Comparison of a blood-free medium and a filtration technique for the isolation of *Campylobacter* spp. from diarrhoeal stools of hospitalised patients in central Australia

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Summary. Single specimens of diarrhoeal stool from 676 patients, mostly aboriginals aged less than 5 years, admitted to Alice Springs Hospital, central Australia, for diarrhoea between Sept. 1988 and Feb. 1989, were examined for *Campylobacter* spp. by culture on a blood-free medium with selective supplement (BFM; Oxoid) and blood agar overlaid with a membrane filter (FM). *Campylobacter* spp. were isolated on either BFM or FM or both from 225 patients. *Campylobacter* spp. were isolated on BFM alone from 75 patients and on FM alone from 213 patients ($p < 0.001$; χ^2 test). Most campylobacters isolated on BFM were *C. jejuni*. All *C. jejuni* subsp. *doylei*, all "*C. upsaliensis*" except one, all *C. laridis*, *C. fetus* subsp. *fetus* and several uncharacterised *Campylobacter* isolates were isolated on FM only. *C. jejuni* was isolated on BFM but not FM from several patients, and *vice versa*. Serotyping of *C. jejuni* and *C. coli* isolated from both media showed the serotypes recovered from the two media to be different in some patients. In some patients concurrent infection with several species or serotypes (up to five) of *Campylobacter*, or both, was shown for the first time by the use of FM. We conclude that the use in combination of a selective medium and a non-selective medium with a filtration technique are better than either medium alone for the isolation of *Campylobacter* spp.

Introduction

Both *Campylobacter jejuni* and *C. coli* have been shown to cause diarrhoea worldwide.^{1,2} Several solid media have been formulated for the isolation of these species from faeces, most of which contain a combination of antibiotics to suppress the normal faecal flora and are incubated at 42°C in a micro-aerophilic atmosphere.^{3–5} There are several reports that *Campylobacter* spp. other than *C. jejuni* and *C. coli* can cause diarrhoea, and these organisms have usually been isolated from diarrhoeal stools when an antibiotic-free medium that makes use of a filtration technique (filter medium, FM) has been used.^{6–8} In this technique, a membrane filter of pore diameter 0.45 or 0.65 µm is applied to the surface of a blood–agar plate and spot inoculated with a faecal suspension. Campylobacters are able to pass through the pores, whereas other organisms are held back. The filter paper is removed after 30 min, and the plate is then incubated in a micro-aerophilic atmosphere.⁹

Another development in the isolation of *Campylobacter* spp., especially of *C. jejuni* and *C. coli*, is the formulation of a blood-free medium (BFM) in which blood is replaced by charcoal.^{10,11} In a study of the aetiology and epidemiology of acute diarrhoea in central Australia, we compared the efficacies of BFM and FM for the isolation of *Campylobacter* spp.

Materials and methods

Isolation of *Campylobacter* spp.

Single samples of diarrhoeal stools from 676 patients, mostly aboriginals aged less than 5 years, were examined. These patients were admitted to hospital between Sep. 1988 and Feb. 1989. For culture of *Campylobacter* spp., BFM with cefoperazone supplement (Oxoid) and FM (sheep blood 5% agar overlaid with a 0.65-µm pore size Sartorius membrane filter) were used. The inoculated BFM was incubated at 42°C in an anaerobic jar with catalyst (BBL) in a micro-aerophilic atmosphere generated by a *Campylobacter* gas-generating sachet (Oxoid). The plate was examined after 48 h and, if no campylobacter was detected, was discarded. Initial evaluation suggested

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Table I. Comparison of isolation of *Campylobacter* spp. on BFM and FM from diarrhoeal stools of 676 patients in hospital in Alice Springs, central Australia for diarrhoea (Sep. 1988 to Feb. 1989)

Category of result	Total number of patients	Number of patients from whom <i>Campylobacter</i> spp. were isolated on BFM								Number of patients from whom <i>Campylobacter</i> spp. were isolated on FM							
		jej	col	doyl	ups	hyoint	lar	fetus	U.C.	jej	col	doyl	ups	hyoint	lar	fetus	U.C.
1. Identical species on both media	19	14	0	0	0	2	0	0	3	14	0	0	0	2	0	0	3
2. One identical species on both media and additional species on either medium	21	18	2	0	1	0	0	0	1	18	4	10	10	0	0	0	3
3. One species on BFM and one or more species on FM	23	19	1	0	0	2	0	0	1	3	0	15	6	0	0	0	6
4. <i>C. jejuni</i> on BFM and no campylobacters on FM	12	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5. No campylobacters on BFM and one or more <i>Campylobacter</i> spp. on FM	150	0	0	0	0	0	0	0	0	22	6	47	35	5	2	2	63
Total	225	63	3	0	1	4	0	0	5	57	10	72	51	7	2	2	75

BFM, blood-free medium with cefoperazone supplement (Oxoid); FM, blood agar overlaid with a membrane filter; jej, *C. jejuni*; col, *C. coli*; doyl, *C. jejuni* subsp. *doylei*; ups, "*C. upsaliensis*"; hyoint, *C. hyointestinalis*; lar, *C. laridis*; fetus, *C. fetus* subsp. *fetus*; U.C., uncharacterised *Campylobacter* sp.

that incubation of plates for longer than 48 h did not affect the isolation rates of *Campylobacter* spp. The inoculated FM was incubated at 37 °C in a micro-aerophilic atmosphere generated as above. The plate was examined for campylobacters after 48 h and daily thereafter for 5 days. Different colony morphotypes were picked from both media for identification.

Campylobacter colonies were identified and speciated by the following tests: morphology after Gram's staining, oxidase and catalase production, nitrate reduction, hippurate hydrolysis, susceptibilities to nalidixic acid and cephalothin, micro-aerophilic growth at 42°C and 25°C, aerobic growth at 37°C, H₂S production in triple sugar iron agar (TSI), tolerance to glycine 1.0% and NaCl 3.5%, and the rapid urease test as described previously.¹² Speciation was further confirmed by electrophoresis of whole-cell proteins as described elsewhere.¹³ *C. jejuni* NCTC 11351, *C. jejuni* subsp. *doylei* NCTC 11937, *C. coli* NCTC 11366, *C. laridis* NCTC 11352, *C. fetus* subsp. *fetus* NCTC 11842, *C. fetus* subsp. *venerealis* NCTC 11354, *C. hyointestinalis* NCTC 11608, "*C. upsaliensis*" NCTC 11926 and NCTC 11541, and *C. cryaerophila* NCTC 11885 were used as reference strains.

O-serotyping

Antigenic extracts of *C. jejuni* and *C. coli* were prepared for O-serotyping according to the procedure described previously¹⁴ and sent to the Department of Microbiology, University of Toronto, Canada, where they were used to sensitise sheep erythrocytes for serotyping by the passive haemagglutination technique (PHA). The panel of antisera reported earlier¹⁵ was extended by including antisera against the recently

defined O-serotypes O 60–O 66. The O-serotype assigned to an isolate was based on the antisera in which PHA titres greater than 40 were observed. Titres of 40 and 80 were considered weak (_w) reactions.

Examination for other enteric pathogens

Parasites were sought by direct microscopy of a wet preparation of faeces and bacterial pathogens such as *Salmonella*, *Shigella*, *Vibrio*, *Yersinia enterocolitica*, *Clostridium difficile*, *Aeromonas* and *Plesiomonas* spp. were identified by standard methods as described elsewhere.¹⁶ Rotavirus, astrovirus, and enteric adenoviruses 40 and 41 were identified by monoclonal antibody-based enzyme-linked immunosorbent assays^{17–19} by Dr J. Herrmann, University of Massachusetts Medical Center, Worcester, MA, USA.

Results

Campylobacter spp. were isolated on BFM or FM or both media from the stools of 225 (33.3%) of the 676 patients (table I). *Campylobacter* spp. were isolated on BFM alone from 75 patients and on FM alone from 213 patients, the difference being highly significant ($p < 0.001$; χ^2 test). Most of the strains isolated on BFM were *C. jejuni*, a few were *C. hyointestinalis*, uncharacterised *Campylobacter* spp., and *C. coli* and one was "*C. upsaliensis*". On the other hand, FM yielded large numbers of uncharacterised *Campylobacter* spp. and "*C. upsaliensis*". *C. jejuni* subsp. *doylei*, *C. laridis* and *C. fetus* subsp. *fetus* were isolated from 72, two and two patients respectively on FM only. Identical organisms were isolated on both media from only 19 patients (category 1). In 12 patients, *C. jejuni* were isolated on

Table II. Patients with more than one species of *Campylobacter* isolated by FM from their diarrhoeal stool in Alice Springs, central Australia (Sep. 1988 to Feb. 1989)

Combination of <i>Campylobacter</i> spp.	Number of patients
<i>C. jejuni</i> + " <i>C. upsaliensis</i> "	6
<i>C. jejuni</i> + <i>C. jejuni</i> subsp. <i>doylei</i>	8
<i>C. coli</i> + <i>C. jejuni</i> subsp. <i>doylei</i>	1
<i>C. coli</i> + " <i>C. upsaliensis</i> "	1
<i>C. jejuni</i> subsp. <i>doylei</i> + " <i>C. upsaliensis</i> "	7
<i>C. hyointestinalis</i> + " <i>C. upsaliensis</i> "	1
<i>C. jejuni</i> subsp. <i>doylei</i> + <i>C. hyointestinalis</i>	1
<i>C. fetus</i> subsp. <i>fetus</i> + " <i>C. upsaliensis</i> "	1
<i>C. jejuni</i> + U.C.	3
" <i>C. upsaliensis</i> " + U.C.	4
<i>C. jejuni</i> subsp. <i>doylei</i> + U.C.	10
<i>C. hyointestinalis</i> + U.C.	1
<i>C. jejuni</i> + <i>C. jejuni</i> subsp. <i>doylei</i> + " <i>C. upsaliensis</i> "	4
<i>C. jejuni</i> + <i>C. coli</i> + <i>C. jejuni</i> subsp. <i>doylei</i>	1
<i>C. jejuni</i> + <i>C. laridis</i> + U.C.	1
<i>C. jejuni</i> + " <i>C. upsaliensis</i> " + U.C.	1
<i>C. jejuni</i> + <i>C. jejuni</i> subsp. <i>doylei</i> + U.C.	1
<i>C. jejuni</i> subsp. <i>doylei</i> + " <i>C. upsaliensis</i> " + U.C.	2
Total	54

U.C., uncharacterised *Campylobacter* sp.

Table III. Comparison of *C. jejuni* and *C. coli* serotypes and concurrent isolates obtained from BFM and FM cultures from diarrhoeal stools of hospitalised patients in Alice Springs, central Australia (Sep. 1988 to Feb. 1989)

Patient no.	Organisms with serotype on BFM	Organisms with serotype on FM
457	<i>C. jejuni</i> O22	<i>C. jejuni</i> O22, 64
488	<i>C. jejuni</i> O8, 17	<i>C. jejuni</i> O8, 17 + <i>C. jejuni</i> subsp. <i>doylei</i>
565	<i>C. jejuni</i> O8, 17	<i>C. jejuni</i> O8, 17 + <i>C. jejuni</i> O58
579	<i>C. jejuni</i> O8	<i>C. jejuni</i> O8, 17 + U.C.
589	<i>C. jejuni</i> O1, 44	<i>C. jejuni</i> O1, 44
590	<i>C. jejuni</i> O1, 44	<i>C. jejuni</i> O1, 44 + <i>C. jejuni</i> subsp. <i>doylei</i>
654	<i>C. coli</i> O54	<i>C. jejuni</i> O3 _w + <i>C. jejuni</i> O66 + " <i>C. upsaliensis</i> " + <i>C. jejuni</i> subsp. <i>doylei</i>
648	<i>C. jejuni</i> O8, 17	<i>C. jejuni</i> O8, 17 + <i>C. jejuni</i> O1, 44 + <i>C. jejuni</i> O22, 64 + " <i>C. upsaliensis</i> "
672	<i>C. jejuni</i> O1, 44	<i>C. jejuni</i> NT
695	<i>C. jejuni</i> O1, 44	<i>C. jejuni</i> O45
724	<i>C. jejuni</i> O19	<i>C. jejuni</i> NT + <i>C. jejuni</i> subsp. <i>doylei</i>
734	<i>C. coli</i> O54	<i>C. coli</i> O54 + <i>C. jejuni</i> subsp. <i>doylei</i>
760	<i>C. jejuni</i> NT	<i>C. jejuni</i> O41 + <i>C. jejuni</i> O5, 31 + <i>C. jejuni</i> subsp. <i>doylei</i>
970	<i>C. jejuni</i> O22	<i>C. jejuni</i> O22

U.C., uncharacterised *Campylobacter* spp.; w, weak reaction with the antiserum; NT, not typable.

BFM only (category 4). In category 3, fewer specimens yielded *C. jejuni* on FM than on BFM and in category 5, this organism was isolated on FM in 22 patients, but on BFM in none. Uncharacterised *Campylobacter* spp. could not be completely identified because all except five isolates were lost after primary isolation; however, all of them had been confirmed as *Campylobacter* spp. because they had characteristic morph-

ology after Gram's stain, were motile, and were catalase and oxidase positive.

Of the 225 patients from whom *Campylobacter* spp. were isolated, the faeces of 121 yielded no other recognised enteric pathogen. Of these 121 patients, "*C. upsaliensis*" was isolated as sole pathogen in 10 and together with another *Campylobacter* sp. in 15; *C. jejuni* subsp. *doylei* was isolated as sole pathogen in 12 and accompanied by another *Campylobacter* sp. in 23; and *C. hyointestinalis* was present as the only pathogen in two and accompanied by another *Campylobacter* sp. in four.

Of the 75 patients from whom a *Campylobacter* sp. was isolated on BFM, only one sample yielded more than one species on this medium (table I, category 2). On the other hand, the faeces of 54 of the 213 patients that were *Campylobacter*-positive on FM, yielded several species on this medium (table II).

Data on serotypes of *C. jejuni* or *C. coli* isolates from both media were available for 14 patients and these serotypes were compared with those of other *Campylobacter* spp. when present (table III). In five patients (nos. 488, 589, 590, 734 and 970) the same serotypes of *C. jejuni* or *C. coli* were isolated from both media, with or without additional *Campylobacter* spp. from FM. In two patients (nos. 565 and 648), *C. jejuni* serotypes other than those present on BFM were isolated. From the remaining seven patients, different serotypes were isolated on BFM and FM. Some patients in this series were infected with between three and five different *Campylobacter* spp. or serotypes.

Discussion

In this study, a significantly higher isolation rate, or more species of *Campylobacter*, or both, were obtained with a filtration technique than with a selective medium, as reported in previous studies.^{5,10} The incubation temperature for BFM recommended by the manufacturers is 42°C, and we are not certain whether incubation of FM at 42°C instead of 37°C would have affected the isolation rate reported in this study. Faeces from 31 patients yielded *C. jejuni* on BFM, of which only three were also detected with FM (table I, categories 3 and 4). A similar but lower isolation rate of *C. jejuni* with FM has been reported previously⁷ when the bacterial count was < 10⁵ organisms/g of faeces. It is likely that, in these patients, the organisms were present in smaller numbers. In the fifth category of patients (table I), faeces from 22 patients yielded *C. jejuni* on FM, none of which were positive with BFM. Either the selective supplement in the medium or the higher incubation temperature could have inhibited their growth on BFM. Such atypical *C. jejuni* strains have been encountered in other studies.^{6,12}

The *Campylobacter* spp. recovered on FM only were all *C. jejuni* subsp. *doylei*, except for one "*C. upsaliensis*" isolate, one *C. laridis* and one *C. fetus* subsp. *fetus*. Some *C. hyointestinalis* strains were

isolated from FM only. Of these species, only *C. laridis* is a known diarrhoeal pathogen,²⁰ the other species are suspected diarrhoeal pathogens.^{7, 8, 20-23} However, increasing importance has been attached to *C. jejuni* subsp. *doylei* and "*C. upsaliensis*" as possible diarrhoeal pathogens. Although the former was present in 72 patients, it was isolated as sole pathogen in 12. The latter was isolated as the only pathogen in 10 of the 51 patients in whose faeces this organism was found.

Case-control studies are needed to establish unequivocally the role of these various species as diarrhoeal pathogens. The 75 unidentified campylobacters isolated from FM were the largest group of organisms to be isolated with this medium. The fact that all except five were lost after primary isolation suggests that they were extremely fragile organisms and great care should be taken in future to define optimal conditions for their isolation and maintenance. The five viable strains could not be assigned to any recognised species of *Campylobacter* and further studies are in progress. We may have encountered new species of *Campylobacter* in this population.

We have demonstrated that several patients were infected with up to five different species or serotypes or both of *Campylobacter*. Moreover, the serotyping data

on *C. jejuni* suggested for the first time that, sometimes, the serotypes of this species recovered from both media of the same patients were different. All these data, together with the high isolation rate of *Campylobacter* spp. (33.3%) suggest that the ecology of *Campylobacter* spp. in this population is unusual and that the level of exposure of this population to this group of organisms is very high. Furthermore, the present study, as the previous studies,^{6, 7} suggested that the use of a single selective medium is not satisfactory for the optimum isolation of *Campylobacter* spp. Although the FM alone yielded a higher isolation rate of *Campylobacter* spp., this medium also failed to isolate several strains of *C. jejuni*, which suggests that, for optimum isolation of *Campylobacter* spp. from diarrhoeal stools, a combination of media, particularly a selective medium and a non-selective medium employing a filtration technique, are needed.

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