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From the Department of Food Hygiene, College of Veterinary Medicine, Helsinki, Finland.

EFFECT OF RECOVERY MEDIUM ON THE ISOLATION OF CAMPYLOBACTER JEJUNI BEFORE AND AFTER HEAT TREATMENT

By

Marja-Liisa Hänninen

HÄNNINEN, MARJA-LIISA: *Effect of recovery medium on the isolation of Campylobacter jejuni before and after heat treatment.* Acta vet. scand. 1982, *23*, 416—424. — The effect of various concentrations of polymyxin B and colistin in Skirrow's or Butzler's type media, respectively, on the recovery rate of 13 strains of C. jejuni before and after heat treatment was studied. Prior to heating, a Butzler's type medium containing 40 I.U. per ml colistin was inhibitory for certain strains of C. jejuni. After heating at 48° C for 30 min, media containing 2.5 I.U. per ml polymyxin B or 10 I.U. per ml colistin were not inhibitory for heat-injured cells. When the concentrations of polymyxin B were increased to 5.0 I.U. per ml or those of colistin to 20 or 40 I.U. per ml in the selective media, the means of the differences of log cell counts between non-selective Brucella blood agar and the selective media was statistically significant ($P < 0.05$). The mean D-value of C. jejuni strains in Brucella broth at 48° C was 18.4±5.4 min.

Campylobacter jejuni; heat treatment; antibiotics; recovery media.

In the cultivation of Campylobacter fetus subsp. jejuni (C. jejuni/coli) from human or animal faecal samples, 2 basic types of selective solid media are used (*Butzler & Skirrow* 1979). Both contain antibiotics as selective components. Only few comparisons between the media in the primary isolation of C. fetus subsp. jejuni from human faecal samples (*Bokkenheuser et al.* 1981) or from animal faecal samples (*Bokkenheuser et al., Patton et al.* 1981) have been published. In these studies the recovery rates of both media have been simliar. In a comparison using the direct cultivation of samples on the selective media or filtration of samples and cultivation on the non-selective media *Bokkenheuser et al., Richardson et al.* (1981) and *Jørgensen* (1981) obtained more positive samples by the filtration technique than by plating on the selective Skirrow's or Butzler's media.

Several modifications of Butzler's and Skirrow's media have been presented. In particular it has been suggested that the concentration of polymyxin B (*Karmali et al.* 1979) in Skirrow's medium and the concentration of colistin (polymyxin E) in Butzler's medium (*Patton et al.* 1981) be increased to 5 μg/ml and 40 I.U./ml, respectively. The increased concentrations of polymyxins are more inhibitory to enteric contaminating flora in faecal samples. The recovery rate of C. fetus subsp. jejuni was not reduced, even though the concentration of polymyxin B in the medium used by *Karmali et al.* was some 20 times higher than in the original Skirrow's medium and the concentration of colistin some 4 times higher in the medium used by *Patton et al.* than in the original Butzler's medium.

Campylobacter infection can be a food-borne epidemic (*Butzler & Skirrow*). C. fetus subsp. fetus has been isolated in particular from poultry and red meat. The consumption of raw cow's milk has been associated with food-borne campylobacteriosis (*Robinson et al.* 1981). The storage and processing conditions of foods can cause stress and injury to Campylobacter cells and can effect their recovery from foods, as has been shown with other bacteria. Heat and cold stresses, for example, have been reported to affect the recovery of the bacteria. Injured bacterial cells are known to be sensitive to secondary stress, e.g. for compounds used in the selective media (*Busta* 1976).

In the present study the efficiency of Skirrow's and Butzler's type media, containing various concentrations of polymyxin B or colistin, for the recovery of uninjured and heat-injured Campylobacter cells were investigated. The thermal death of Campylobacter cells at 48° C was also observed.

MATERIALS AND METHODS

Bacterial strains and cultural conditions

Thirteen strains of C. fetus subsp. jejuni were used in the studies. The strains were isolated from the intestinal contents of cattle (N 120, N 104 and N 191), swine (S 12 and S 20), and broiler chicken (B 33, B 85, B 42 and B 102) (*Hänninen & Raevuori* 1981). The two strains isolated from humans were NCTC 11168 and strain 5616 isolated by T. U. Kosunen, Department of Bacteriology, University of Helsinki. Two sheep strains were isolated from the caecal contents of 2 sheep during the autumn

of 1981 and were identified by the method of *Veron & Chatelain* (1973). All strains were hippurate positive and were thus C. jejuni (*Skirrow & Benjamin* 1980b). The strains were stored at —70° C in semifluid (0.12 % agar) Brucella broth (Difco Laboratories, Detroit, Michigan, U.S.A.). When a strain was required, the suspension was thawed, inoculated into Brucella broth with 5 % calf blood and incubated microaerophilically at 42° C for 2 days in evacuated replacement jars containing 85 % N_2, 10 % CO_2 and 5 % O_2. The broth cultures were subcultured on blood agar plates, which were incubated as described above.

Media

Brucella agar (Difco) with 7 % calf blood (BA) was used as a control medium in the studies of the growth of non-heated or heated cells of C. jejuni. The following media were used as selective media in counting the recovery rates of C. jejuni:

1. Skirrow's type medium, containing Brucella agar with 7 % calf blood, 10 µg vancomycin per ml, 5 µg trimethoprim per ml and 2.5 I.U. polymyxin B per ml.
2. Same composition as medium 1, with the modification that it contained 5.0 I.U. polymyxin B per ml.
3. Butzler's medium, containing thioglycollate medium (Difco) with 2 % agar and 15 % calf blood, colistin 10 I.U. per ml, novobiocin 5 µg per ml, bacitracin 25 I.U. per ml, cefazolin 15 µg per ml and cycloheximide 50 µg per ml.
4. Same composition as medium 3, with the modification that it contained colistin 20 I.U. per ml.
5. Same composition as medium 3, with the modification that it contained 40 I.U. colistin per ml.

All plates were prepared 1 day before the experiment and stored overnight at room temperature in order to evaporate some of the moisture from the plates. On moist agar plates Campylobacter colonies have a tendency to spread, and these spread colonies are difficult to count.

Thermal death and injury procedure

To prepare the inoculum for the thermal death and injury experiments, a loopful of fresh blood agar culture of a C. jejuni strain was inoculated into Brucella broth, which was incubated microaerophilically for 24 h at 42° C. Heat stress was brought

about by adding 2 ml of the Brucella broth culture to 98 ml preheated Brucella broth (48° C) in a 200 ml glass bottle; the mixture was then blended for 15 sec. The zero-time sample was withdrawn, serially diluted and plated on BA and on media 1, 2, 3, 4 and 5. The Brucella broth was inoculated at 48° C in a thermostatically controlled water bath for 30 min. After this time the Campylobacter count was determined, using the same media as at the beginning of the experiment. The counts were done on duplicated plates of the media. As a dilution fluid 0.1 % peptone water was used. With 3 of the strains the experiment was carried out twice, in order to test the repeatability of the method. All the above plates were incubated microaerophilically, as described above, for 48 h. Negative cultures were incubated for an additional 24 h. An injured population was defined by the log difference in the counts between the non-selective and the selective medium. The decimal death time (D-value) was determined using the results obtained on BA from the zero-time and 30 min samplings (*Stumbo* 1965).

In the statistical evaluation of the results the paired sample t-test was used (*Steel & Torrie* 1960) with a 5 % significance level.

RESULTS

When the cells of 13 strains of C. jejuni were incubated in Brucella broth at 48° C for 30 min, the mean D-value was 18.4 min ± 5.44 (mean ± s) (range 8.7 min—27.7 min). The size of the inoculum in the Brucella broth before heating varied with different strains from 6.5×10^6 to 1.2×10^7 per ml. The efficiency of the different media in the enumeration of non-heated and heated cells of C. jejuni is given in Table 1 as the means of the differences between the log cell counts on BA and on the selective media. The mean of the difference obtained for non-heated cells was statistically significant only for medium 5, containing 40 I.U. colistin per ml. On this medium 2 of the strains did not grow. For the other Campylobacter strains investigated, the cell counts were 0—98 % lower on medium 5 than on BA.

When the heated cells were plated on BA and on the selective media, the means of the differences of log cell counts were statistically significant on media 2, 4 and 5. The means of the differences of log cell counts between BA and Skirrow's medium containing 2.5 I.U. per ml polymyxin B or Butzler's medium containing 10 I.U. per ml colistin were not significant.

Table 1. Efficiency of different cultivation media in detection of Campylobacter jejuni before and after heat treatment at 48° C for 30 min.

Medium	Mean[1] of differences (±s) of log cell counts before heat treatment	Mean[1] of differences (±s) of log cell counts after heat treatment
Brucella blood agar		
1. with 2.5 I.U. polymyxin B, 10 μg vancomycin, 5 μg trimethoprim/ml	0.10±0.24	0.17±0.41
2. with 5.0 I.U. polymyxin B (other antibiotics are same as in Medium 1)	0.14±0.24	0.40±0.40*
Thioglycollate blood agar		
3. with 10 I.U. colistin 15 μg cefazolin, 25 I.U. bacitracin, 5 μg novobiocin, 50 μg cycloheximide/ml	0.01±0.18	0.10±0.23
4. with 20 I.U./ml colistin (other antibiotics are same as in Medium 3)	0.038±0.095	0.85±0.70*
5.[2] with 40 I.U./ml colistin (other antibiotics are same as in Medium 3)	0.36±0.38*	0.94±0.92*

[1] log cell count on Brucella blood agar — log cell count on the selective medium of 13 strains of C. jejuni.
[2] results are from 11 strains (strains NCTC 11168 and B 85 did not grow on this medium).
* significant at 5 % level.

DISCUSSION

Campylobacter fetus subsp. jejuni is thermophilic in comparison with C. fetus subsp. fetus and C. fetus subsp. intestinalis, which cannot grow at 42—43° C (*Smibert* 1974). An incubation temperature of 45.5° C is recommended by *Skirrow & Benjamin* (1980) for the biotyping of C. jejuni and C. coli. The mean D-value at 48° C obtained in the present study is higher than that presented by *Doyle & Roman* (1981). One reason for this may be the dissimilarity of the test conditions. The age of the culture in the present work, for instance, was 24 h, while in Doyle's and Roman's work it was 16—18 h. Another factor, observed both in the present study and in the work of *Doyle &*

Roman, is that at 48° C, there may evidently be wide variation between strains in the time required to inactivate 1 $\log_{10}$ (D-value) of the population.

The results further showed that those cells which remained viable after heating were probably injured. This cell injury was reflected in the lower counts observed on the selective media containing higher concentrations of polymyxin B or colistin than recommended by *Skirrow* or *Butzler,* respectively, when compared with the counts on the non-selective BA. This effect was not observed prior to the heat treatment, except on medium 5. The site of the damage caused by the heating of Campylobacter cells at sublethal temperature is not known, but 2 general effects are observed with other bacteria: (a) the degradation of bacterial RNA, and (b) lesions in the cytoplasmic membrane (*Witter* 1978). The cell injury in the present work was more evident when the concentration of polymyxins was increased. The site of action of polymyxins in the bacterial cell is also known to be the bacterial cell membrane; they have an effect on cell permeability (*Franklin & Snow* 1981). Polymyxins in the cultivation medium probably have an additive effect for the damage caused by sublethal heat treatment.

This study showed that the media developed by *Skirrow* or *Butzler* were not inhibitory for heat-stressed cells of C. jejuni. Both media are widely used in the detection of human campylobacteriosis. Butzler's medium has been proved to be more selective than Skirrow's medium in the inhibition of competing faecal flora, especially Proteus sp., coliform bacteria and Pseudomonas sp. (*Patton et al.* 1981). When the concentration of colistin in Butzler's medium was increased to 40 I.U. per ml, it was found to be inhibitory also to unstressed cells of C. jejuni, although this inhibition was apparent only with certain strains. It has been shown by others (*Gilchrist et al.* 1981) that colistin concentrations of 0.75 µg per ml (∞ 17 I.U.) and 1.5 µg per ml (∞ 36 I.U.), together with bacitracin 25 I.U. per ml, novobiocin 5 µg per ml, and cephalothin 5 µg per ml, inhibited growth in 11.9 % and 30.0 % of C. fetus subsp. jejuni strains, respectively, when tested in Mueller Hinton agar at 42° C. It has further been shown that the activity of colistin (*Gilchrist et al.*) and polymyxin B (*Karmali et al.* 1981) against C. jejuni is temperature-dependent; at 35—36° C they are more inhibitory than at 42° C. In the present study an incubation temperature 42° C was used.

There is probably variability between different strains of C. jejuni in their tolerance to polymyxins; this has been observed in the present study as well. Antimicrobial susceptibility tests made by different authors also display various minimal inhibitory concentrations (MIC) for colistin; e.g. MIC_{90} % 3.12 µg per ml (*Vanhoof et al.* 1981), MIC_{95} % 16 µg per ml (*Bokkenheuser et al.* 1978) and MIC_{90} % 50 µg per ml (*Vanhoof et al.* 1978). *Karmali et al.* (1981) presented MIC_{90} % 16 µg per ml for polymyxin B. The activity of polymyxin B and colistin is dissimilar; smaller concentrations of polymyxin B than of colistin are needed to inhibit the growth of contaminating Gram-negative flora on the selective media (*Gilchrist et al.* 1981). No direct conclusions can be drawn from the minimal inhibitory concentration studies of the optimal concentration of polymyxins in the selective isolation medium for campylobacters, because of differences in the cultivation media, in the size of the inoculum, the age of the culture, etc. It has been shown that Campylobacter strains which are originally isolated on a medium containing more polymyxin B (5 µg per ml) than in Skirrow's medium cannot grow at this concentration in secondary cultivation (*Karmali et al.* 1981). In the present study the effect of other antibiotics included in Skirrow's or Butzler's medium was not investigated. It has been confirmed by *Gilchrist et al.* (1981) that in Butzler's medium other antibiotics enhance the activity of colistin against C. jejuni. Bacitracin in particular enhances the inhibition of polymyxin B to Vibrio fetus (C. fetus subsp. fetus) (*Shepler et al.* 1964).

All the media contained blood, which is known to support the growth of C. fetus (*Border et al.* 1974). Blood contains the catalase enzyme, which has been proved to be an effective supplement in the recovery of stressed catalase-positive bacteria (*Martin et al.* 1976), since the inactivation of catalase is probably a universally obsedved lesion in injured bacteria.

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SAMMANFATTNING

Effekten av växtmedium på isolationen av Campylobacter jejuni före och efter värmebehandling.

Effekten av olika koncentrationerna av polymyxin B och colistin i Skirrows eller Butzlers media på återhämtningsgraden hos 13 stammar av Campylobacter jejuni före och efter värmebehandling undersöktes. Före upphettning hade Butzlers medium innehållande 40 I.U. colistin/ml en inhiberande effekt på vissa C. jejuni stammar. Efter upphettning vid 48°C under 30 min. var media innehållande 2.5 I.U./ml polymyxin B eller 10 I.U./ml colistin inte inhiberande för värmeskadade celler. När polymyxin B koncentrationen höjdes till 5.0 I.U./ml och colistin koncentrationen till 20 eller 40 I.U./ml i selektiva media, var medeltalen av skillnaderna i log cell uträkningarna mellan nonselektiva Brucella blodagar och de selektiva medierna statistiskt signifikanta ($P < 0{,}05$). Medeltalet av D-värdet för C. jejuni stammar vid 48°C var 18.4 ± 5.4 min.

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Reprints may be requested from: Marja-Liisa Hänninen, the Department of Food Hygiene, College of Veterinary Medicine, Hämeentie 57, 00550 Helsinki 55, Finland.