ORIGINAL ARTICLE

Detection of campylobacter species: a comparison of culture and polymerase chain reaction based methods

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Aims: To investigate the optimal method for the detection of campylobacters from stool samples by comparing selective culture with membrane filtration and the polymerase chain reaction (PCR).
Methods: Three hundred and forty three stool samples were investigated by each of the three methods mentioned above. Selective culture was performed with charcoal cefoperazone desoxycholate agar plates. Membrane filtration was performed using cellulose triacetate membranes with 0.45 μm pores placed on blood agar plates. Enteropathogenic campylobacters were detected using a PCR identification algorithm, consisting of screening PCRs and species identification using a PCR enzyme linked immunosorbent assay (PCR-ELISA), both based on the 16S rRNA gene.
Results: Of the 343 samples tested, 23 were positive by one or more method. Of these, 17 were positive by selective culture, 12 by membrane filtration, and 20 by the PCR identification algorithm. A total of 18 of 23 positives were identified as *C jejuni* and/or *C coli* by the PCR identification algorithm, compared with 14 identified to the genus level by selective culture, and 10 by membrane filtration. Among the remaining five positive samples, one *C hyointestinalis* was detected only by the PCR identification algorithm; one *C upsaliensis* was detected only by the PCR identification algorithm; one *Campylobacter* sp was detected by membrane filtration and selective culture and later identified as *C concisus*; one *Campylobacter* sp was detected by membrane filtration alone and later identified as *Arcobacter* sp; and one *Campylobacter* sp detected only by selective culture was lost to study and therefore not speciated. There was no significant difference between detection by selective culture and the other two methods. However, detection by PCR was significantly better than by membrane filtration (0.05 > p > 0.02).
Conclusion: The PCR identification algorithm can detect and identify *Campylobacter* spp to the species level and the result is obtained on the same day. However, PCR is expensive, labour intensive, and does not provide an isolate for further identification or typing. Selective culture is as good as the PCR identification algorithm for the detection of the two most common species, *C jejuni* and *C coli,* and it is cheap and practical. However, it does miss the less common species, results take 48 hours, and identification is only to the genus level. Membrane filtration showed a low sensitivity compared with the other methods and is not appropriate for the diagnostic laboratory, although it was the only method to detect the *Arcobacter* sp. The optimum method for the detection of campylobacters from stool samples in the diagnostic laboratory remains selective culture.

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Campylobacters were first isolated from humans in 1938 from the blood cultures of patients suffering from diarrhoea. They could not be isolated from the faeces because of the overgrowth of plates by commensal faecal flora and because of their own fastidious nature.

The breakthrough of their successful isolation from faeces came in 1972 when Butzler used the technique of membrane filtration.[1] This was followed in 1977 by the development of a selective medium by Skirrow, which enabled the isolation of campylobacters with greater ease.[2] The selective medium was designed to isolate the two species known at the time to cause gastroenteritis, *Camylobacter jejuni* and *C coli.* Today, by current isolation and culture methods, these two species are estimated to cause approximately 99% of campylobacter infections in England and Wales and the USA.[3 4]

> "The combination of not being able to detect the unusual species by the current method, and not identifying to the species level the common species that are isolated, has contributed to a limited understanding of the epidemiology of campylobacter gastroenteritis"

Since 1977, membrane filtration,[5 6] modified selective media,[7] and molecular methods[8–12] have discovered other species that are rarely implicated as causes of disease. These species, namely *C hyointestinalis*,[13–15] *C upsaliensis*,[16–24] and *C fetus*,[25] are inhibited by the high amount of cefoperazone contained in the selective medium.[7 26–28] Because most laboratories in the UK routinely use selective culture, these less common species are being missed.

Moreover, biochemical identification to the species level is limited and unreliable for campylobacters; hence they are identified only to the genus level. The combination of not being able to detect the unusual species by the current method, and not identifying to the species level the common species that are isolated, has contributed to a limited understanding of the epidemiology of campylobacter gastroenteritis.

MATERIALS AND METHODS
Our study was performed over a 10 week period from August to October 1998. All 343 stool samples received in the laboratory during that time were included. Every morning, samples received the previous afternoon (which had been stored at

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Abbreviations: CCDA, charcoal cefoperazone desoxycholate agar; ELISA, enzyme linked immunosorbent assay; PCR, polymerase chain reaction
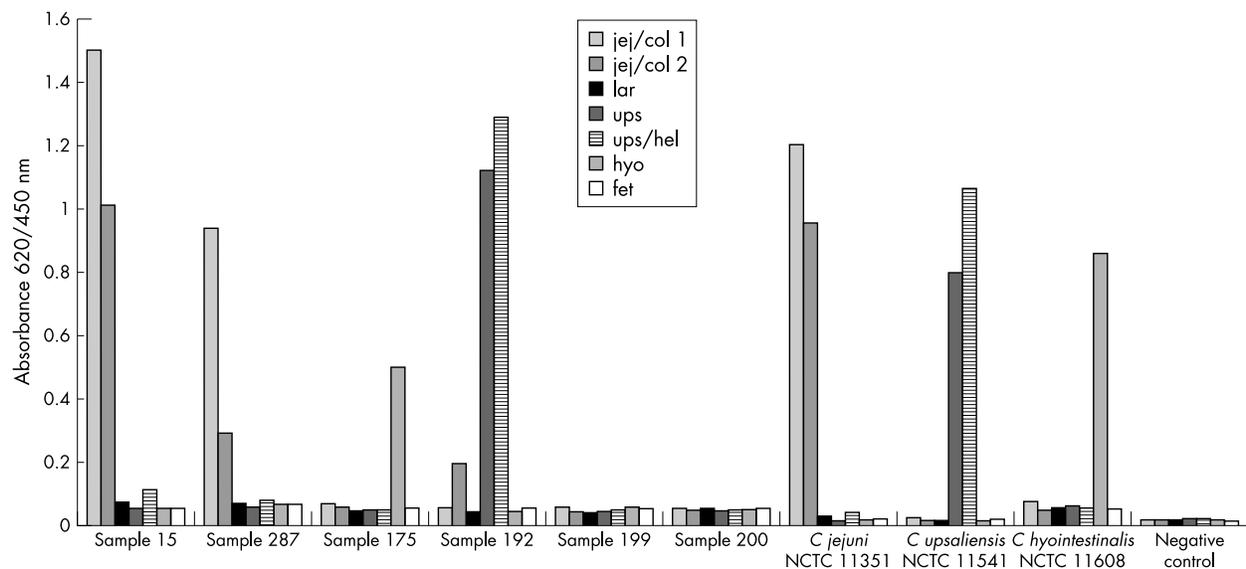
Figure 1 Polymerase chain reaction enzyme linked immunosorbent assay results for six representative samples, with positive and negative controls. The absorbance at 450/620 nm was considered negative if below 0.1 and positive if above 0.2 units.

4°C) and those received on the same morning, were cultured in parallel by selective culture and membrane filtration. Culture and DNA extraction were performed on all samples within 24 hours of receipt by the laboratory.

Culture using selective agar

The selective agar used in our study was a commercially available preparation, charcoal cefoperazone desoxycholate agar (CCDA; Oxoid, Basingstoke, UK) containing 32 mg/litre of cefoperazone. Plates were incubated microaerobically at 37°C for two days. Suspect colonies (moist, translucent colonies, sometimes with a silver sheen) were identified to the genus level by a positive oxidase reaction and a typical Gram stain appearance (slender, curved, "seagull wing shaped", Gram negative rods).

Culture using membrane filtration

A cellulose triacetate membrane with 0.45 μm pores was placed on the surface of a blood agar plate. A pasteur pipette was used to place eight to 10 drops of the sample on to the surface of the membrane. The membrane was left on the agar surface until all the fluid had passed through; this took 20 to 30 minutes.[5] The pores allow the relatively slender campylobacters to pass through, whereas facultative anaerobes and other bacteria that might grow in a microaerobic atmosphere are excluded. This method should detect all cultivable campylobacter species because there is no antibiotic in the medium used. The plates were incubated under the same conditions as the CCDA plates but were incubated for five days to isolate the less common, slower growing species. Identification was to the genus level as described above for selective culture.

PCR based methodology

Campylobacter detection and species identification by PCR, PCR enzyme linked immunosorbent assay (ELISA), and supplementary PCR tests was implemented as described by Lawson *et al*.[27] The steps involved in this PCR identification algorithm are described below.

Nucleic acid extraction from faeces

DNA extraction was performed at the same time as culture, as described previously.[26] DNA extracts were then stored at −20°C until required for PCR screening.

Screening PCR assays

Two PCR assays based on the 16S rRNA gene were used to facilitate large scale screening surveys by reducing the number of samples to be tested by the species specific PCR-ELISA (see below).[27] The first assay, termed pathgroup, detected the *C jejuni*, *C coli*, *C lari*, *C upsaliensis*, and *C helveticus* group of thermophilic campylobacters.[27] The second assay, termed fet/hyo, detected both *C hyointestinalis* and *C fetus*.[10]

A total of 2.5 μl of DNA extract was amplified in a 25 μl reaction volume, as described previously,[28] using reference strains of *C jejuni* (NCTC 11351[T]), *C hyointestinalis* (NCTC 11608[T]), and *C fetus* (NCTC 10842[T]) as appropriate positive controls and sterile water as a negative control. Amplification conditions were denaturation at 94°C for one minute, annealing at 66°C (pathgroup) or 65°C (fet/hyo) for one minute, and extension at 72°C for one minute. This was repeated for 30 cycles in a RoboCycler thermocycler with a hot top assembly (Stratagene, California, USA). The preparation of the reagents for PCR, addition of the DNA extracts to the mix, and the PCR thermocycling were performed in three separate rooms to prevent crossover contamination by extraneous nucleic acids. Amplicons were analysed using a 96 well format gel electrophoresis (on a 1% (wt/vol) agarose gel with 100 V for 90 minutes). The gels were stained with SYBR green I (Flowgen Instruments Ltd, Lichfield, UK)[29] for 30 minutes and viewed under ultraviolet light. Any samples where the bands were of the correct size expected from the screening assays, irrespective of their band intensity, were recorded as positive.

Identification of screening PCR positives by PCR-ELISA

All the samples that were positive by the screening PCRs were identified using a PCR-ELISA assay.[30] Briefly, capture probes based on the 16S rRNA gene, specific for *C jejuni-C coli*, *C upsaliensis*, *C hyointestinalis*, *C lari*, *C fetus*, and *C helveticus* were immobilised via their 5′ biotin ends to a strepavidin coated microtitre plate. The screening PCR positive amplicons were amplified in a second round asymmetric PCR, which used a single, genus specific 5′ fluorescein labelled primer. The conditions were as for the screening PCR except that the annealing temperature was 60°C. This produced predominantly single stranded amplicons, which were then applied to the wells of a microtitre plate containing the specific capture probes. Hybridisation between probe and amplicon was detected colorimetrically using an antifluorescein enzyme conjugate,

Table 1 Comparison of the detection of campylobacter species by membrane filtration, selective culture, and a PCR identification algorithm

Sample number	Membrane filtration	Selective culture	PCR algorithm identification	Identification of culture positive PCR algorithm negative
1	+	+	C jejuni	
2	+	+	C jejuni	
3	+	+	C jejuni	
4	+	+	C jejuni	
5	+	+	C jejuni	
6	+	+	C jejuni	
7	+	+	C jejuni	
8	+	+	C jejuni	
9	–	+	C jejuni	
10	–	+	C jejuni	
11	–	+	C jejuni	
12	–	+	C jejuni	
13	–	+	C jejuni	
14	+	–	C jejuni	
15	–	–	C jejuni	
16	–	+	C jejuni/C coli	
17	–	–	C jejuni/C coli	
18	+	+	C coli	
19	–	–	C upsaliensis	
20	–	–	C hyointestinalis	
21	+	+	–	Isolate C concisus PCR positive
22	+	–	–	Isolate Arcobacter sp PCR positive
23	–	+	–	Isolate lost to the study
Totals	12/23	17/23	20/23	

where the optical density was measured at 450/620 nm by an automated plate analyser, and a graphical printout produced (fig 1). An advantage of the PCR-ELISA is that it allows six species to be detected using a single assay format; however, the 16S rRNA genes of *C jejuni* and *C coli* show a high percentage of sequence similarities, which precludes their differentiation based on this gene.[30]

Speciation of *C jejuni* and *C coli*

Samples identified as *C jejuni*/*C coli* by PCR-ELISA were speciated by additional PCRs specific for the hippuricase (hip) gene of *C jejuni* and the aspartokinase (asp) gene of *C coli*.[31] In cases where both hip and asp species specific PCRs were negative, a more sensitive PCR assay (termed cc/cj) for the multicopy 16S rRNA gene[31] was performed to confirm the PCR-ELISA result.

Statistical analysis

The results of campylobacter identification to the genus level by selective culture and by membrane filtration culture and campylobacter detection to the species level using the PCR identification algorithm were compared by means of McNemar's test.[33]

Identification of culture positive PCR algorithm negatives

When either culture method detected a campylobacter to the genus level but the PCR algorithm gave a negative result, this indicated that a pathogenic campylobacter species was unlikely to have been isolated. In these cases, isolates were further investigated using three PCRs specific for the commensal species *C concisus*,[34] *C rectus*,[35] and *C hominis*,[36] and the arcobacter species was identified using a PCR based on the 16s rRNA gene.[37]

RESULTS

Please refer to table 1. Of the 343 samples tested in our study, 320 samples were negative by all three methods. There were 23 samples positive by one or more method; 17 were positive by selective culture, 12 by membrane filtration, and 20 using the PCR identification algorithm.

Of the 23 positive samples, nine were positive by all three methods (eight *C jejuni* and one *C coli* by PCR); six were positive by PCR and selective culture (five *C jejuni* and one *C jejuni*/ *C. coli** by PCR); one was positive by PCR and membrane filtration (one *C jejuni* by PCR); four were positive by PCR alone (one *C jejuni*, one *C jejuni*/*C coli**, one *C upsaliensis* and one *C hyointestinalis*); and the remaining three samples were negative by the PCR identification algorithm, but yielded isolates by culture based methods and were provisionally identified as "*Campylobacter* sp".

In two instances, the PCR algorithm identified samples as *C jejuni*/*C coli* (marked * above). Here, the 16S rDNA (campylobacters typically have three copies of this gene) based PCR-ELISA detects but does not speciate *C jejuni* from *C coli* because of sequence constraints, but the species specific hip and asp (both single copy genes) PCRs were negative.

The three culture positive/PCR algorithm negative isolates were later investigated further using species specific PCRs for commensal campylobacters and an arcobacter genus PCR: one isolate, detected by both selective culture and membrane filtration, was identified as *C concisus*; another detected by selective culture alone was lost to the study and could not be tested; whereas a third, detected by membrane filtration alone, was speciated as an *Arcobacter* sp.

The sensitivity of detection of the three protocols (selective culture, membrane filtration, and PCR identification algorithm) for campylobacters was compared using McNemar's test. For selective culture and the PCR identification algorithm, p = 0.5 > p > 0.1; for selective culture and membrane filtration, p = 0.5 > p > 0.1; for membrane filtration and the PCR identification algorithm, p = 0.05 > p > 0.02.

DISCUSSION

In our present study, the PCR identification algorithm detected campylobacter species including the uncommon species, *C hyointestinalis* and *C upsaliensis*, with the greatest sensitivity of the three methods examined. In most cases, the PCR identification algorithm was to the species level. This is an advantage over the culture methods, where identification was only to the genus level. PCR results were available on the same day as the assays were performed.

The PCR identification algorithm detected all (confirmed) pathogenic species that were detected by both culture methods. This was in contrast to the study by Lawson et al,[27] where an equal number of culture negative/PCR positive samples as culture positive/PCR negative samples was found. A possible explanation could be the difference in the timing of the DNA extraction in the two studies. In our present study, DNA extraction was done within 24 hours of receipt of the specimens, whereas in the study by Lawson et al extraction was done within 10 days of receipt by the reference laboratory. This delay might have resulted in degradation of campylobacter DNA and hence failure of the PCR to detect it. Further work would be needed to confirm this hypothesis.

The PCR identification algorithm consisted of initial screening PCRs, which were designed to be as inclusive as possible and used lowered stringency, high sensitivity PCR cycling conditions to facilitate the maximal detection of Campylobacter spp, although still screening out most Campylobacter spp negative samples.[27] Any screening PCR mismatch products were eliminated by the high specificity PCR-ELISA. Therefore, the screening PCRs produce a proportion of amplicons that are negative by PCR-ELISA—in our study 13 samples—but which at the same time greatly reduced the number of samples examined by PCR-ELISA, here 33 samples were tested rather than all 343 stool samples.

Nevertheless, using PCR in the enteric laboratory for the detection of campylobacters is labour intensive and not cost effective. A further disadvantage of PCR based methods is the lack of an isolate and hence the inability to perform simple antibiotic sensitivity testing and in some cases further identification or detailed typing for epidemiological purposes.

There was no significant difference between selective culture and the PCR identification algorithm for the detection of the most common pathogenic species, C jejuni and C coli ($0.5 > p > 0.1$). Selective culture missed three of the total 18 (one positive by membrane filtration and PCR, two positive by PCR alone) positive samples that were detected in our study. A single Campylobacter sp was detected by selective culture alone, but unfortunately was lost to the study before further identification was possible (although it was highly probable that this was either C jejuni or C coli).

> "A further disadvantage of PCR based methods is the lack of an isolate and hence the inability to perform simple antibiotic sensitivity testing and in some cases further identification or detailed typing for epidemiological purposes"

However, selective culture did not isolate the less common species, C upsaliensis and C hyointestinalis. This is not unexpected because the non-C jejuni/C coli species may be inhibited by the 32 mg/litre of cefoperazone contained in the selective medium.[7 26] Surprisingly, an isolate identified as C concisus did grow on CCDA, although this species is thought to be inhibited by conventional selective culture medium (see below). Modified selective medium containing cefoperazone at lower concentrations may better support the growth of non-C jejuni/C coli campylobacter.[7] A cost effective alternative to PCR might be a combination of two media, as is currently used for the detection of salmonellae. For epidemiological purposes, all culture positive samples could then be tested by PCR to identify the isolates to the species level.

Further work, comparing the sensitivity of two culture media methods with PCR methods, is planned.

In theory, the membrane filtration method could have isolated all the Campylobacter spp seen in our study because it does not depend on selective antibiotics. However, in practice not all the campylobacter cells are able to pass through the filter pores and its sensitivity is limited to 10^5 colony forming units/g of faeces.[26] In our study, the detection rate by

Take home messages

- The advantages of the PCR identification algorithm are that it can detect and identify Campylobacter spp to the species level and the result is obtained on the same day
- The disadvantages are that it is expensive, labour intensive, and does not provide an isolate for further identification or typing
- Selective culture is as good as the PCR identification algorithm for the detection of the two most common species, C jejuni and C coli, and it is cheap and practical, but it misses the less common species, results take 48 hours, and identification is only to the genus level
- Modified selective medium containing cefoperazone at lower concentrations may better support the growth of non-C jejuni/C coli campylobacter
- Membrane filtration is less sensitive than the other methods and is not appropriate for the diagnostic laboratory, although it was the only method to detect the Arcobacter sp
- Thus, currently selective culture is the optimum method for the detection of campylobacters from stool samples in the diagnostic laboratory, although increased automation of PCR methods in the future may make it the best choice

membrane filtration was found to be less than that of selective culture, although the results were not significant ($0.5 > p > 0.1$). It failed to isolate six of the 15 Campylobacter spp detected by selective culture (although a positive by PCR and membrane filtration was missed by selective culture). Membrane filtration also failed to isolate the C hyointestinalis and C upsaliensis detected by PCR and there was a significant difference between the detection rates of PCR and membrane filtration in general ($0.05 > p > 0.02$). Apart from its relative insensitivity, the poor performance of this method may be caused by inexperience in processing the samples, which resulted in a considerable number of plates being overgrown with commensal faecal flora. This method is labour intensive and time consuming, which is why it has only been used in a limited number of centres. The strength of membrane filtration is its ability to isolate many different Campylobacter spp and campylobacter-like bacteria, which are not detected by selective isolation media or PCR assays designed for specific bacterial species.

In our present study, C concisus was detected by membrane filtration and selective culture. This last fact is noteworthy because C concisus is generally sensitive to cefoperazone and does not usually grow on CCDA medium. Campylobacter concisus is usually associated with the oral cavity and its role in human disease remains unclear.[37] Membrane filtration alone isolated a campylobacter-like organism later identified as Arcobacter sp. Members of this genus, most notably A butzleri, have been associated with diarrhoea in humans and animals. Nevertheless, the role of Arcobacter sp in human gastroenteritis remains to be determined.[37]

In conclusion, selective culture is currently the optimal method for the isolation of enteropathogenic campylobacters. PCR based methods are more sensitive than selective culture in detecting the less common, non-C jejuni/C coli species, and would therefore increase our understanding of the epidemiology of campylobacter gastroenteritis. PCR is more rapid than culture and is being increasingly automated. However, currently PCR is more expensive and labour intensive than culture and the advantages do not outweigh the expense.

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