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 10⁵ 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 foodborne 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), cceU (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.
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 ampiclon, 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, 1 bp DNA maker.
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.

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 preparations were prepared for the analysis of $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ cells per PCR of C. jejuni and C. coli. The multiplex-PCR method was able to detect the presence of $10^6$–$10^5$ 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 $10^2$ 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**

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

**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, $10^5$; lanes 2 and 9, $10^2$; lanes 3 and 10, $10^4$; lanes 4 and 11, $10^5$; lanes 5 and 12, $10^2$; lanes 6 and 13, $10^1$. 
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, 165 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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REFERENCES


