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Pre-Enrichment and Enrichment Methods for Isolating Small Number of Campylobacteria from Contaminating Flora

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Aho, M., M. Kauppi and J. Hirn: Pre-enrichment and enrichment methods for isolating small number of campylobacteria from contaminating flora. Acta vet. scand. 1988, 29, 443-449. - A method was developed to detect fewer than 100 CFU of campylobacteria from SIFF transport medium to which thawing drip from deep frozen broiler carcasses was added as a source of contamination and which was then stored at room temperature for 20 h. The method was made possible by using pre-enrichment in 1% buffered peptone water under a microaerophilic atmosphere for 5 h at 43°C before selective enrichment either in brucella enrichment broth and on brucella blood selective agar supplemented with Skirrow antibiotics or in CCD enrichment broth and on blood free CCD selective agar. The other pre-enrichment broth studied was alkaline peptone water with reducing agents (RAPW) and the other enrichment broths and selective agars were Preston broth and agar, THAL broth and alkaline tryptose broth (ATB) and brucella agar with ATB antibiotics. Contaminating flora can be a problem when using enrichment broths and selective agars with limited antibiotic supplementation.

microbiological methods; food hygiene; diagnostic procedures; selective media.

Introduction

Campylobacteria have very narrow optimum growth limits. Various physical and chemical factors control their growth: temperature, oxygen concentration, water activity, NaCl, pH and disinfectants (Doyle 1984). Campylobacteria can withstand various stress-inducing factors if there is a high protein concentration in their environment (Hänninen 1981, Blankenship & Craven 1982, Park *et al.* 1983, Svedhem *et al.* 1981).

Stressed, supplethally injured campylobacteria are sensitive to the antibiotics used in selective enrichment broths and agars, in particular to polymyxin B, rifampicin and ce-

phalothin (Hänninen 1982, Ray & Johnson 1984a, Ray & Johnson 1984b, Humphrey & Cruickshank 1985, Lai-King *et al.* 1985). If the primary sample contains a large number of campylobacteria, some of them may survive selective enrichment. Difficulties arise, however, with samples containing small number of campylobacteria and high levels of other bacteria. Special demands must be ascribed to methods for detection of campylobacteria because the infective dose to humans can be as low as only 500 bacteria (Robinson 1981).

Pre-enrichment is one possible method of improving detection sensitivity. The International Standardization Organization stan-

dard of 6579-1981 recommends the use of pre-enrichment in *Salmonella* detection, especially for food samples. Pre-enrichment in buffered peptone water is applied to revive sublethally injured bacteria before selective enrichment. *Humphrey & Cruickshank* (1983) found that recovery of the cold- and freeze-stressed campylobacteria is complete in broth after incubation for 4 h at 43°C and *Palumbo* (1984) reported that heat-injured *Campylobacter jejuni* were revived in brucella broth supplemented with $\text{Na}_2\text{S}_2\text{O}_3$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and sodium pyruvate within 4 h at 37°C. Later *Humphrey et al.* (1985) recommended inoculation of samples containing small number of sublethally injured campylobacteria to refrigerated broth (refrigeration for increased viscosity) and incubation of these broths for 2 h at 37°C before addition of the selective agents. *Sjögren et al.* (1985) recommended pre-enrichment of one of the duplicate samples in semi-solid motility test medium (*Chan & Mackenzie* 1982) for 24 h.

In the present study we compared the sensitivity of 2 low cost pre-enrichment broths with the method without pre-enrichment. We attempted to simulate real conditions of sample transport and compared the 5 most used enrichment broths and 4 most used selective agars in detecting small number of stressed campylobacteria with and without pre-enrichment.

Materials and methods

Bacteria

Campylobacter jejuni biotype 1 (NCTC 11385), *C. jejuni* biotype 2 (NCTC 11392), *C. coli* (NCTC 11353) and *C. laridis* (NCTC 11352) were obtained from the Public Health Laboratory Service, London, U.K. *C. jejuni* B33 (broiler origin) and *C. jejuni* KH3, 5616 and 9000 (human origin) were obtained from Dr. M.-L. Hänninen, Depart-

ment of Food and Environmental Hygiene, College of Veterinary Medicine, Helsinki, Finland. *C. jejuni* 100 (bovine origin) was isolated by the authors at the National Veterinary Institute, Helsinki, Finland.

Preparation of cultures

Organisms were grown for 20 h at 43°C in brucella broth (Difco Laboratories, Detroit, Mich., U.S.A.) supplemented with FBP (ferrous sulfate, sodium metabisulfite and sodium pyruvate all at 0.5 g/l). Strains were preserved in the same broth supplemented with 10% glycerol at -70°C. Frozen and freeze-dried strains were cultured twice in brucella broth before use.

Transport and cultured media

Transport medium. SIFF medium (*Sandven et al.* 1982) – Portions of 10 ml medium were added to test tubes.

Pre-enrichment broths. 1% buffered peptone water (BBL Microbiology Systems, Cockeysville, Md., U.S.A.) and Alkaline peptone water with reducing agents (RAPW) (*Wang et al.* 1983) – Media portions were 10 ml in test tubes for the first test and 90 ml in 200 ml flasks for the second.

Enrichment broths and selective agars. Modified Skirrow enrichment broth and selective agar (*Skirrow* 1977, *Balser et al.* 1979, *Hänninen* 1981) – The modification was addition of 2 mg/l amphotericin B (E. R. Squibb et Sons Ltd., London, U.K.) to the media. Preston enrichment broth and selective agar (*Bolton & Robertson* 1982) – Modified Thioglycolate-horse blood-actidone-laurylsulfate enrichment broth (THAL) (*Oosterom et al.* 1983) – The modification was addition of fresh defibrinated bovine blood to the media instead of lysed horse blood. Alkaline tryptose enrichment broth (ATB) and brucella agar with selective agents (ATB selective agar) (*Wesley et al.*

1983). Charcoal-cefazolin-deoxycholate enrichment broth (CCD enrichment broth) and selective agar (CCD agar) (Bolton *et al.* 1984).

All broths were portioned 90 ml in 200 ml flasks and agars 25 ml on Ø 9 cm Petri dishes with nodules.

Incubation and identification

All enrichment broths and selective agars were incubated under a microaerophilic atmosphere, broths for 20 h and selective agars for 44 h at 43°C. The microaerophilic atmosphere above the liquid (90 ml in a 200 ml flask) and agars was achieved by evacuating gas boxes to -800 mbar and then filling them with a gas mixture containing 5% O₂, 10% CO₂ and 85% N₂. Evacuation and filling was carried out twice.

Campylobacteria were recognized by examining wet mounts by microscopy for curved rods exhibiting darting motility and by catalase and oxidase tests. After the incubation period of the test strains when the campylobacteria were in the early stationary phase, brucella broth was diluted in 0.1% buffered

peptone water (BBL). For quantitative examination the brucella broth dilutions were plated on Skirrow selective agar. Thawing drip from deep-frozen broiler carcasses, used as a source of natural contaminating flora, was examined for campylobacteria using Skirrow enrichment broth and selective agar with negative results.

Test procedure

The pre-enrichment broths comparison test: 1 ml aliquots of tenfold brucella broth dilutions (representing from < 10 to < 10 000 CFU of campylobacteria) were added to 10 ml of both 1% buffered peptone water and RAPW. Five different *C. jejuni* strains were used in this test. After incubation in a microaerophilic atmosphere for 5 h and 20 h at 43°C the whole pre-enrichment culture was poured into a Skirrow broth, enriched and plated on Skirrow selective agar. Another experiment with contaminating flora was made by adding 1 ml of thawing drip from a deep-frozen broiler carcass to the pre-enrichment broth before incubation (see Fig. 1).

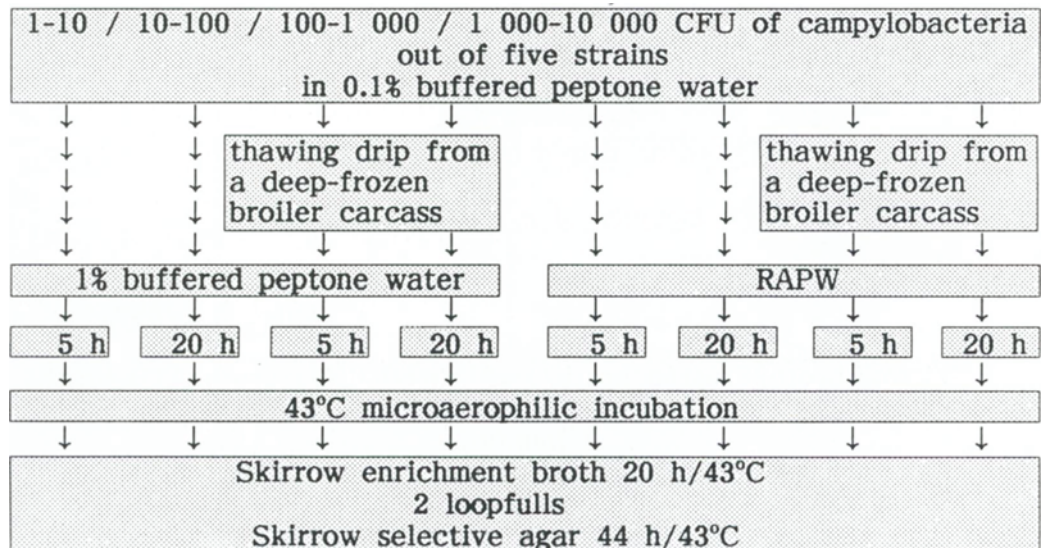


Figure 1. Test procedure for the pre-enrichment broth comparison test.

The test simulating sample transport consisted of three experiments. Four NCTC type strains were used in this test.

In the first "transport" experiment 1 ml aliquots of tenfold brucella broth dilutions (representing from < 10 to < 10 000 CFU of campylobacteria) were added to five tubes containing 10 ml of SIFF medium. Inoculated SIFF tubes were sealed and stored for 20 h at room temperature in the dark, transferred to Skirrow, Preston, THAL, CCD and ATB enrichment broths, incubated and plated (two loopfulls of broth) on Skirrow, Preston, Skirrow, CCD and ATB selective agars, respectively.

The second "transport with contaminating flora" experiment was carried out by adding 1 ml of thawing drip from a deep-frozen broiler carcass to SIFF tubes before inoculation with campylobacteria. Enrichment and plating were carried out as in the first experiment.

The third "transport with contaminating flora and pre-enrichment" experiment was carried out by adding contaminating flora to

SIFF tubes and pre-enriching whole stored SIFF medium in 90 ml of 1 % buffered peptone water under a microaerophilic atmosphere for 5 h at 43°C. After incubation 10 ml of pre-enrichment broth was transferred to enrichment broths and plated as in the first experiment (see Fig. 2).

Results and discussion

The results of the pre-enrichment broth comparison test are presented in Tables 1 and 2. Results of the test simulating sample transport are shown in Fig. 3.

The sensitivity of the campylobacter detecting methods was improved by using pre-enrichment with 1 % buffered peptone water. Another pre-enrichment broth, alkaline peptone water with reducing agents (RAPW), was inhibitory to 4 of the strains used in this study. The concentration of cysteine (0.25 g/l) and sodium thioglycolate (0.5 g/l) used in the RAPW medium could have created an anaerobic environment under reduced oxygen atmosphere and partly explain this phenomenon. When contaminating

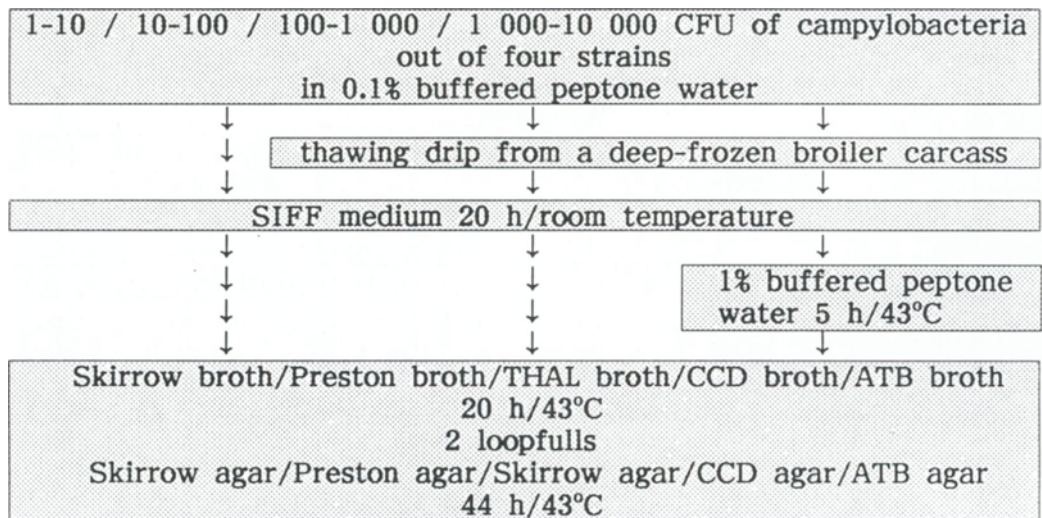


Figure 2. Test procedure for the test simulating sample transport.

ating flora (thawing drip from a deep-frozen broiler carcass) was added, it was still possible to detect smaller number of campylo-

Table 1. Smallest number of campylobacteria out of 5 strains which could be detected using brucella enrichment broth and brucella blood agar supplemented with Skirrow antibiotics. The sample was first pre-enriched in 1% buffered peptone water (1% PW) or in alkaline peptone water with reducing agents (RAPW) under a microaerophilic atmosphere for 5 h and for 20 h at 43°.

strain	control	pre-enrichment for 5 h		pre-enrichment for 20 h	
		1% PW	RAPW	1% PW	RAPW
100	2 700	400	>4 000	60	- ^a
9000	70	n.t. ^b	n.t.	70	-
KH3	4 200	>30 000	>30 000	60	-
5616	3 200	n.t.	n.t.	320	-
B33	360	1 500	1 500	90	-

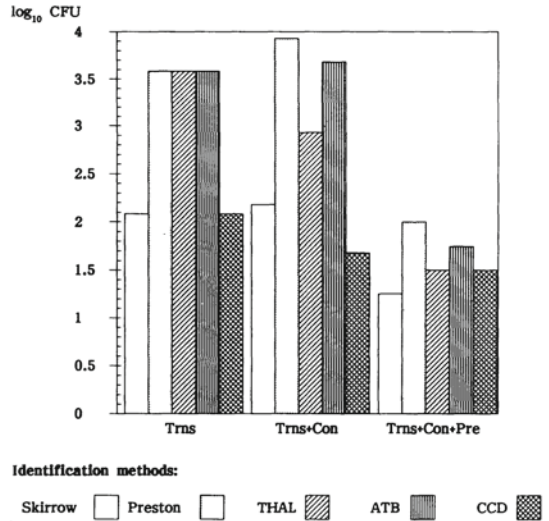
^a - = not grown in this pre-enrichment broth (required inoculation was > 10 000 CFU)

^b n.t. = not tested

Table 2. Smallest number of campylobacteria out of 5 strains which could be detected from contaminating flora using brucella enrichment broth and brucella blood agar supplemented with Skirrow antibiotics. The sample was first pre-enriched in 1% buffered peptone water (1% PW) or in alkaline peptone water with reducing agents (RAPW) under a microaerophilic atmosphere for 5 h and for 20 h at 43° and thawing drip from a deep-frozen broiler carcass as a source of contaminating flora was added to samples.

strain	control	pre-enrichment for 5 h		pre-enrichment for 20 h	
		1% PW	RAPW	1% PW	RAPW
100	800	1 700	1 700	C ^a	C
9000	100	1	1	C	C
KH3	1 100	1 100	>1 000	C	C
5616	9 400	1 600	>1 600	C	C
B33	730	130	>1 300	C	C

^a C = plate contaminated completely



Identification methods:

Skirrow Preston THAL ATB CCD

Figure 3. Test simulating sample transport. Smallest number of campylobacteria detected by five different methods with and without contaminating flora or pre-enrichment. Transport simulation (Trns) was 20 h storage in SIFF media at room temperature. Source of contaminating flora was 1 ml of thawing drip from a deep-frozen broiler carcass (Con). Pre-enrichment (Pre) was carried out 5 h in 1% peptone water.

See text for test procedure.

bacteria after 5 h pre-enrichment than without pre-enrichment.

Microbiological laboratories face another special problem with environmental samples which contain low number of campylobacteria. *Rollins & Clowell* (1986) showed that campylobacteria change into a nonculturable state within 2-3 weeks in aquatic environments at 25 or 37°C. This change can also be seen in the morphology of the bacteria: *Moran & Upton* (1987) reported loss of spiral morphology after prolonged incubation on solid media. The nonculturable state of campylobacteria has serious epidemiological implications because the cells are still viable. Change to the nonculturable state was not observed in pure cultures cultivated

in stationary biphasic culture systems but it was seen in the same system after 3 days if the culture medium was shaken. Shaking of the medium appears not to be favourable to campylobacteria.

Our results show that 5 h pre-enrichment in inexpensive stationary liquid media under a microaerophilic atmosphere is sufficient to revive stressed campylobacteria. If the pre-enrichment incubation time is prolonged, contaminant bacteria reach the exponential growth phase and easily overgrow the sample.

In the test simulating sample transport, pre-enrichment improved the sensitivity of all the isolation methods used (paired Student t-test, $p < 0.01$). The most sensitive methods tested were those with limited antibiotic supplementation, CCD and Skirrow (paired Student t-test, $p < 0.01$, with the exception of the Skirrow and THAL methods comparison, in which the Skirrow method was more sensitive with $p < 0.05$). The contaminating flora also appeared most often on these agars. Low concentrations of polymyxin B (2 500 IU/l) and the absence of rifampicin in the Skirrow antibiotic supplementation and the only antibiotic, cefazolin (10 mg/l), in CCD antibiotic supplementation do not restrict the growth of campylobacteria but are also insufficient to inhibit contaminating flora.

When a pre-enrichment step in 1% buffered peptone water under a microaerophilic atmosphere for 5 h at 43°C was added to the method, it was possible with CCD and Skirrow methods to detect fewer than 100 CFU of campylobacteria from SIFF medium which was stored for 20 h at room temperature after inoculation.

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Sammanfattning

Preinkuberings- och anrikningsmetod till ett litet antal campylobakterier med kontaminering.

Färre än 100 CFU av campylobakterier kunde isoleras ur SIFF transport medium som hade kontaminerats med smältvatten från djupfrysta broiler och som förvarats i rumstemperatur för 20 h. Metoden bestod av 5 timmar preinkubering i 1 % peptonvatten i mikroaerofil atmosfär i 43°C före det selektive anrikandet antingen i brucella buljong och på brucella blod agar med Skirrow antibiotika eller i kol-cefazolin-desoxycholate (CCD) anrikningsbuljong och på CCD agar. Den andra preinkuberingsbuljongen studerats var alkalisk pepton vatten med reducerande medel (RAPW) och andra buljonger och plattor var Preston-buljong och -medium, THAL-buljong och alkalisk trypton buljong (ATB) och brucella agar med ATB-antibiotika. Kontaminering av plattorna kan vara ett problem när man använder anrikningsbuljonger och selektiva medier med begränsad dosering av antibiotika.

(Received April 5, 1988).

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