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Detection of campylobacter in gastroenteritis: comparison of direct PCR assay of faecal samples with selective culture

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SUMMARY

The prevalence of campylobacter gastroenteritis has been estimated by bacterial isolation using selective culture. However, there is evidence that certain species and strains are not recovered on selective agars. We have therefore compared direct PCR assays of faecal samples with campylobacter culture, and explored the potential of PCR for simultaneous detection and identification to the species level. Two hundred unselected faecal samples from cases of acute gastroenteritis were cultured on modified charcoal cefoperazone deoxycholate agar and subjected to DNA extraction and PCR assay. Culture on CCDA indicated that 16 of the 200 samples contained '*Campylobacter* spp.'. By contrast, PCR assays detected campylobacters in 19 of the 200 samples, including 15 of the culture-positive samples, and further identified them as: *C. jejuni* (16), *C. coli* (2) and *C. hyointestinalis* (1). These results show that PCR offers a different perspective on the incidence and identity of campylobacters in human gastroenteritis.

INTRODUCTION

Campylobacter enteritis is probably the most frequent cause of acute bacterial diarrhoea world-wide. While *Campylobacter jejuni* and *C. coli* account for the majority of these infections, other species have also been shown to be enteropathogenic for humans [1]. Phenotypic identification of campylobacters can be difficult since they have fastidious growth requirements, are asaccharolytic and possess few distinguishing biochemical characteristics. Consequently many clinical laboratories do not pursue identification to the species level [2, 3].

Antibiotics are incorporated into media used for the selective isolation of *C. jejuni* and *C. coli* and these media may inhibit growth, especially of less commonly encountered *Campylobacter* species such as *C. upsaliensis*, *C. hyointestinalis* and *C. fetus*. As a consequence certain *Campylobacter* species may be under-reported in the epidemiological literature on human gastrointestinal illness [2, 3]. Precise identification to the species level is a prerequisite to accurately define the disease spectrum and microbial ecology of campylobacters. In this report, we have used polymerase chain reaction (PCR) assays for *C. jejuni* and *C. coli* and of other *Campylobacter* species [4, 5] to detect and identify these enteropathogens directly in faecal samples, submitted to a clinical laboratory. We have compared the data obtained by PCR assays with those obtained by standard selective culture of 200 faecal samples from cases of acute gastroenteritis.

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MATERIALS AND METHODS

Bacteriological investigation of clinical samples

Faecal samples submitted from 200 cases of acute gastroenteritis were examined for the presence of *Campylobacter*, *Salmonella* and *Shigella* species by standard laboratory culture. Examination for the presence of campylobacters was made by culture on modified charcoal cefoperazone deoxycholate agar

(CCDA, Unipath) for 48 h at 37 °C under micro-aerobic conditions (by volume: 5% O_2, 5% CO_2, 2% H_2 and 88% N_2). The identity of colonies with characteristic campylobacter morphology was confirmed by Gram stain and positive cytochrome oxidase test. These culture data were stored until completion of 'blind' PCR assays which took place (at Central Public Health Laboratory) not later than one week after receipt of specimen and initial culture (by Central Middlesex Public Health Laboratory).

Reference strains and culture conditions

The following type strains were used as positive controls, and in seeding experiments: *C. jejuni* subsp. *jejuni* NCTC 11351, *C. coli* NCTC 11366, *C. lari* NCTC 11352, *C. upsaliensis* NCTC 11541, *C. helveticus* NCTC 12470, *C. hyointestinalis* subsp. *hyointestinalis* NCTC 11608 and *C. fetus* subsp. *fetus* NCTC 10842. All were cultured at 37 °C on 5% (v/v) horse blood agar plates under micro-aerobic conditions (as above).

Seeding experiments

Log phase cultures of seven *Campylobacter* species type strains (see above) were suspended in diluent (Brucella broth, Unipath) equivalent to McFarland standard 0·5 ($1{\cdot}5 \times 10^8$ colony forming units (c.f.u.) ml^{-1}). These suspensions were further diluted to give a range from 10^8 to 10^2 c.f.u. ml^{-1}. The estimated concentrations were confirmed by viable count on 5% (v/v) horse blood agar, incubated at 37 °C in a micro-aerobic atmosphere for 48 h. Nine ml of each dilution (for each species) was used to homogenize 1 g of faecal material (from a healthy, campylobacter culture-negative individual). In this way the seeding bacteria were introduced and the faecal sample was liquified to facilitate pipetting.

To determine the relative sensitivity of PCR assay versus culture for the detection of *Campylobacter* species type strains, 100 μl aliquots of the seeded samples and an unseeded control were (*a*) used in the DNA extraction procedure described below and (*b*) inoculated on CCDA plates. Three replicates of each seeded sample were tested.

Extraction of nucleic acids from faeces

One gram of each clinical faecal sample was homogenized in 9 ml of Brucella broth. Nucleic acid was extracted from a 100 μl aliquot of the faecal suspension following the method of Boom and colleagues [6] for recovery of nucleic acid from cell-rich sources with guanidinium thiocyanate and diatomaeous silica. In order to reduce the effect of substances inhibitory to PCR that might be co-extracted from faeces, a 50 μl aliquot of the nucleic acid extract was treated with 150 μl of a 10% (w/v) solution of polyvinyl pyrrolidone as previously described by Lawson and colleagues [7]. DNA was recovered by isopropanol precipitation.

PCR assays

All primer sequences used in this study are given in Table 1. Co-detection of *C. jejuni* and *C. coli* was made by PCR assay for the 16S rRNA gene, while specific detection of *C. jejuni* was made by PCR primers targeting the hippuricase gene [4]. Specific detection of *C. coli* was made by PCR primers targeting a species-specific cloned fragment encoding part of the aspartokinase gene [4]. The species *C. lari*, *C. upsaliensis*, *C. helveticus*, *C. fetus* and *C. hyointestinalis* were detected and differentiated by PCR assays based on the 16S rRNA gene [5]. Subspecies of *C. jejuni*, *C. fetus* and *C. hyointestinalis* were co-detected, but not differentiated, by these assays.

A total of 2·5 μl of the nucleic acid sample obtained from the faecal extract was amplified (Stratagene RoboCycler) in a 25 μl reaction volume containing 20 mM Tris-HCl, pH 8·4; 50 mM KCl; 2·5 mM $MgCl_2$; 0·625 units *Taq* polymerase; 0·2 mM of each deoxynucleotide; 0·4 μM of each primer and an overlay of 25 μl of mineral oil. Amplification conditions were: denaturation temperature 94 °C for 1 min; annealing temperature dependent on the primer set used (see Table 1) for 1 min; extension temperature 72 °C for 1 min; 30 cycles. For each PCR a 10 μl aliquot of each reaction was analysed by electrophoresis in a 1% w/v agarose gel.

Hybridization with digoxigenin-labelled probes

DNA–DNA hybridization was performed to maximize the sensitivity of the PCR assays, and to confirm the identity of amplicons. For the 16S rDNA-based primers, a probe was produced from *C. jejuni* NCTC 11351 with previously described *Campylobacter* genus-specific primers [4]. These primers produced a 780 bp amplicon from a region of 16S rDNA conserved among all seven *Campylobacter* species

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Table 1. *PCR primers*

Target gene	Forward primer	Reverse primer(s)	Annealing temperature	Product size (bp)	Species detected	Ref.
16SrRNA	5′-GGGACAACACTTAGAAATGAG	5′-CACTTCCGTATCTCTACAGA	60 °C	878	*C. upsaliensis**	[4]
		5′-CCGTGACATGGCTGATTCAC		1225 or 1375	*C. helveticus**	
	5′-GCAAGTCGAACGGAGTATTA	5′-GCAGCACCTGTCTCAACT	65 °C	997	*C. fetus**	[4]
		5′-GCGATTCCGGCTTCATGCTC		1287	*C. hyointestinalis**	
	5′-CAAGTCTCTTGTGAAATCCAAC	5′-ATTTAGAGTGCTCACCCGAAG	64 °C	561	*C. lari*	[4]
	5′-AATCTAATGGCTTAACCATTA	5′-GTAACTAGTTTAGTATTCCGG	58 °C	854	*C. jejuni, C. coli*	[5]
cj hip	5′-GGAGAGGGTTTGGGTGGTG	5′-AGCTAGCTTCGCATAATAACTTG	66 °C	735	*C. jejuni*	[5]
cc asp	5′-GGTATGATTTCTACAAAGCGAG	5′-ATAAAAGACTATCGTCGCGTG	60 °C	500	*C. coli*	[5]

* PCR assays for *C. upsaliensis* and *C. helveticus*, or *C. fetus* and *C. hyointestinalis*, are duplex reactions using a common forward primer with species-specific reverse primers.

which overlapped the area amplified by the species-specific primers. Probes for the hippuricase and aspartokinase PCR amplicons were prepared by using the original assay primers to amplify probe DNA from *C. jejuni* NCTC 11351 and *C. coli* NCTC 11366 respectively.

The probes were labelled with digoxigenin-labelled dUTP by random priming (Boehringer–Mannheim). PCR products were analysed by electrophoresis in 2% agarose gels, transferred to Hybond N+ membranes (Amersham International) by Southern blotting with Vacugene apparatus (Pharmacia), and cross-linked with a UV Stratalinker (Stratagene). Blots were prehybridized for 2 h at 65 °C in hybridization buffer (5 × SSC; 0·1% *N*-lauroylsarcosine; 0·02% SDS; 1% Boehringer–Mannheim blocking reagent). Hybridization was for 18 h at 65 °C with hybridization buffer containing 250 ng of digoxigenin-labelled probe per 100 cm^2 of membrane. Following hybridization, membranes were washed twice in 2 × SSC; 0·1% SDS for 5 min at room temperature and twice in 0·5 × SSC; 0·1% SDS for 15 min at 68 °C. Bound digoxigenin probe was detected with the DIG nucleic acid detection kit (Boehringer–Mannheim). 1 × SSC is 0·15 M NaCl; 0·015 M sodium citrate.

RESULTS

Sensitivity of PCR assays

Each of the species-specific PCR assays detected the appropriate target *Campylobacter* species in artificially-seeded faeces with a sensitivity of 10^5 c.f.u. g^{-1} faeces. Given the initial 1:10 dilution of faecal material, this was equivalent to approximately 25 c.f.u. present in each 25 μl PCR volume. Following Southern blotting and hybridization with digoxygenin labelled DNA probes, the threshold of detection was lowered to between 10^4 and 10^3 c.f.u. g^{-1} faeces (between 2·5 and 0·25 c.f.u. per 25 μl PCR volume).

Sensitivity of culture on CCDA

The sensitivity of detection of different *Campylobacter* species type strains on CCDA (inoculated with 100 μl aliquots of a 1:10 dilution of seeded faeces) was between 10 and 100 c.f.u. g^{-1} faeces (type strains of *C. jejuni*, *C. coli*, *C. lari* and *C. hyointestinalis*). It was less sensitive for *C. helveticus* and *C. fetus*; these type strains were detectable at 10^3 c.f.u. g^{-1} faeces. The

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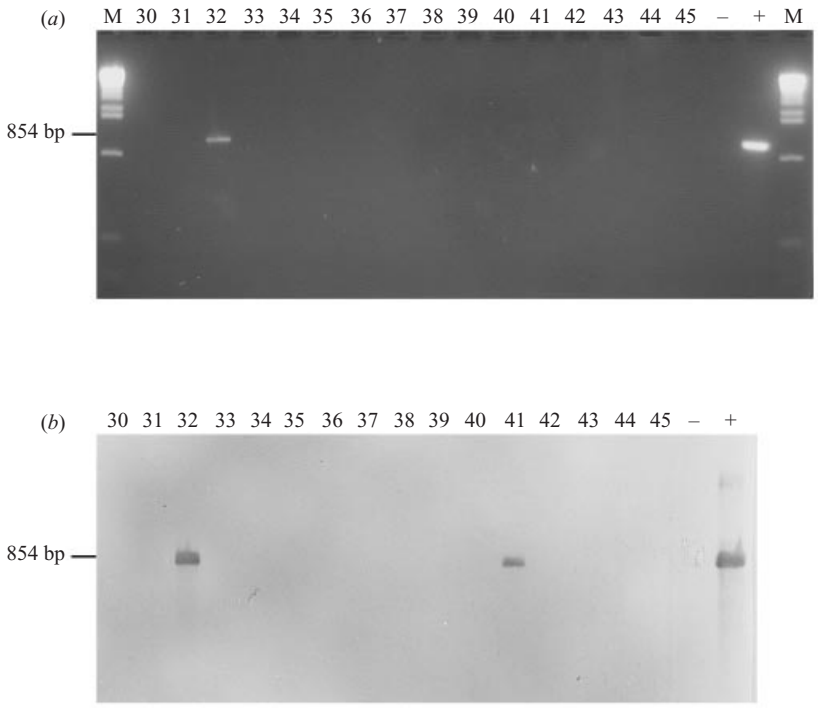

Fig. 1. Example of results obtained by PCR assay. Data obtained from clinical faecal samples (numbered as in Table 2) with *C. coli*/*C. jejuni* specific primers. Lanes M, λBstEII molecular size marker, lanes; 30–45, faecal samples tested; −, negative control (extracted from control faeces); +, positive control (genomic DNA of NCTC 11168). (*a*) Amplicons visualized on agarose gel by ethidium bromide staining. Sample 32 and the positive control show specific amplicons. (*b*) Same gel transferred by Southern blotting and hybridized with 16S rDNA-specific probe. This assay is positive for samples 32 and 41 as well as the positive control.

type strain of *C. upsaliensis* was not detectable on CCDA ($> 10^8$ c.f.u. g^{-1} faeces).

PCR assays applied to clinical samples

PCR amplicons were produced by *C. jejuni*/*C. coli* 16S rRNA primers from 18 of 200 faecal DNA extracts. Of these PCR-positive samples, 16 also gave an amplicon with *C. jejuni*-specific (hippuricase gene) primers, while two gave an amplicon with primers specific for *C. coli*. One of the *C. jejuni* and one of the *C. coli* PCR-positives were detected only following hybridization. One of the 200 samples yielded an amplicon specific for *C. hyointestinalis* (species-specific primers based on 16S rDNA). PCR assays for *C. lari*, *C. upsaliensis*, *C. helveticus* and *C. fetus* were negative. An example of the results obtained with the PCR assays is illustrated in Figure 1.

Comparison with culture detection from clinical samples

Culture on CCDA yielded colonies identified as *Campylobacter* spp. from 16 of the 200 samples. Three samples which were PCR-positive for *C. jejuni* and one sample PCR-positive for *C. hyointestinalis* were culture-negative for *Campylobacter* spp. One campylobacter culture-positive sample was negative by PCR (Table 2). The difference in the rates of detection between culture and PCR evaluated by McNemar's test (*P* value 0·18) was not statistically significant.

DISCUSSION

We have investigated whether PCR detection may offer a different perspective on the frequency and role of various *Campylobacter* species in gastrointestinal illness. In this study, 16 samples (8%) of 200 unselected faecal samples sent for testing at a clinical laboratory proved to be culture-positive for *Campylobacter* species while 19 (9·5%) were positive by PCR. Moreover, PCR assay simultaneously provided precise identifications to the species level that was lacking from the culture data. It showed that 16 samples contained *C. jejuni*, 2 contained *C. coli* and 1 contained *C. hyointestinalis*.

In contrast to culture, the sensitivity of the PCR assays of faecal samples was identical for all tested

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Table 2. Campylobacter-*positives among 200 acute diarrhoeal stool samples: identification and speciation by PCR and culture*

Sample no.	Culture (CCDA)	PCR assays† cj/cc 16S	cj hip	cc asp	cl 16S	cf 16S	chy 16S	cu 16S	ch 16S	PCR speciation
6	+	−	−	−	−	−	−	−	−	—
14	+	+	−	+	−	−	−	−	−	*C. coli*
27	+	+	+	−	−	−	−	−	−	*C. jejuni*
28	−	+	+	−	−	−	−	−	−	*C. jejuni*
29	+	+	+	−	−	−	−	−	−	*C. jejuni*
32	+	+	+	−	−	−	−	−	−	*C. jejuni*
41	+	+*	−	+*	−	−	−	−	−	*C. coli*
55	+	+	+	−	−	−	−	−	−	*C. jejuni*
59	+	+	+	−	−	−	−	−	−	*C. jejuni*
60	+	+	+	−	−	−	−	−	−	*C. jejuni*
69	+	+	+	−	−	−	−	−	−	*C. jejuni*
79	+	+	+	−	−	−	−	−	−	*C. jejuni*
99	+	+	+	−	−	−	−	−	−	*C. jejuni*
119	+	+	+	−	−	−	−	−	−	*C. jejuni*
121	−	−	−	−	−	−	+	−	−	*C. hyointestinalis*
160	−	+	+	−	−	−	−	−	−	*C. jejuni*
164	−	+	+	−	−	−	−	−	−	*C. jejuni*
171	+	+	+	−	−	−	−	−	−	*C. jejuni*
180	+	+	+	−	−	−	−	−	−	*C. jejuni*
196	+	+*	+*	−	−	−	−	−	−	*C. jejuni*
Total /200	16	18	16	2	0	0	1	0	0	19

* Positive only after Southern blotting and hybridization (cf. Fig. 1).
† The PCR assays were as described in Materials and Methods and references [4] and [5].

species. It was 10^5 c.f.u. g^{-1} or from 10^4 to 10^3 c.f.u. g^{-1} after Southern blotting and hybridization. In seeding experiments employing the type strains of *C. jejuni* and *C. coli*, for which CCDA was designed [8], the sensitivity of the PCR assay may appear relatively low by comparison with culture. We note that this is in part due to the difference in sample size examined: while plates were inoculated with 100 μl of diluted faecal material, the sample volume for PCR was fortyfold less. However, certain *Campylobacter* strains or species may be susceptible to the antibiotics incorporated in selective media [2, 3]. In practice, the sensitivity of PCR assay for actual clinical samples is probably greater than that achieved in seeding experiments with type strains. Campylobacter cells in faecal material will exist in a variety of metabolic states, some of which may not be amenable to culture; direct PCR will detect both culturable and non-culturable cells.

Three samples which were PCR-positive for *C. jejuni* were negative by culture. Since PCR detection/identification was achieved by sequential assays targeted at two different genes (the 16S rRNA gene of *C. jejuni*/*C.coli*, followed by hippuricase gene) there is little likelihood of false PCR-positivity due to contamination. These PCR-positives may represent strains which were non-viable at the time of culture, or strains which were susceptible to components of the selective isolation medium. It is also possible that these *C. jejuni* amplicons might have been produced from strains of subspecies *doylei*, since our PCR assays for *C. jejuni* detects both subspecies. This nitrate-negative subspecies grows more slowly at 37 °C than subspecies *jejuni*. *C. jejuni* subspecies *doylei* has been isolated from cases of gastroenteritis in children and from gastric biopsies in adults. However its exact role as an agent of human disease is not yet understood [9].

C. hyointestinalis was detected by PCR in one sample, but was not found by culture. This species is associated with proliferative enteritis in pigs and has only rarely been cited as a cause of gastroenteritis in humans [10–12]. In those cases *C. hyointestinalis* was isolated on conventional selective agar, as could be done for the type strain in our seeding experiments.

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While some such strains are evidently resistant to the antibiotics used in selective media, they may not be representative of the species as a whole [13]. This interpretation would be consistent with our results, since our clinical sample failed to yield colonies on CCDA, but gave a strong positive signal by species-specific PCR assay for *C. hyointestinalis*. We conclude that further investigation of the role and incidence of *C. hyointestinalis* in human disease is called for. In general, it seems that certain strains and species of *Campylobacter* that fail to grow on selective agar would be detectable by PCR. For example, the type strain of *C. upsaliensis* is susceptible to cefoperazone and will not grow on CCDA. Other strains of *C. upsaliensis* are less susceptible to the antibiotic and a selective agar containing a lowered concentration of cefoperazone is recommended for their isolation [14].

In one case, *Campylobacter* sp. was detected by culture but not by PCR. This isolate was unfortunately not recovered from storage. It is likely to have been *C. jejuni*, *C. coli* or *C. lari* since it was isolated on CCDA selective medium. Among the possible explanations for the negative PCR result are that campylobacter cells may have been present at less than the detection threshold of the PCR assay. Again, the cells may have lysed *in situ* in the interval between culture and nucleic acid extraction, leaving genomic DNA susceptible to degradation by the diverse nucleases present in faecal material. Alternatively, there may have been sufficient sequence diversity in the 16S rDNA of this particular isolate to have introduced primer mismatching.

There was no evidence of campylobacter infection by either culture or PCR for 180 of the 200 faecal samples. Individual PCR assays for *C. lari*, *C. fetus*, *C. upsaliensis* and *C. helveticus* were all negative. Two of these species, *C. lari* and *C. upsaliensis*, have been described as potentially significant causes of human gastroenteritis [1, 13].

In this study we have compared PCR assay(s) with culture on CCDA medium, a standard method employed for the isolation of campylobacter in the United Kingdom [3]. This medium is designed for the isolation of the thermophilic campylobacters (*C. jejuni*, *C. coli*, *C. lari*) and is not suitable for reliable isolation of other *Campylobacter* species such *C. hyointestinalis* or *C. upsaliensis*. The alternative membrane filtration culture technique [15] may be employed to enhance the isolation of other campylobacters, but has been considered inconvenient for most diagnostic laboratories, so that most continue to work only with selective agar methods [2]. A recently developed selective agar CAT [14], designed for enhanced isolation of *C. upsaliensis*, has yet to find widespread application in the diagnostic laboratory.

Detection of *Campylobacter* sp. by culture requires 48 h to isolate the organism and assign putative identification to the genus level. Identification to the species level, which requires further investigation, is not normally undertaken by clinical laboratories. By contrast, direct PCR assay of faecal DNA simultaneously detects and identifies *Campylobacter* species, and a typical batch of 48 samples can be processed (without blotting and hybridization) within 8 h. As presently configured, PCR is relatively labour intensive, and costly compared to culture, and thus is as yet unlikely to provide an alternative to culture diagnosis for *C. jejuni* and *C. coli*. However, in several areas it will find immediate application – they include recommended studies [3] of the incidence, epidemiology and role in human disease of viable but non-cultivable campylobacters, of non-*jejuni*/non-*coli* campylobacters, and of strains susceptible to the antibiotics incorporated in selective agars.

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