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Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and directly on stool samples

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A multiplex-PCR method, specifically designed for application in routine diagnostic laboratories, was developed for the detection of *Campylobacter coli* and *Campylobacter jejuni*. Primers were directed towards the following loci: the hippuricase gene (*hipO*) characteristic of *C. jejuni*, a sequence partly covering an aspartokinase gene characteristic of *C. coli*, and a universal 16S rDNA gene sequence serving as an internal positive control for the PCR. The method was tested on 47 *C. coli* strains and 88 *C. jejuni* strains, and found to be almost 100 % in concordance with biochemical analyses (all except for one *C. coli* strain), regardless of whether the DNA was prepared from colonies by a simple boiling procedure or by DNeasy Tissue Kit. Pure cultures of *C. coli* and *C. jejuni* were identified at 10–100 cells per PCR. When the multiplex-PCR method was used on spiked human stool samples, both strains were identified at 10^5 cells per ml stool. This sensitivity limit was the same whether the DNA was purified by the method of KingFisher mL or QIAamp DNA Stool Kit. When the same spiked stools were grown on modified charcoal cefoperazone deoxycholate agar (mCCDA) plates before PCR, the sensitivity was 100 cells per ml stool, indicating that culturing of campylobacters on mCCDA plates is superior to direct DNA extraction at least when fresh stool samples are analysed by PCR.

INTRODUCTION

Campylobacters are one of the most frequent causes of food-borne gastroenteritis in developing as well as developed countries (Allos, 2001; Blaser, 1997; Mead *et al.*, 1999; Tauxe, 1997). Campylobacter diagnostics and determination of antibiotic resistance are important for the treatment of infected individuals, and the distinction between the two most prevalent species in humans, namely *Campylobacter coli* and *Campylobacter jejuni*, is important for epidemiological surveillance. The only biochemical test for discriminating between *C. coli* and *C. jejuni* is based on hippurate hydrolysis, which is time consuming, cumbersome and sometimes difficult to interpret when the enzymic activity is impaired under the methodological conditions (Rautelin *et al.*, 1999; Totten *et al.*, 1987). Therefore, different molecular strategies and genetic targets have been applied for the identification of *C. coli* and *C. jejuni* in the literature. Examples of these include: PCR on *asp* and *hipO* (Lawson *et al.*, 1998), *ceuE* (Gonzalez *et al.*, 1997), *cadF* (Englen & Fedorka-Cray, 2002), and *hipO* and 16S rRNA (Bang *et al.*, 2002), PCR-RFLP on 23S rRNA (Engvall *et al.*, 2002) and *cdt* (Eyigor *et al.*, 1999), PCR/ELISA on *glyA* (Al Rashid *et al.*, 2000), real-time PCR on *hipO* and *glyA* (LaGier *et al.*, 2004), and microarray detection of *fur*, *glyA*, *cdtABC*, *ceuB–E* and *fliY* (Volokhov *et al.*, 2003).

This report describes a three-gene multiplex-PCR-based method for the detection of *C. coli* and *C. jejuni*. The method is based on the aspartokinase (*asp*) primers specific for *C. coli* developed by Linton *et al.* (1997), novel primers designed towards the hippuricase gene (*hipO*) characteristic of *C. jejuni*, and a universal 16S rDNA sequence serving as an internal positive control for the PCR. Compared to the previously described methods, the specific gene combination, the one-step analysis by multiplex PCR and the incorporation of the carry-over prevention system uracil *N*-glycosylase (UNG) (Longo *et al.*, 1990) makes this method especially suited for routine diagnostic laboratories.

Diagnostic PCR on template DNA extracted directly from the primary source offers attractive advantages including reduced time of analysis and detection of non-viable and non-cultivable bacteria contained in the sample. Therefore, the PCR method was tested on both plate-grown stools and on DNA purified directly from stools.

Abbreviation: UNG, uracil *N*-glycosylase.

METHODS

Strain origin and DNA preparation. Campylobacter strains were grown on modified charcoal cefoperazone deoxycholate agar (mCCDA) plates (SSI Diagnostica) or on 5 % (v/v) defibrinated horse blood agar plates with yeast (SSI Diagnostica) or in Bolton Broth (Oxoid) without antibiotics. Cultures were grown under microaerobic conditions, 6 % O_2, 6 % CO_2, 3 % H_2 and 85 % N_2, for 24 h at 37 °C. Bacterial colonies were prepared for PCR either by DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions, or by 8 min boiling in 10 % Chelex 100 (Bio-Rad) in 10 mM Tris/HCl, 1 mM EDTA, pH 8, followed by centrifugation and 10-fold dilution of the supernatant in PCR-grade water.

The present study included 47 *C. coli*, 88 *C. jejuni* and one *Campylobacter upsaliensis* strains isolated from humans with diarrhoea from 2001 to 2003 by the National Reference Laboratory for Enteric Pathogens, Unit of Gastrointestinal Infection, Statens Serum Institut, Denmark. The following 14 campylobacter reference strains were also included (kindly provided by Dr Eva Møller Nielsen, Statens Serum Institut, Denmark): *C. coli* (ATCC 33559), *Campylobacter fetus* subsp. *fetus* (CCUG 6823), *C. fetus* subsp. *venerealis* (CCUG 538), *Campylobacter hyointestinalis* subsp. *hyointestinalis* (CCUG 14169), *C. jejuni* subsp. *doylei* (CCUG 24567), *C. jejuni* subsp. *jejuni* (CCUG 11284), *Campylobacter lari* (CCUG 18267), *C. lari* (CCUG 23947), *Campylobacter mucosalis* (CCUG 6822), *Campylobacter rectus* (CCUG 20446), *Campylobacter showae* (CCUG 30254), *Campylobacter sputorum* subsp. *bubulus* (CCUG 11290), *C. upsaliensis* (CCUG 14913) and *C. upsaliensis* (CCUG 23626).

Cell densities of liquid cultures were estimated by colony counts of 10-fold diluted cultures plated on semi-dried blood-agar plates, and by counting cells in a Bürker-Türk counting chamber. Cell densities, for sensitivity experiments, were used to construct 10-fold serially diluted cultures of 2×10^9 to 2×10^4 cells per ml. Each of these dilutions was 10-fold diluted in 10 % Chelex 100 (Bio-Rad), 10 mM Tris/HCl, 1 mM EDTA, pH 8, boiled and centrifuged as described above, and 5 µl of the supernatant was used directly in the PCR, resulting in 10^6 to 10^1 cells per PCR.

Spiked stool experiments. Two bloody and two non-bloody campylobacter-negative stool samples were selected for a spiking experiment. Liquid campylobacter cultures were added to the stools, resulting in final campylobacter concentrations of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 per ml stool of either *C. coli* or *C. jejuni*. DNA was extracted from the stools by either KingFisher mL (Thermo Labsystems) or QIAamp DNA Stool Kit (Qiagen), according to the manufacturer's instructions. Ten microlitres of the spiked stools was also grown on mCCDA plates as described above. Template DNA was purified from plates containing visible growth by the simple boiling procedure described above.

Multiplex PCR. PCRs were performed in a total reaction volume of 25 µl containing 1× PCR buffer [50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, pH 8·3], 2·6 mM $MgCl_2$, 260 µM dATP, dGTP and dCTP, 520 µM dUTP, 0·15 U UNG (Applied Biosystems), 1·25 U FastStart Taq Polymerase (Roche Diagnostics), 0·4 µM *asp*-primers CC18F and CC519R (Linton *et al.*, 1997), 0·2 µM *hipO* primers hipO-F (5′-GACTTCGTGCAGATATGGATGCTT) and hipO-R (5′-GCTATAAC TATCCGAAGAAGCCATCA), and 0·05 µM 16S rDNA primers 16S-F (5′-GGAGGCAGCAGTAGGGAATA) and 16S-R (5′-TGACGGGCG GTGAGTACAAG). Template volumes were 5 µl when PCRs were performed on cultured campylobacters prepared by the simple boiling method or the DNeasy Tissue Kit or when stool samples were extracted with QIAamp DNA Stool Kit. When stool samples were extracted with KingFisher mL, 1 µl was used as the template volume. Thermocycler conditions were 94 °C for 6 min, followed by 35 cycles of 94 °C for 50 s, 57 °C for 40 s and 72 °C for 50 s, and finally 72 °C for 3 min. Completed PCRs were analysed by 1·5 % agarose gel electrophoresis under standard conditions and stained by ethidium bromide.

Biochemical identification. *Campylobacter* speciation was performed by standard biochemical tests including hippurate hydrolysis, indoxyl acetate hydrolysis, resistance to nalidixic acid and cephalothin, H_2S (TSI), catalase and oxidase (Nachamkin, 2003).

RESULTS AND DISCUSSION

A multiplex PCR was developed for the identification of *C. coli* and *C. jejuni*. Included in the method are the *C. coli*-specific *asp*-primers developed by Linton *et al.* (1997), which result in a 500 bp amplicon, novel primers designed to amplify a 344 bp fragment of the *hipO* gene characteristic of *C. jejuni*, and universal primers used to amplify a 1062 bp fragment of the 16S rDNA gene, serving as an internal positive control for the PCR.

The method specificity was tested on fourteen different campylobacter reference strains and showed that the *C. coli* and *C. jejuni* strains resulted in the expected amplicons, while all other campylobacter reference strains produced only the 16S rDNA amplicon (data not shown). Also, 47 *C. coli*, 88 *C. jejuni* and one *C. upsaliensis* strains (biochemically identified) of human origin were subjected to the multiplex-PCR method and biochemical species identification. All isolates gave the same results by both methods, except for one strain that initially was identified as *C. coli* by the biochemical tests but was found to be *C. jejuni* upon repeated PCR testing. This strain is believed to represent a *C. jejuni* strain not expressing hippurate hydrolysis activity *in vitro*, which has also been observed by others (Rautelin *et al.*, 1999; Totten *et al.*, 1987), further legitimizing PCR analyses for this diagnostic purpose. Fig. 1 shows the PCR results of three *C. jejuni*, four *C. coli* and one *C. upsaliensis* strains. The biochemically identified *C. upsaliensis* could not be identified by the PCR method, but, as expected, showed a *C. coli*/*C. jejuni*-negative result (Fig. 1, lane 5).

All strains tested were easily prepared for PCR by a simple boiling procedure of the bacterial colonies, and required no special treatment to extract useful DNA for the PCR analysis. Others have found heat-resistant campylobacter strains that could not produce template DNA by simple boiling unless

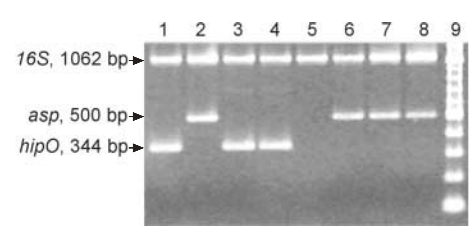

Fig. 1. Multiplex PCR on eight mCCDA-plate-grown campylobacter strains. Lanes 1, 3 and 4, *C. jejuni*; lanes 2, 6, 7 and 8, *C. coli*; lane 5, *C. coli*/*C. jejuni* negative, biochemically identified as *C. upsaliensis*; lane 9, 100 bp DNA marker.

treated with phenol/chloroform, proteinase K or SDS (Englen & Kelley, 2000; Mohran *et al.*, 1998; Nachamkin *et al.*, 1993). The reason why no such observations were found in the present study, cannot be determined, but could be due to differences in growth conditions, DNA preparation or PCR method. For the evaluation of the specific PCR conditions, the present method contains a 16S rDNA internal positive control, which always needs to be present if a negative result is to be trusted. This will eliminate false negatives, at least when the difference in copy number between the internal positive control locus and the diagnostic loci is not critical. In most diagnostic laboratories at least 95 % of human campylobacter isolates belong to either *C. coli* or *C. jejuni* when a selective medium is applied (Endtz *et al.*, 1991; Engberg *et al.*, 2000). Hence, the present method based on simple boiling of plate cultures and multiplex PCR will allow a fast identification of these samples, which is clearly an advantage for a routine diagnostic laboratory setting.

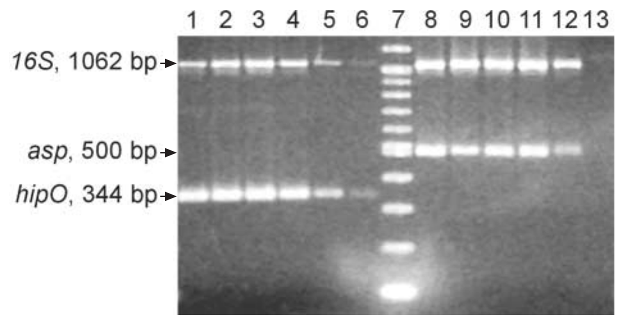

Fig. 2. Multiplex-PCR sensitivity study by 10-fold dilutions of bacterial DNA derived from pure culture of *C. jejuni* (lanes 1–6) and *C. coli* (lanes 8–13). Lane 7, 100 bp DNA marker. DNA concentrations (cells per 25 µl PCR): lanes 1 and 8, 10^6; lanes 2 and 9, 10^5; lanes 3 and 10, 10^4; lanes 4 and 11, 10^3; lanes 5 and 12, 10^2; lanes 6 and 13, 10^1.

The sensitivity of the multiplex-PCR method was tested on different preparations and the results are summarized in Table 1. First, the sensitivity was investigated by extracting DNA from serially diluted pure cultures. DNA templates were prepared for the analysis of 10^6, 10^5, 10^4, 10^3 10^2 and 10^1 cells per PCR of *C. jejuni* and *C. coli*. The multiplex-PCR method was able to detect the presence of 10^6–10^2 bacteria per PCR for both *C. coli* and *C. jejuni*, and for *C. jejuni* a weak signal was observed at 10^1 cells per PCR (Fig. 2).

Next, both bloody and non-bloody campylobacter-negative stool samples were spiked with 10-fold serial dilutions of either *C. coli* or *C. jejuni* cultures, resulting in final concentrations of 10^7–10^2 campylobacters per ml stool. Template DNA from each stool sample was purified by either KingFisher mL or QIAamp DNA Stool Kit, and analysed by the multiplex-PCR method. Different eluate volumes from the two purification procedures were tested by the PCR method for highest sensitivity. The optimal volumes were found to be 1 µl and 5 µl eluate for KingFisher mL and QIAamp DNA Stool Kit, respectively. Both DNA extraction methods had a sensitivity limit of 10^5 campylobacters per ml stool for both species, regardless of whether the stool contained blood or not (data not shown), and therefore PCR inhibitors that are known to be present in blood (Al-Soud & Radstrom, 1998, 2001; Fredricks & Relman, 1998) were not interfering with the PCR at and above 10^5 campylobacters per ml stool. For both the KingFisher mL and QIAamp DNA Stool Kit procedures, the DNA was eluted in the same volume as the stool volume entering the extraction procedure. Thus, if 100 % of the DNA was recovered during the extraction procedure, 10^5 cells per ml stool would yield 10^5 cells per ml eluate. Given that 1 µl or 5 µl of the eluate was used in the PCRs, 10^5 cells per ml stool equals 100 or 500 cells per PCR, which is comparable to the sensitivity limit of the DNA extraction from pure cultures (10–100 cells per PCR). Hence, both methods perform well with respect to the recovery of DNA.

When the same spiked stool samples were grown on mCCDA plates before PCR, the sensitivity limit was 100 cells per ml stool. When stool samples are grown on mCCDA plates the growth of campylobacters is selectively favoured. This selectivity is a powerful way of elevating the sensitivity level of campylobacter from the complex bacterial and chemical nature of faeces. However, the success of this growth step is solely dependent on the viability of campylobacter in the sample. Campylobacters are known to have a low survival rate if exposed to room temperature and atmospheric air (Holler *et al.*, 1998; Wang *et al.*, 1983). This, in combination with a potential long transport time from sample collection to sample analysis, may reduce the viability of routine diagnostic samples. It should be emphasized that, in the present spiking experiments, fresh campylobacter cultures were added to the stool samples just prior to the culturing

Table 1. Sensitivity limits for the multiplex-PCR method on different starting materials prepared by different DNA extraction methods

Starting material	Template DNA preparation method	Sensitivity limit
Pure cultures	Simple boiling	10–100 cells per PCR
Spiked stools	KingFisher mL	10^5 cells (ml stool)$^{-1}$
	QIAamp DNA Stool Kit	10^5 cells (ml stool)$^{-1}$
Colonies (mCCDA plates) from spiked stools	Simple boiling	100 cells (ml stool)$^{-1}$

step, favouring this experimental outcome compared to daily procedures on routine diagnostic samples. Therefore, the observed 10^3-fold higher sensitivity of culturing compared to direct DNA purification is expected to be less pronounced on routine diagnostic samples, and the direct DNA purification should be considered advantageous with respect to the analysis of samples containing dead and non-cultivable bacteria that may constitute a significant proportion of the bacteria in a given stool sample (Maher *et al.*, 2003). For a further test of the routine diagnostic applicability, the direct DNA purification should be compared to culturing when applied on a number of routine laboratory stool samples.

In short, the present method offers a fast and robust identification of *C. coli* and *C. jejuni*. The intense validation with respect to sensitivity and specificity, 16S rDNA internal PCR control and inclusion of the carry-over prevention system UNG makes this method especially suited for routine laboratories performing diagnostics on human specimens, where these two species constitute the vast majority of campylobacters.

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