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COMPARISON OF FOUR ENRICHMENT MEDIA IN THE RECOVERY OF CAMPYLOBACTER JEJUNI

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HÄNNINEN, MARJA-LIISA: *Comparison of four enrichment media in the recovery of Campylobacter jejuni.* Acta vet. scand. 1982, 23, 425—437. — The effectiveness of 4 enrichment media for the recovery of low levels of inoculated cells of Campylobacter jejuni was evaluated. The media contained antibiotics or antibiotics and bile acids as selective compounds. Three of the media recovered most of the inoculated low numbers of 6 C. jejuni strains. In the 3 media the growth rate of 3 strains, indicated by the increase in the log number of cells during 24 h or 48 h incubation at 42° C, was about the same as in the control medium without selective compounds. The same 3 media also recovered a low number of Campylobacter cells from artificially contaminated raw milk or ground meat samples. The enrichment medium B containing 40 I.U. colistin, 5 µg novobiocin, 2 mg Na-cholic acid and 50 mg cycloheximide per ml was inhibitory for most Campylobacter strains studied.

Campylobacter jejuni; enrichment; milk; ground meat.

Enteritis caused by Campylobacter jejuni/coli (C. fetus subsp. jejuni) is a zoonotic disease (*Butzler & Skirrow* 1979). Foods of animal origin, e.g. raw cow's milk (*Robinson & Jones* 1981) and poultry (*Brouwer et al.* 1979) have been suspected of causing certain epidemics. C. jejuni/coli has been frequently isolated from various foods, e.g. red meat (*Turnbull & Rose* 1982), poultry meat (*Smith & Muldoon* 1974, *Simmons & Gibbs* 1979, *Svedhem & Kaijser* 1981, *Notermans et al.* 1981), lamb carcasses (*Stern* 1981 b) and swine liver (*Rosef* 1981). The contamination of meat or organs takes place during slaughtering. Milk is contaminated by faeces or infected udders (*Lander & Gill* 1981). The number of C. jejuni/coli cells in a contaminated foodstuff is

probably very low compared to that found in human or animal faeces. The number of C. jejuni/coli cells in faecal samples from diseased humans has been reported to be 10^6—10^8 per gram (*Pellerin* 1981), and that in the rectal contents of broiler chickens 5.6×10^4—1.2×10^7/g (*Grant et al.* 1980). There have been only few reports dealing with the quantitative number of campylobacters in foods; most studies present their results in qualitative terms only. *Notermans et al.* (1981) studied broiler carcasses and found approximately 1.8 log units of campylobacters per g of skin. Since C. jejuni/coli is thermophilic, the number of campylobacters cannot increase in food if stored under proper conditions (*Butzler & Skirrow*). The methods developed for the detection of human campylobacteriosis are based on the direct cultivation of faecal samples on solid media containing antibiotics as selective compounds (*Butzler & Skirrow*). The indigenous flora of the samples may also be removed by filtration of samples through a 0.65 μm pore size membrane (*Dekeyser et al.* 1972).

For the recovery of low numbers of C. jejuni from foods, an enrichment procedure is needed. In the present study, 4 different enrichment media were compared as to their effectiveness in the recovery of low numbers of campylobacters from artificially contaminated raw milk or ground meat. The selective compounds contained in the media were antibiotics, cholic acid or deoxycholic acid. The growth of certain strains of C. jejuni as pure cultures in the 4 enrichment media was also studied.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

Six strains of C. jejuni were used. Two (N 120, N 104) were isolated from bovine and two (B 85, B 102) from broiler intestinal contents (*Hänninen & Raevuori* 1981). Strain L 32A was isolated from the caecal contents of a sheep during the autumn of 1981 and was identified by the method of *Véron & Chatelain* (1973). The sixth strain used was NCTC 11168. All the strains were hippurate positive and were thus regarded as C. jejuni (*Skirrow & Benjamin* 1981). The strains were stored frozen in semifluid Brucella broth (0.12 % agar) at —70° C. The thawed suspension of cells was grown in Brucella broth with 5 % calf blood at 42° C for 48 h and subcultured onto Brucella blood

agar, which was incubated at 42° C for 48 h. All the incubations took place in an atmosphere containing 85 % N_2, 10 % CO_2 and 5 % O_2.

Media

All the enrichment media were of the same basic composition: Brucella broth (Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 0.05 % each of $FeSO_4 \times 7H_2O$, $Na_2S_2O_5$, and Na-pyruvate (FBP) as recommended by *Hoffman et al.* (1979). The following selective compounds were used: in enrichment medium A 10 I.U. polymyxin B (Sigma Chemical Co., St. Louis, Mo, U.S.A.), 10 μg vancomycin (Eli Lilly & Comp., Indianapolis, U.S.A.), 5 μg trimethoprim (A/S Rosco, Copenhagen, Denmark), 5 μg cefazolin (Eli Lilly & Comp.) and 2 μg amphotericin B (Squibb & Sons Ltd., London, England) per ml; in enrichment medium B, 40 I.U. colistin (Orion, Pharmaceuticals Comp., Finland), 5 μg novobiocin (Sigma), 2 mg Na-cholic and 50 μg cycloheximide (Sigma); in enrichment medium C, 5 I.U. polymyxin B, 10 μg vancomycin, 5 μg trimethoprim, 2 mg Na-deoxycholic acid (Sigma) and 2 μg amphotericin B per ml; in enrichment medium D, the same compounds as medium C, except that Na-deoxycholic acid was replaced by 2 mg Na-cholic acid (Sigma) per ml.

Brucella agar (Difco) with 7 % calf blood was used to count the number of cells in the growth studies of strains in the enrichment media. A modified Skirrow's medium (*Skirrow* 1977) was used for the recovery of Campylobacter cells from artificially contaminated milk and ground meat samples. Skirrow's medium was modified by using calf blood instead of horse blood and Brucella agar (Difco) instead of blood agar (Oxoid). In counting the cells 0.1 % peptone water was used as a dilution fluid for the samples.

Growth of low levels of C. jejuni in the enrichment media

Six strains of C. jejuni were grown in Brucella broth with FBP supplement at 42° C for 24 h. One ml of the broth was serially diluted in 0.1 % peptone water to 10^{-8}. Series of 10 tubes (9 ml) of each enrichment medium were inoculated with 1 ml of 10^{-8} dilution (2—4 cells) of Campylobacter strains. Each strain was inoculated separately. Similar series of tubes were

also inoculated with 1 ml of 10^{-7} (20—40 cells) and 10^{-6} (200—400 cells) dilution of the Campylobacter strains. After incubation for 48 h at 42° C, the growth was evaluated visually and compared with the growth in control tubes containing Brucella broth with FBP supplement.

The growth of C. jejuni strains in the enrichment media

The strains used in the growth studies were NCTC 11168, N 120 and B 102. The strains were grown in Brucella broth with FBP supplement for 24 h at 42° C. Aliquots of the broth were then diluted in 0.1 % peptone water to 10^{-5}. One ml of this dilution was added to 99 ml of each enrichment medium. The number of cells in the inoculum was determined by plating 0.1 ml of duplicated serial dilutions on Brucella agar plates. The inoculated enrichment media were incubated at 42° C. After incubation periods of 24 h and 48 h the numbers of C. jejuni cells were similarly determined by serial dilution. Duplicated experiments were always performed.

Artificially contaminated meat samples

Twenty samples (100 g) of fresh ground meat were purchased at local retail stores. Each 100 g sample was divided into two 50 g samples. Ten 50 g samples were inoculated with the 24 h Brucella broth (+FBP) culture of strain N 120 using an inoculum size of approximately 30 cells per g ground meat (5 ml of 10^{-6} dilution). The inocula were carefully mixed with the meat samples and 1 g of each inoculated ground meat samples was then added to 9 ml of each enrichment medium. The size of the inoculum was determined on Brucella blood agar plates. Approximately 300 cells (5 ml of the 10^{-5} dilution) per g of the same strain were inoculated into 10 more samples, which were treated as above. All the enrichment media were incubated at 42° C for 24 h. After incubation, 0.1 ml of each enrichment medium was streaked onto Skirrow's medium, which was then incubated at 42° C for 48 h. The growth of C. jejuni and competing organisms on the media was evaluated. C. jejuni was confirmed by colonial morphology and Gram staining.

The same procedure was carried out with strain NCTC 11168. The sizes of the inocula used were approximately 20 cells or 200 cells per g, respectively.

Artificially contaminated milk samples

The inoculum for the artificial contamination of milk samples was prepared similarly to that described above for the meat samples.

Forty raw milk samples originating from different farms in southern Finland were used. Approximately 20 cells per ml of strain N 120 were inoculated into 10 ml of 10 milk samples and approximately 200 cells per ml of the same strain were inoculated into 10 more samples. One ml of the artificially contaminated milk was added to 9 ml of each enrichment media and incubated and analyzed as described in the case of ground meat. The same procedure was carried out with strain NCTC 11168. The sizes of the inocula were 13 or 130 cells per ml of milk, respectively.

RESULTS

The results of experiments concerning the growth and recovery of different low levels of inoculated cells of 6 C. jejuni strains in 4 different enrichment media are presented in Table 1. In the recovery of very low cell numbers (2—4 cells), enrichment media A and C were about equally effective. With enrichment medium D, vely low numbers of inoculated cells were also recovered, except for strains N 104 and NCTC 11168, which started to grow when 20—40 cells or 200—400 cells per tube, respectively, were used as the inoculum. Enrichment medium B was inhibitory for most strains. Three of the 6 strains grew in this medium when the inoculum consisted of 200—400 cells per tube.

When the growth of the strains in the enrichment media was evaluated by counting the cells after 24 h and 48 h incubation, the results obtained in the above experiments were confirmed. Strains N 120, NCTC 11168 and B 102 were used. The results are presented in Table 2. The growth of strains was poorest in medium B. In media A, C and D the log numbers of cells obtained were only slightly lower than those of the control medium, except for strain NCTC 11168. Since strain N 120 did not grow in medium B and strain NCTC 11168 did not grow in media B and D with the inoculum level at about 10^1 cells per ml, the growth of these strains was tested using a higher inoculum level. With the inoculum level at about 10^2 cells per ml, strain N 120

Table 1. Recovery of low levels of Campylobacter jejuni inoculated into 4 different enrichment media (A, B, C, D).

Strain of C. jejuni	Inoculum level[1]	Enrichment medium[3]			
		A	B	C	D
N 120	2—4	8/10[2]	0/10	10/10	9/10
NCTC 11168		10/10	0/10	10/10	0/10
B 102		8/10	1/10	10/10	10/10
N 104		8/10	0/10	19/10	3/10
B 85		10/10	0/10	10/10	9/10
L 32A		8/10	0/10	10/10	9/10
N 120	20—40	10/10	0/10	—	10/10
NCTC 11168		—	0/10	—	0/10
B 102		10/10	0/10	—	—
N 104		10/10	0/10	—	10/10
B 85		—	0/10	—	10/10
L 32A		10/10	10/10	—	10/10
N 120	200—400	—	10/10	—	—
NCTC 11168		—	0/10	—	10/10
B 102		—	5/10	—	—
N 104		—	0/10	—	—
B 85		—	0/10	—	—
L 32A		—	10/10	—	—

[1] Cells per tube.
[2] Number of positives/number of samples after 48 h at 42° C.
[3] A: Brucella broth (supplemented with 0.05 % each of Na-pyruvate, $FeSO_4 \times 7H_2O$, $Na_2S_2O_5$; FBP) with 10 I.U. polymyxin, 10 μg vancomycin, 5 μg trimethoprim, 5 μg cefazolin and 2 μg amphotericin B per ml;
B: Brucella FBP broth with 40 I.U. colistin, 5 μg novobiocin, 2 mg Na-cholic acid and 50 μg cycloheximide per ml;
C: Brucella FBP broth with 5 I.U. polymyxin B, 10 μg vancomycin, 5 μg trimethoprim, 2 mg Na-deoxycholic acid and 2 μg amphotericin B per ml;
D: Same composition as medium C, except that instead of Na-deoxycholoc acid 2 mg Na-cholic acid per ml was used.

grew in medium B and strain 11168 in medium D, but not in medium B.

The results concerning the recovery of campylobacters by enrichment from artificially contaminated ground meat or milk samples are presented in Table 3. Two inoculation levels were used. When the samples were investigated prior to artificial contamination by enrichment in medium A, none were found to

Table 2. Growth of strains N 120, NCTC 11168 and B 102 of Campylobacter jejuni in different enrichment media at 42° C.

Strain of C. jejuni	Inoculum level[1]	Incubation time (h)	Growth (log number of cells/ml medium) Enrichment medium[3]				
			Control medium[2]	A	B	C	D
N 120	18	24	7.54	7.42	<1	6.88	7.35
		48	9.30	9.1	3.47	8.0	9.10
	200	24	7.40		5.69		
		48	9.15		8.45		
NCTC 11168	10	24	7.38	7.60	<1	2.90	<1
		48	8.90	9.0	<1	5.76	<1
	120	24	7.56		<1		2.0
		48	9.0		<1		5.98
B 102	30	24	7.30	7.40	5.0	6.41	7.14
		48	9.07	9.06	5.0	8.61	7.80

[1] Cells/ml of the medium.
[2] Brucella broth with 0.05 % each of $FeSO_4 \times 7H_2O$, $Na_2S_2O_5$, Na-pyruvate.
[3] Abbreviations are the same as in Table 1.

Table 3. Recovery of Campylobacter jejuni from artificially contaminated raw milk or ground meat samples by enrichment.

Product	Strain of C. jejuni	Inoculum level (cells/g or ml of product)	Enrichment medium[1]			
			A	B	C	D
Ground meat	N 120	30	10/10[2]	8/10	9/10	10/10
	NCTC 11168	20	9/10	0/10	8/10	10/10
	N 120	300	10/10	10/10	10/10	10/10
	NCTC 11168	200	10/10	0/10	10/10	10/10
Raw milk	N 120	20	9/10	10/10	9/10	9/10
	NCTC 11168	13	9/10	6/10	10/10	10/10
	N 120	200	10/10	10/10	10/10	10/10
	NCTC 11168	130	9/10	10/10	10/10	10/10

[1] Abbreviations are the same as in Table 1.
[2] Number of positive samples/number of samples after 24 h at 42° C.

be naturally contaminated. After artificial contamination, none of the samples were again positive when cultivated directly by the swab method on Skirrow's medium. The effectiveness of media A, C and D was almost identical; most of the samples inoculated with 13—30 cells per g or ml were found positive. Although medium B did not support the growth of pure strains, strain N 120 was in most instances recovered from the ground meat or raw milk samples using this medium, and strain NCTC 11168 from milk but not from meat samples.

The growth of campylobacters on Skirrow's medium after enrichment was in most cases predominant; they were spread over the plate. The negative results after enrichment in medium A were probably due to overgrowth of the Skirrow's medium by competing organisms.

When the artificially contaminated meat samples (strain NCTC 11168, lower inoculum level) were stored at 4° C for 2 days in order to evaluate the survival or low levels of campylobacters, 8 of the samples were found to be positive after enrichment in the present enrichment media.

DISCUSSION

In comparing detection of C. fetus subsp. jejuni from clinical specimens and food, it is important to note that the bacterial flora and other characteristics of these 2 types of sample differ markedly. In clinical specimens, for instance, large population of physiologically active campylobacters facilitate detection, whereas the food sample may be contaminated by comparatively few, inactive campylobacters.

With all the enrichment media tested except medium B, low lewels of inoculated C. jejuni cells were recovered. The effect of the composition of the base medium was not investigated, but FBP supplement has been confirmed to increase the oxygen tolerance of C. fetus subsp. jejuni (*George et al.* 1978). This supplement probably also promotes the growth of C. jejuni from low inocula, by scavenging possible toxic products which may form in a microbial cultivation medium (*Hoffman et al.* 1979). The FBP supplement in the Brucella broth probably also increases the tolerance of low numbers of Camplyobacter cells to antibiotics in enrichment media (author's unpublished results).

The concentrations of antibiotics used in the present work were derived from earlier works on Campylobacter recovery from faecal samples (*Skirrow* 1977, *Blaser et al.* 1979, *Patton et al.* 1981). A concentration of colistin 40 I.U. per ml, combined with bacitracin (25 I.U. per ml), novobiocin (5 μg per ml), cefazolin (15 μg per ml) and cycloheximide (50 μg per ml) in a cultivation medium has been confirmed to be more inhibitory to faecal flora than a similar medium with 10 I.U. colistin per ml in the detection of C. jejuni/coli from faecal samples (*Patton et al.*). In the present work, enrichment medium B (40 I.U. colistin per ml) effectively inhibited the growth of the indigenous flora of milk and meat samples.

A colistin concentration of 40 I.U. per ml together with other antibiotics in the Butzler's type medium (*Butzler & Skirrow* 1979), however, has been confirmed to be inhibitory also for certain strains of C. fetus subsp. jejuni (*Gilchrist et al.* 1981, *Hänninen* 1982 b). This inbitory effect becomes more pronounced when the growth starts from low inocula. This is evident from the present study and also from the studies of *Karmali et al.* (1981). Polymyxin B at a concentration of 10 I.U. per ml combined with vancomycin, cefaxolin, trimethoprim and amphotericin B in the enrichment medium A did not inhibit the growth of campylobacters from low inocula. Polymyxin B is known to be more effective against Gram-negative bacteria; thus a lower concentration of polymyxin B is needed for the inhibition of competing organisms (*Nord & Hoeprich* 1964).

Cholic acid and deoxycholic acid are known to suppress the growth of Gram-positive microorganisms (*Floch et al.* 1971) and bile acids are widely used in the detection of Gram-negative enteric organisms (*Mac Conkey* 1908, *Leifson* 1935). Campjlobacters are known to tolerate bile acids in the growth medium (*Schneider & Morse* 1955, *Hänninen* 1982 a).

A high incubation temperature markedly affects the productivity of selective enrichment media by suppressing the growth of competing organisms, but not the growth of C. jejuni. An incubation temperature of 42° C was chosen, because at this temperature C. jejuni will grow faster than e.g. at 35° C (*Stern* 1981 a), and because at this temperature C. jejuni has been confirmed to tolerate 2—4 times more polymyxins than at 36° C (*Karmali et al.* 1981). Although an incubation temperature of 42° C is rather high, many competing organisms, such as Pseu-

domonas aeruginosa, Bacillus sp. and coliform bacteria can grow at this temperature.

The dominant indigenous flora of raw milk or meat is known to be one of Gram-negative, psychothrophic microorganisms, and raw milk is also sometimes contaminated with psychothropic Bacillus sp. (*Ayres et al.* 1980). A suitable enrichment medium will have to be one which suppresses these bacterial species. The addition of antibiotics or antibiotics and cholic or deoxycholic acid to the enrichment media suppressed the growth of these species considerably. When contaminating organisms of Pseudomonas aeruginosa, Enterobacter sp. and Bacillus sp. isolated from enrichment media were inoculated as a pure culture into enrichment media, none of the species grew as indicated by visual evaluation after 2 days incubation at 42° C, although they survived in the enrichment media.

The effect of incubation time on the recovery rate was not investigated in the present study. In these media (A, C and D) where the growth of strains was usually similar to their growth in the control medium, 1 day of incubation is probably adequate. In medium B, where most strains grew poorly from low inocula, more positive results could have been obtained if the incubation time has been lengthened to 48—72 h.

Although the pure strains grew poorly from low inocula in enrichment medium B, the same strains inoculated into food enriched frequently in this medium. The colonies of campylobacters grown from this medium on Skirrow's plate were smaller than those of the other enrichment media. The number of colonies per plate was also lower than that from the other media, indicating the toxicity of this medium to C. jejuni.

The present study showed that C. jejuni can be isolated even when the initial number of Campylobacter cells is low and when competing microorganisms are present. Only artificially contaminated samples or raw milk or ground meat were used, among other reasons because contamination of milk with campylobacters has been suspected to be quite sporadic (*Robinson & Jones* 1981). The situation with respect to the natural contamination of foods of animal origin is not well known and further research is needed to find out how well C. jejuni can be recovered from various foods using enrichment techniques. Recently *Turnbull & Rose* (1982) showed that the enrichment procedure enhances the recovery of campylobacters from raw red meat samples;

42 % of 98 positive meat samples were recovered by enrichment only. Probably several compositions of enrichment media are needed for the analysis of foods.

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SAMMANFATTNING

En jämförelse mellan fyra anrikningsmedier för påvisning av Campylobacter jejuni.

Fyra olika anrikningsmedier för påvisning av små mängder av C. jejuni har jämförts. Medierna innehöll antimikrobiella ämnen eller antimikrobiella ämnen och gallsyror som selektiva faktorer. I tre av dessa medier var både växten av C. jejuni stammar i renkulturer och påvisningen av C. jejuni från artificiellt kontaminerat malet kött eller rå mjölk bra. I det fjärde anrikningsmediet, som innehöll 40 I.U. kolistin, 5 μg novobiocin, 2 mg Na-cholin syra och 50 mg cyclohexamid per ml, inhiberades tillväxten av de flesta undersökta C. jejuni stammarna.

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