{-5}, 10^{-8} and 10^{-9} of this standardized suspension were prepared.

Viable count method

The method of Miles, Misra & Irwin (1938) was used to determine the viable count of the 10^{-5} dilution of the standardized suspension of *C. jejuni*. The culture medium used was non-selective agar containing 2 % New Zealand agar in order to give discrete colonies. Plates were incubated microaerobically at 42 °C for 48 h.

The MPN method

1 ml portions of the 10^{-8} and 10^{-9} dilutions of the standardized suspension were pipetted into 50 bijou (7 ml) bottles each containing 5 ml of non-selective broth (Table 1) and 50 bijou bottles containing 5 ml of Preston enrichment broth (Table 1). The 200 inoculated bottles (100 for each dilution) were incubated with caps screwed down tight at 42 °C for 24 h. Subcultures (five broths per plate) were then made onto both non-selective agar and Preston Medium (Bolton & Robertson, 1982) (Table 1). Plates were incubated microaerobically at 42 °C for 48 h and the number of campylobacter-negative broths in each group was recorded. The MPN of campylobacters per ml was calculated according to the formula of Campbell (1974).

The testing of raw water samples

Forty-nine coastal and estuary water samples and 44 river-water samples were collected in $2\frac{1}{2}$ l Winchester bottles and transported in an ice-cooled box to the laboratory where tests were performed on the same day.

Table 1. *Ingredients of non-selective broth, non-selective agar, Preston enrichment broth and Preston medium (quantities per litre)*

Ingredients	Non-selective broth	Non-selective agar	Preston enrichment broth	Preston medium
Nutrient Broth (Oxoid CM 67)	25 g	25 g	25 g	25 g
New Zealand agar	—	12 g	—	12 g
Saponin lysed horse blood	50 ml	50 ml	50 ml	50 ml
		FBP supplement*		
Ferrous sulphate	0·25 g	—	0·25 g	—
Sodium metabisulphite	0·25 g	—	0·25 g	—
Sodium pyruvate	0·25 g	—	0·25 g	—
		Antibiotics		
Polymyxin B sulphate	—	—	5000 i.u.	5000 i.u.
Trimethoprim lactate	—	—	10 mg	10 mg
Rifampicin	—	—	10 mg	10 mg
Actidione	—	—	100 mg	100 mg

* Aerotolerant supplement of George *et al.* (1978).

MPN method

1 ml portions of each water sample, which had first been mixed thoroughly, were pipetted into 10 bijou bottles each containing 5 ml of Preston enrichment broth (Table 1). These were incubated and subcultured as above onto Preston medium, and the number of campylobacter-negative broths recorded. The MPN of campylobacters present in each sample was read off from Table 2 which was derived from the formula of Campbell (1974):

$$m = -log_e\, x/10,$$

where m is the mean number of organisms in 1 ml of water and x is the number of campylobacter-negative broths.

Filtration method

200 ml of each raw water sample were filtered through 7·0 cm Glass Microfibre Filters (Whatman GF/F) in a Buchner funnel connected to a vacuum pump via a conical flask. Each filter was placed in 25 ml of Preston enrichment broth and incubated microaerobically at 42 °C for 24 h. Subcultures were then made onto plates of Preston medium which were incubated microaerobically at 42 °C for 12 h.

RESULTS

Evaluation of the MPN method with graded suspensions of C. jejuni

The results are summarized in Table 3. According to the Miles, Misra & Irwin (1938) method the 10^{-5} dilution of the standardized suspension contained 2600

Table 2. *MPN of campylobacters in water samples estimated from the number out of ten 1 ml portions found to be campylobacter-negative*

Number of negative broths (x)	MPN	
	Organisms per 100 ml	95% confidence limits*
0	>230	118–>600
1	230	81–600
2	160	59–368
3	120	43–270
4	92	30–211
5	69	21–168
6	51	13–134
7	36	7–106
8	22	3–81
9	10	0·25–59
10	0	<0–37

* Diem (1962).

organisms per ml (the mean colony count of 16 0·02 ml drops was 52). Thus the estimated viable count of the 10^{-9} dilution was 26 per 100 ml.

With the MPN method 27 of the 50 Preston enrichment broths and 28 of the 50 non-selective broths were negative with the 10^{-9} dilution, which gave estimated counts of 62 and 58 organisms per 100 ml respectively. Subcultures on Preston and non-selective agars gave identical results. None of the broths inoculated with the 10^{-8} dilution were campylobacter-negative on subculture which gives an estimated campylobacter count of > 230 per 100 ml.

Tests on raw water samples

Eleven of the 49 coastal and estuary samples were campylobacter-positive by the MPN method with counts ranging from 10–230 per 100 ml. An additional 15 samples were shown to contain campylobacters by the filtration method. Similarly, 7 of the 44 river samples were positive by the MPN method with counts ranging from 10–36 campylobacters per 100 ml; an additional nine were positive by the filtration method. All 18 samples found positive by the MPN method were also positive by filtration.

DISCUSSION

The results obtained by the MPN method and the viable count method of Miles, Misra & Irwin (1938) show close agreement; in fact the figures lying within the 95% confidence limits almost overlap (Table 3). Moreover, the most selective system to which the *C. jejuni* test strain was exposed, i.e. Preston enrichment broth in conjunction with Preston medium, was no more inhibitory than the combination of non-selective media. The method will detect as few as 10 campylobacters per 100 ml water when 10 × 1 ml amounts are used, which is what we suggest for routine use, but the 50 × 1 ml regimen is more sensitive and accurate owing to the larger sample size. Alternatively, larger volumes could be accommodated by the use of double-strength broth, but not in the case of sea-water, because some *C. jejuni* and *C. coli* strains are inhibited by salt concentrations greater than 1·5%.

Table 3. *Comparison of counts of* C. jejuni *in a suspension estimated by an MPN method and determined by the viable count method of Miles, Misra & Irwin* (1938)

Method	Media	Counts/100 ml of different dilutions of the suspension		
		10^{-5}	10^{-8}	10^{-9}
MPN	Preston enrichment broth/ Preston medium	—	>230	62 (38–94)*
	Preston enrichment broth/ non-selective agar	—	>230	62 (38–94)*
	Non-selective broth/ Preston medium	—	>230	58 (36–89)*
	Non-selective broth/ non-selective agar	—	>230	58 (36–89)*
Viable count	Non-selective agar (containing 2% agar)	$2{\cdot}6 \times 10^5$	—	26 (17–35)†

* 95% confidence limits (Diem, 1962)
† 95% confidence limits (Wilson & Miles, 1975)

The MPN method allows the possibility of strain identification, since the broths are subcultured onto solid medium which enables selection of single colonies. We used the scheme of Skirrow & Benjamin (1980) to biotype the strains isolated from river, estuary, and coastal water samples and found that *C. jejuni*, *C. coli*, and nalidixic acid-resistant thermophilic campylobacter (NARTC), were all represented, and there were also strains untypable by this scheme. A few of the water samples contained several different strains. Complementary tests for the presence of *E. coli* type 1 by established methods (H.M. Stationery Office 1969) showed that campylobacters were found only in the presence of *E. coli*, as previously observed by other workers (Pearson *et al.* 1977; Knill, Suckling & Pearson 1978), but there was no apparent correlation between numbers of campylobacters and *E. coli* present.

Membrane filtration can be used to detect campylobacters in water (Pearson *et al.* 1977; Knill, Suckling & Pearson 1978) but it is unsuitable for their enumeration, because colonies developing on a membrane spread and coalesce; also waters containing particulate matter, such as sea water, block the membrane. The MPN method described in this paper is quick and easy to perform and is suitable for testing river, coastal, and estuary water samples; it should also be applicable to fluids other than water.

REFERENCES

Blaser, M., Powers, B. W., Cravens, J. & Wang, W. L. (1978). *Campylobacter* enteritis associated with canine infection. *Lancet* ii, 979–981.

Bolton, F. J. & Robertson, L. (1982). A selective medium for isolating *Campylobacter jejuni/coli*. *Journal of Clinical Pathology* **35**, 462–467.

Brouwer, R., Mertens, M. J. A., Siem, T. H. & Katchaki, J. (1979). An explosive outbreak of *Campylobacter* enteritis in soldiers. *Antonie van Leeuwenhoek* **45**, 517–519.

Campbell, R. C. (1974). *Statistics for Biologists*, 2nd ed. Cambridge University Press.

Diem, K. (ed.). (1962). *Documenta Geigy: Scientific Tables*, Manchester: Geigy Pharmaceutical.

George, H. A., Hoffman, P. S., Smibert, R. M. & Kreig, N. R. (1978). Improved media for growth and aerotolerance of *Campylobacter fetus*. *Journal of Clinical Microbiology* **8**, 36–41.

HER MAJESTY'S STATIONERY OFFICE. (1969). The bacteriological examination of water supplies.

KNILL, M., SUCKLING, W. G. & PEARSON, A. D. (1978). Environmental isolation of heat-tolerant *Campylobacter* in the Southampton area. *Lancet* ii, 1002–1003.

MENTZING, L. O. (1981). Waterborne outbreaks of *Campylobacter* enteritis in central Sweden. *Lancet* ii, 352–354.

MILES, A. A., MISRA, S. S. & IRWIN, J. O. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**, 732–749.

PEARSON, A. D., SUCKLING, W. G., RICCIARDI, I. D., KNILL, M. & WARE, E. (1977). *Campylobacter*-associated diarrhoea in Southampton. *British Medical Journal* ii, 955–956.

ROBINSON, D. A. & JONES, D. M. (1981). Milk-borne *Campylobacter* infection. *British Medical Journal* **282**, 1374–1376.

SKIRROW, M. B. & BENJAMIN, J. (1980). Differentiation of enteropathogenic *Campylobacter*. *Journal of Clinical Pathology* **33**, 1122.

SVEDHEM, A. & NORKRANS, G. (1980). *Campylobacter jejuni* enteritis transmitted from cat to man. *Lancet* i, 713–714.

VOGT, R. L., SOURS, H. E., BARRETT, T., FELDMAN, R. A., DICKINSON, B. S. & WITHERELL, L. (1982). *Campylobacter* enteritis associated with contaminated water. *Annals of Internal Medicine* **96**, 292–296.

WILSON, G. S. & MILES, A. A. (1975). *Topley and Wilson's Principles of Bacterioloy, Virology and Immunity*, 6th ed., vol. II, p. 2651. Edward Arnold.