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Blood-Free Selective Medium for Isolation of *Campylobacter jejuni* from Feces

F. J. BOLTON,* D. N. HUTCHINSON, AND D. COATES
Public Health Laboratory Service, Preston Infirmary, Preston, Lancashire, England

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A blood-free selective agar is described which contains charcoal, ferrous sulfate, sodium pyruvate, casein hydrolysates, cefazolin, and sodium deoxycholate (CCD agar). CCD agar was compared with Preston medium for isolation of *Campylobacter jejuni* from human feces, and isolation rates were similar on both media, but CCD agar was less selective. Temperature studies at 37 and 42°C confirmed that incubation of direct plates at 42°C for 48 h was necessary for maximum isolation of *C. jejuni*.

Campylobacter jejuni and *Campylobacter coli* grow satisfactorily on basal media, but optimal isolation from routine specimens is only achieved when both selective and nonselective supplements are incorporated. The nonselective supplement common to most campylobacter media is animal blood. Butzler medium (10) and Blaser-Wang medium (1) contain whole sheep blood, whereas lysed horse blood is the choice of Skirrow (12), Lander and Gill (9), and Bolton and Robertson (4). George et al. (6) reported that a combination of ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP supplement), when added to brucella medium, enhanced the growth and aerotolerance of *Campylobacter* species. This supplement has been used in combination with animal blood in selective agars (5, 7) and in a selective broth (3) for the isolation of *Campylobacter* species from human feces. Because blood is of variable quality, liable to be contaminated, and relatively expensive, a blood-free medium would be advantageous.

We have investigated various compounds including those in the FBP supplement with a view to replacing blood in campylobacter isolation media. As a result of our studies, we have recently described a medium in which blood is adequately replaced by a mixture of charcoal, ferrous sulfate, and sodium pyruvate (CFP) (2) and which supports the growth of the majority of thermophilic campylobacter strains. However we have found that the addition of casein hydrolysates to the original CFP medium was necessary as it improved the growth of some environmental nalidixic acid-resistant thermophilic campylobacter (NARTC) strains. The growth of *C. jejuni* and *C. coli* strains is not affected by the absence or presence of this ingredient.

A great variety of antimicrobial agents and other substances have been used in selective media. Therefore, in a search to find selective agents which could be incorporated into the modified CFP medium for isolation purposes, we have screened 11 dyes, 17 chemical compounds, 8 surface-active agents, and 14 chemotherapeutic agents for their ability to inhibit a wide selection of gram-positive and gram-negative bacteria yet which allowed the growth of all *Campylobacter* biotypes. As a result of these tests sodium deoxycholate and cefazolin were chosen as selective agents.

This paper describes the formulation and preparation of the campylobacter blood-free selective agar and investigates the ability of the medium to support the growth of pure cultures of *C. jejuni*, *C. coli*, and NARTC strains. We also report the findings of a trial comparing this new medium with our blood-containing medium (4) for the direct culture of campylobacter strains from feces.

* Corresponding author.

MATERIALS AND METHODS

Blood-free selective medium. The following ingredients were added to 1 liter of deionized water: nutrient broth no. 2 (Oxoid Ltd., London, England) 25 gm; New Zealand agar (Davis Gelatine, N.Z. Ltd., Christchurch, New Zealand) 12 gm; bacteriological charcoal (Oxoid) 4 gm; and casein hydrolysates (Oxoid) 3 gm. Ten milliliters of 10% aqueous sodium deoxycholate (BDH, Poole, England), 5 ml of 5% aqueous ferrous sulfate, and 5 ml of 5% aqueous sodium pyruvate were then added to the basal medium to give final concentrations of 0.1, 0.025, and 0.025%, respectively. The medium was adjusted to pH 7.4 and sterilized by autoclaving at 121°C for 15 min. A 1-ml amount of a 10,000 mg/liter aqueous solution of cefazolin (Eli Lilly & Co., Indianapolis, Ind.) was added to the cooled molten agar to give a final concentration of 10 mg/liter. The charcoal-cefazolin-sodium deoxycholate agar will be known as CCD agar.

Selective blood medium. This was prepared to the following formulation: nutrient broth no. 2 (Oxoid) 25 gm/liter and New Zealand agar (Davis) 12 gm/liter, pH 7.4. The medium was sterilized by autoclaving at 121°C for 15 min. Saponin lysed horse blood 5%, polymixin sulfate 5,000 IU/liter, trimethoprim lactate 10 mg/liter, rifampin 10 mg/liter, and cyclohexamide 100 mg/liter were added to the cooled molten agar before pouring. This agar will be known as Preston agar (4).

Quantitative tests with pure cultures of *Campylobacter* species. The test organisms were *C. jejuni* biotype 1 (13) NCTC 11168, *C. jejuni* biotype 2 (13) NCTC 11392, *C. coli* NCTC 11353, and NARTC NCTC 11352. The organisms were grown on Columbia horse blood agar plates (Oxoid) incubated at 42°C for 24 h in an atmosphere containing ca. 10% O_2, 10% CO_2, and 80% N_2. Bacteria were harvested into 10 ml of 0.1% peptone water and standardized to a density of ca. 2×10^8 CFU/ml with a Perkin-Elmer 6/20 spectrophotometer at a wavelength of 450 nm. Tenfold dilutions in 0.1% peptone water were prepared to give suspensions ranging from 10^8 to 10^3 CFU/ml. CCD agar medium, Preston agar medium, and a nonselective control medium (nutrient broth no. 2 [Oxoid] plus New Zealand agar and 5% lysed horse blood) were all prepared with a concentration of 2% agar to facilitate counting. These media were inoculated with a 50-dropper pipette by the method of Miles

TABLE 1. Mean counts of four campylobacter strains tested on two selective agars

Medium	Campylobacter counts × 10^8 CFU/ml (SE)			
	C. jejuni NCTC 11168	*C. jejuni* NCTC 11392	*C. coli* NCTC 11353	NARTC NCTC 11352
CCD agar	3.3 (0.6)	1.5 (0.3)	4.3 (0.4)	0.9 (0.3)
Preston agar	4.5 (0.5)	2.1 (0.4)	4.2 (0.6)	1.1 (0.3)
Control (nonselective)	5.1 (0.4)	2.3 (0.4)	5.2 (0.5)	2.1 (0.4)

et al. (11), and the plates were incubated microaerobically at 42°C for 42 h. All tests were done in triplicate, and the mean count (of 18 drops) for each selective medium was compared with that of the control.

Tests with human fecal samples. Initially 350 specimens were cultured on the day of arrival in the laboratory. These samples, selected as high-risk specimens, included feces submitted by general medical practitioners, feces from known campylobacter cases or their contacts, and other fluid feces. Fecal specimens were inoculated onto two plates of CCD agar and two plates of Preston agar with cotton-tipped swabs, and the inoculum was spread out to produce discrete colonies. One pair of each medium was incubated at 37°C and the other at 42°C, both in a microaerobic atmosphere obtained by evacuating anaerobic jars to 500 mmHg and refilling them with 10% CO_2 in H_2. Plates of the two media were examined independently by different staff after 24 and 42 h of incubation. Suspect colonies were identified by oxidase test and characteristic morphology and motility under dark-field microscopy.

In a further trial 660 additional fecal samples were tested on the two selective agars but were incubated microaerobically at 42°C and were examined only after 42 h of incubation.

RESULTS

Colonial morphology of *Campylobacter* strains on the blood-free selective agar. The majority of *C. jejuni* strains produced grey, moist, flat, spreading growth on CCD agar after 42 h of incubation at 42°C. However, some strains may have a green hue, or a dry appearance, with or without a metallic sheen. Discrete colony formation is not common. *C. coli* strains tend to be creamy-grey in color, moist, slightly raised, and often produce discrete colonies. NARTC strains are varied in their morphology; some produce an appearance like the *C. jejuni* and *C. coli* strains, others produce grey, discrete colonies ca. 1 mm in diameter.

Quantitative tests. Table 1 gives the mean counts of the four test campylobacter strains on the two selective media and on nonselective control medium. The differences were analyzed by a two-sample Student t test for each strain separately at the 5% level of significance. In comparison with the counts on the nonselective medium, counts were significantly reduced in the following cases: the *C. jejuni* NCTC 11168 and NARTC strains on CCD agar and the NARTC strain on Preston agar. However, there was no significant difference between counts on CCD agar and those on Preston agar.

Direct culture. A total of 54 campylobacter isolations were made from 350 feces examined by direct culture (Fig. 1). All 54 strains were isolated at 42°C, whereas only 40 strains were recovered from culture at 37°C. All the strains isolated at 37°C were also isolated from the 42°C cultures. At the higher temperature, the recovery rate was the same (52 isolates) on both media: 50 strains were isolated on both media, 2 strains on Preston agar and 2 on CCD agar. At 37°C Preston agar was much more selective, and 39 of the 40 isolations were made on that medium compared with only 32 on CCD agar.

The optimum incubation period at both temperatures was 42 h, but CCD agar showed a better recovery rate than Preston agar when cultures were examined at 24 h (Fig. 1).

A crude estimate of the quantitative growth of campylobacter strains and contaminants was made (Table 2). At 37°C both media were relatively poor at restricting the growth of contaminants, i.e., 52% of the Preston agar plates and 75% of the CCD agar plates showed growth. At both 37 and 42°C, Preston medium showed less contamination than the blood-free medium.

Table 2 illustrates the qualitative growth of campylobacter strains and contaminants from direct culture at 42°C, on a further 660 fecal specimens. These results confirm that CCD agar is less selective than Preston agar and that the two media are comparable for the isolation of campylobacter strains. In this survey CCD agar isolated two campylobacter strains which failed to grow on Preston agar, whereas the latter medium isolated one strain which was not recovered on CCD agar. All three of these specimens showed a scanty growth of campylobacter strains, and these minor differences in isolation are probably due to sampling.

DISCUSSION

The development of a reliable selective medium for the isolation of *Campylobacter* species is an essential requirement for the total understanding of the clinical and epidemiological aspects of the organism. Modifications and variations of original media formulations are needed as the metabolic requirements of an organism become better understood or as new or alternative selective compounds, e.g., antibiotics, are described. The eventual choice, quantity, and variety of

Total number of Isolates
54
a. 42°C 54 | 37°C 40
b. CCD agar 52 | Preston agar 52 | CCD agar 32 | Preston agar 39
c. 24 h 34 | 42 h 52 | 24 h 28 | 42 h 52 | 24 h 14 | 42 h 32 | 24 h 13 | 42 h 39

FIG. 1. Isolation of campylobacter strains from 350 feces on two selective agars incubated at 37 and 42°C which were read after 24 and 42 h of incubation. (a) Temperature of incubation, (b) direct plating medium, (c) time of reading of direct plates.

TABLE 2. Qualitative growth of campylobacter strains and contaminants at 37 and 42°C on direct plates inoculated with human fecal specimens

n	Agar	Incubation temp (°C)	Growth[a] of: Campylobacter strains					Contaminants				
			+++	++	+	±	No. of strains (%)	+++	++	+	±	No. of strains (%)
350	CCD	37	18	9	4	1	32 (9)	119	61	45	37	262 (75)
	CCD	42	33	12	2	5	52 (15)	20	33	29	41	123 (35)
	Preston	37	25	8	2	4	39 (11)	44	37	52	50	183 (52)
	Preston	42	33	10	4	5	52 (15)	5	15	26	27	73 (21)
660	CCD	42	46	7	6	5	64 (10)	78	39	55	57	229 (35)
	Preston	42	39	10	10	4	63 (10)	12	22	35	45	114 (17)

[a] +++, Growth over all of the inoculated area; ++, growth over two-thirds of the inoculated area; +, >10 colonies on the primary inoculum area; ±, <10 colonies on the primary inoculum area.

selective agents incorporated into media depend upon the organisms to be isolated and the type of specimens to be cultured. The modified CFP basal medium (nonselective) supports the growth of *Campylobacter fetus* subspecies *fetus*, *C. fetus* subspecies *venerealis*, and *C. fetus* subspecies *intermedius* strains, but the selective agents in the definitive CCD agar are inhibitory to most strains of *C. fetus* subspecies *venerealis* and *C. fetus* subspecies *intermedius* but less so to *C. fetus* subspecies *fetus* strains. Although we developed CCD agar primarily for the isolation of *C. jejuni* and *C. coli* from human feces, the modified CFP basal medium with different selective agents could also be developed as an isolation medium for *C. fetus* strains.

It was decided to compare CCD agar with Preston agar medium because the latter has been shown to give recovery rates of campylobacter strains equal to or superior to other selective campylobacter agars (3, 4). In most laboratories direct plating onto a selective agar with incubation in a microaerobic atmosphere at 42°C for 42 to 48 h is the routine technique for the isolation of *C. jejuni* and *C. coli*. Under these standard conditions, CCD agar performed as well as Preston agar. In the two surveys seven campylobacter strains were isolated on only one or other of the two media, and this is most probably due to sampling at the time of plating. In practice the more selective agars are easier to interpret, whereas plates that contain contaminants require greater technical expertise since the colonial morphology of *C. jejuni* is so variable. Although Preston agar proved to be more inhibitory than CCD agar, the growth of contaminants on this latter medium did not interfere with the recovery of campylobacter strains from positive specimens.

The original object of including cultures at 37°C was to explore the possibility that the media may allow strains to grow which were different from those isolated at 42°C. As we did not isolate additional strains at the lower temperature, culture at 37°C was discontinued after 350 specimens had been examined. The effect of temperature on the isolation rate in this study is in agreement with the findings of Lauwers et al. (10) and Janssen and Helstad (8). These latter workers found that incubation of a modified Skirrow agar at 42°C was necessary for maximal isolation of *C. jejuni* from fecal specimens, and in the present study only 74% of the campylobacter strains isolated at 42°C were simultaneously detected at 37°C.

Contrary to the observations of Janssen and Helstad, who reported that plates incubated at 35°C and read after 24 h failed to show growth of campylobacter organisms, we found in our first study (Fig. 1) that at 37°C, 44 and 33% of isolations could be detected at 24 h on CCD agar and Preston agar, respectively. Although it is customary to read plates for campylobacter isolation at 48 h, speedier isolation may, on occasion, be an advantage. Of the 52 strains isolated after 48 h at 42°C, 65% could be detected on CCD agar with 24 h of incubation, compared with 54% on Preston agar.

We feel that the blood-free selective agar is as efficient as other media for the isolation of campylobacter strains from feces. Furthermore its advantage over blood-containing media is that, being more defined, it should be suitable in situations where the supply of animal blood is erratic or the quality variable.

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