Multiplex PCR for direct identification of *Campylobacter* spp. in human and chicken stools

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Differentiation between *Campylobacter jejuni* and *Campylobacter coli* is problematic in clinical specimens due to fastidious growth requirements and limited biochemical tests. This study describes a rapid, multiplex PCR protocol for the direct detection and differentiation of *C. jejuni* and *C. coli* in stools. An evaluation was carried out of this multiplex protocol based on the detection of *cadF* (genus specific), and *hipO* (*C. jejuni*) and *asp* (*C. coli*) genes, using stool from patients with *Campylobacter* enteritis and chicken. Protocol sensitivity was assessed and specificity determined using a panel of enteric bacteria, and evaluation of 30 diarrhoeic stool specimens culture negative for *Campylobacter*. Of the 114 specimens (54 human and 60 chicken) evaluated by the protocol, 70 (61.4 %) were identified as *C. jejuni*, 35 (30.7 %) as *C. coli* and 9 (7.9 %) as a mixed infection/colonization with both species. All mixed infections were identified as *C. jejuni* by culture. Among the stool specimens that were culture negative for *Campylobacter*, two (6.7 %) were *C. jejuni* positive by multiplex PCR. The protocol sensitivity limit was 0.015–0.016 ng *C. jejuni* and *C. coli* DNA μl^{-1} in the specimen. There was no cross-reaction with the reference strains assessed. Comparison of hippurate test and multiplex PCR demonstrated 17 isolates with false-positive hippurate enzymic activity and 7 with false-negative activity. This rapid protocol (turnaround time 6 h) is highly sensitive and specific for direct evaluation of stool for these pathogens. It has significant application for routine clinical diagnostic and epidemiological purposes.

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INTRODUCTION

Campylobacter spp. are a major cause of bacterial gastroenteritis worldwide (Moore *et al.*, 2005; Ismaeel *et al.*, 2002). The relatively low infective dose, the potentially serious sequelae (Moore *et al.*, 2005), as well as the association between certain *Campylobacter* virulence genes and the pattern of clinical infection (Al-Mahmeed *et al.*, 2006; Rozynek *et al.*, 2005), confirm the importance of this zoonotic infection as a significant health hazard. Conventional diagnostic methods utilizing a combination of culture and biochemical testing require that suspected stool specimens are cultured on selective agar at 42 °C under microaerophilic conditions for up to 72 h before a negative report is issued. Only culture plates with colonies showing characteristic *Campylobacter* morphology and oxidase positivity are reported as *Campylobacter* spp. Further identification to the species level requires other

tests, including growth temperature preferences, antibiotic sensitivity to cephalothin and nalidixic acid, and biochemical tests. The sodium hippurate hydrolysis reaction is the only biochemical test used to differentiate *Campylobacter jejuni* and *Campylobacter coli*. The extended and tedious nature of these procedures has stimulated research into molecular diagnostic approaches. Several workers have investigated the application of multiplex PCR for *Campylobacter* detection and speciation (Aquino *et al.*, 2002; Chuma *et al.*, 2000; Klena *et al.*, 2004; LaGier *et al.*, 2004) with these protocols being applied to isolates from pure cultures. However, the application of multiplex PCR on bacterial colonies meant that conventional cultures were still needed for the initial identification. To evaluate the multiplex approach directly on stool specimens, Persson & Olsen (2005) artificially spiked stool specimens with bacteria. However, only two reports have described the direct application of a multiplex protocol on stools obtained from patients with enteritis (Houng *et al.*, 2001; LaGier *et al.*, 2004) and both used primers targeting the *ceuE* gene.

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Various combinations of genus-specific and species-specific genes, as well as combinations of species-specific sequences of *ceuE* or *lpxA* genes, have been applied in multiplex protocols (Jensen *et al.*, 2005; Liu *et al.*, 2005; Best *et al.*, 2003; Gebreyes *et al.*, 2005). In this study, we have developed a multiplex PCR protocol using a novel combination of species-specific and virulence genes, and applied it directly to stool specimens of both human and poultry origin.

METHODS

Collection of human and poultry stool specimens. Human stool specimens ($n=91$) were obtained from the diagnostic microbiology laboratories of three tertiary hospitals in the eastern province of Saudi Arabia and two hospitals in the Kingdom of Bahrain. These specimens were from patients with gastroenteritis and included those subsequently determined to be due to *Campylobacter* infection ($n=61$) according to the routine diagnostic method applied in the hospitals. *Campylobacter* detection in these hospitals was carried out using selective media (blood based), and incubation at 42 °C under microaerophilic conditions for a minimum of 48 h. Only one tertiary hospital routinely carried out speciation of *Campylobacter* isolates using the hippurate hydrolysis test. All stool specimens were stored at 4 °C until collection by the research team. The participating centres were provided with cryopreservation tubes containing preservative medium (10% fetal bovine serum, 60% nutrient broth, 30% glycerol) used for storing isolated *Campylobacter* colonies at -20 °C until collection by the research team. Chicken stool specimens ($n=60$) were obtained directly from three slaughter houses, two in Saudi Arabia and one in Bahrain.

DNA extraction from stool. DNA from 180–220 mg stool specimens (manufacturer's recommended minimum 180 mg stool) was extracted using a QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's recommended procedures. As some researchers had reported incubation at 95 °C with the lysing buffer for DNA release (Inglis & Kalischuk, 2003; Persson & Olsen, 2005), in preliminary experiments some specimens were subjected to incubation at both 70 °C and at 95 °C to see if there was any effect on the sensitivity of the extraction method. As no differences were observed between the two temperatures, incubation at 70 °C in lysing buffer, as recommended by the manufacturer, was used for all the specimens. The DNA obtained was stored at -20 °C.

Multiplex PCR protocol. Three genes were targeted in the multiplex PCR protocol, namely *cadF* (genus-specific virulence gene) (Nayak *et al.*, 2005), *hipO* (hippuricase gene for *C. jejuni*) (Linton *et al.*, 1997) and *asp* (aspartokinase gene for *C. coli*) (Linton *et al.*, 1997). The primer sets used, as described by Nayak *et al.* (2005) and Linton *et al.* (1997), were: *cadF* (F 5'-TTG AAG GTA ATT TAG ATA TG-3' and R 5'-CTA ATA CCT AAA GTT GAA AC-3') giving a 400 bp product, *hipO* (F 5'-GAA GAG GGT TTG GGT GGT G-3' and R 5'-AGC TAG CTT CGC ATA ATA ACT TG-3') giving a 735 bp product and *asp* (F 5'-GGT ATG ATT TCT ACA AAG CGA G-3' and R 5'-ATA AAA GAC TAT CGT CGC GTG-3') giving a 500 bp product (Thermo Electron).

The multiplex protocol used a reaction mixture at a final volume of 50 µl, consisting of 25 µl multiplex master mix (Qiagen), 0.5 µl *cadF* primer (50 pmol µl⁻¹), 0.3 µl *asp* primer (30 pmol µl⁻¹), 1.0 µl *hipO* primer (100 pmol µl⁻¹), 0.5 µl 10 mg BSA ml⁻¹ (Promega), 4.5 µl eluted DNA (0.1 µg) and sterile water. The PCR amplification cycle included initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for

1 min and extension at 72 °C for 1 min. The final stage was an extension cycle at 72 °C for 7 min. PCR cycles were carried out in a GeneAmp (Perkin-Elmer 9700; Applied BioSystems). *C. jejuni* ATCC 33291 and a well-characterized in-house *C. coli* strain, AGU 12305 (isolated from a clinical specimen), were used as positive controls, and the negative control did not include any template DNA. These controls were included in every PCR run. Following gel electrophoresis, the amplified PCR product was stained with ethidium bromide (Sigma) and visualized with a UV transilluminator (Bio-Rad). The sizes of PCR products were estimated by comparison with 100 bp DNA molecular mass markers (Bio-Rad; EZ Load) and the amplified control strains. This protocol was used for the identification and speciation of *Campylobacter* in the human and stool specimens obtained. Where specimens showed mixed infection with *C. jejuni* and *C. coli*, single PCR assays were carried out to confirm the finding.

Bacterial suspensions of the control strains were prepared in a Tris-EDTA buffer equivalent to 0.5 McFarland, and DNA was extracted by the boiling method as previously described (Al-Mahmeed *et al.*, 2006). The DNA concentration was measured; serial dilutions (1:10, 1:100, 1:1000 and 1:10000) were used together with the concentrated DNA to determine the minimal amount of DNA detectable by the multiplex PCR protocol. A panel of enteric bacteria that are found in stool specimens normally or under pathological conditions was used for the assessment of protocol specificity. Table 1 shows the list of bacterial reference strains tested. To assess the ability of the multiplex PCR protocol to correctly identify *Campylobacter*-negative specimens, 30 stool specimens obtained from patients with gastroenteritis due to non-*Campylobacter* infection (as determined by culture methods) were tested.

Comparison of the multiplex protocol with the conventional method. A comparative assessment of the multiplex protocol and the conventional diagnostic method was carried out. As hippurate test remains the main biochemical test for differentiating between *C. jejuni* and *C. coli*, an evaluation of this test versus the multiplex approach was undertaken. The hippurate enzyme activity of 74 isolates (29 human and 45 chicken) was carried out to differentiate *C. jejuni* from *C. coli*, and the findings compared with the result obtained by multiplex PCR. In addition, an evaluation of the turnaround time and the cost of both tests was conducted.

Table 1. Reference strains of enteric bacteria tested using the multiplex protocol

Species	Reference strain
<i>Helicobacter pylori</i>	NCTC 11637
<i>Salmonella enterica</i> serovar Typhimurium	ATCC 14028
<i>Salmonella enterica</i> serovar Typhi	S17893
<i>Shigella boydii</i>	ATCC 9207
<i>Shigella sonnei</i>	ATCC 9290
<i>Shigella flexneri</i>	ATCC 12022
<i>Vibrio cholerae</i>	ATCC 9459
<i>Vibrio parahaemolyticus</i>	ATCC 17802
<i>Yersinia enterocolitica</i>	ATCC 23715
<i>Aeromonas hydrophila</i>	ATCC 7965
<i>Plesiomonas shigelloides</i>	ATCC 51903
<i>Enterococcus faecalis</i>	ATCC 29212
<i>Klebsiella pneumonia</i>	ATCC 700603
<i>Escherichia coli</i> (O157:H7)	NCTC 12900
<i>Proteus mirabilis</i>	ATCC 7002
<i>Pseudomonas aeruginosa</i>	ATCC 27853

RESULTS AND DISCUSSION

For the assessment of the multiplex protocol, DNA extraction was carried out for a total of 91 human stool specimens and 60 chicken stool specimens. The human stool specimens provided by participating centres were from patients with gastroenteritis, and included both bloody and mucoid specimens. A total of 61 specimens were identified as *Campylobacter* culture positive at the participating diagnostic laboratories and the remaining 30 were *Campylobacter* negative. Seven *Campylobacter*-positive specimens were also reported as being positive for *Salmonella enterica* serovar Typhimurium. Stool represents a heterogeneous specimen consisting of micro-organisms, dietary by-products, bile salts, complex polysaccharides and fat (McOrist *et al.*, 2002), while under pathological conditions, blood, pus and mucous may also be present. This heterogeneity makes DNA extraction technically challenging and this has hindered the direct application of molecular techniques on faecal material. In a report of a comparative assessment of DNA extraction kits, the QIAamp DNA stool mini kit was found to be the most sensitive kit, particularly for Gram-negative bacteria (McOrist *et al.*, 2002). Furthermore, it has been utilized for *Campylobacter* spp. with satisfactory results (Inglis & Kalischuk, 2003; LaGier *et al.*, 2004), hence its selection for use in this study.

We developed a multiplex PCR protocol using a novel combination of genus-specific virulence genes and species-specific genes that has been validated on stool specimens from humans and poultry. Culture-positive stool specimens (61 human and 60 chicken) were evaluated. Fig. 1 shows a representative electrophoresis gel of samples from a direct application of the multiplex PCR protocol using human and chicken stool specimens. Multiplex PCR is particularly attractive as it enables the simultaneous detection and speciation of the micro-organism. Unlike other molecular techniques, such as PCR-RFLP and PCR-nested PCR, no manipulation of the post-amplification product is required before gel electrophoresis, thus reducing the risk of false-positive results likely associated with amplicon contamination.

The results of seven human specimens were excluded from the study because of the insufficient quantity of faecal material provided by the participating centres (less than the minimum 180 mg required). The assays of the seven excluded specimens were negative. Of the remaining 114 specimens, 70 (61.4%) were *C. jejuni*, 35 (30.7%) were *C. coli* and 9 (7.9%) were a mixed infection/colonization with both species (Table 2). The majority of the mixed infections were in chicken specimens (77.8% $n=7/9$). Single PCRs of all the specimens showing mixed infection/colonization confirmed the presence of both *C. jejuni* and *C. coli*. The ability to detect such mixed infections has important therapeutic implications in view of the high level of resistance of *C. coli* to erythromycin, which is usually the drug of choice for *C. jejuni*.

Fig. 1. Representative electrophoresis gels of multiplex PCR samples for identification and speciation of *C. jejuni* and *C. coli*. (a) *C. jejuni* and *C. coli* in chicken stool specimens: lane 1, *C. coli*; lanes 2–6, *C. jejuni*; lanes 7 and 8, positive controls of *C. jejuni* and *C. coli*, respectively; M, 100 bp marker. (b) *C. jejuni* and *C. coli* in human stool specimens: lane 1, 3 and 6, *C. jejuni*; lane 2, mixed infection with both *C. jejuni* and *C. coli*; lanes 4 and 5, positive controls of *C. jejuni* and *C. coli*, respectively; M, 100 bp marker.

In our protocol, three genes, namely *cadF* (genus-specific virulence gene), and *hipO* and *asp* (species specific for *C. jejuni* and *C. coli*, respectively), were targeted. These genes and the primers used for their identification have been studied independently and reported on by other workers (Nayak *et al.*, 2005; Linton *et al.*, 1997). However, this is the first study using such a combination of virulence and species-specific genes in a multiplex protocol for *Campylobacter*. The *cadF* gene, a putative virulence gene associated with adhesion, is 100% conserved among isolates of diverse sources (Datta *et al.*, 2003; Rozynek *et al.*, 2005). The *hipO* gene is highly conserved in *C. jejuni* strains and represents the most widely validated gene for the identification of *C. jejuni* (Linton *et al.*, 1997; Sinha *et al.*, 2004). The *asp* gene encodes aspartokinase and is highly specific for *C. coli* (Linton *et al.*, 1997). Although several studies have targeted the rRNA genes for genus and species identification, they may lack specificity due to the high level of conservation among closely related species (On & Jordan, 2003; Linton *et al.*, 1996). Thus the choice of target genes used in this study clearly overcomes this drawback. However, a possible limitation of our multiplex protocol is the absence of an internal PCR control, which

Table 2. Assessment of human and chicken stool specimens using the multiplex PCR protocol

	<i>C. jejuni</i>	<i>C. coli</i>	Mixed infection (<i>C. jejuni</i> and <i>C. coli</i>)
Human stool specimens (n=54)	36 (66.7 %)	16 (29.6 %)	2 (3.7 %)
Chicken stool specimens (n=60)	34 (56.6 %)	19 (31.7 %)	7 (11.7 %)

could be important when analysing stools that may be *Campylobacter* negative. In this study, all culture-positive specimens were correctly identified by our protocol. In addition, 30 human specimens identified as *Campylobacter* negative at the diagnostic laboratories were examined and 2 (6.7 %) were found to be *C. jejuni* positive by the multiplex protocol. These findings are indicative of the usefulness of this protocol to correctly detect the presence of these *Campylobacter* spp. in stools.

The presence of more than one primer pair in the multiplex PCR tends to increase the chances of non-specific PCR products being amplified resulting in inaccurate results. In this study, use of the multiplex master mix significantly increased protocol performance and eliminated non-specific bands. The multiplex PCR master mix contains pre-optimized concentrations of HotStar *Taq* DNA polymerase with balanced combination of salts and additives, which enables comparable efficiencies for annealing and extension with maximal yield of targeted PCR products. However, the multiplex master mix is about three times more expensive than the regular master mix and contributed to the higher cost of the multiplex assay.

Sensitivity and specificity

Using serial dilutions of DNA extracted from cultures of *C. jejuni* and *C. coli*, the level of bacterial DNA detectable using this protocol was as low as 0.015–0.016 ng μl^{-1} (15–16 ng ml^{-1}), which is equivalent to 10 bacterial cells per PCR tube (Fig. 2). Thus the sensitivity of the protocol for detection of *Campylobacter* DNA directly from stool is very high and quite similar to published data from studies using artificially spiked stool specimens (Persson & Olsen, 2005). This ability to detect very low numbers of *Campylobacter* is of diagnostic importance, particularly in patients with convalescent-phase excretion or in those with post-infectious manifestations such as Guillain-Barré syndrome. To assess the specificity of the protocol, 16 reference strains representing a cross-section of enteric pathogenic and non-pathogenic bacteria were tested. None of these enteric reference strains showed a positive cross-reaction with the multiplex PCR protocol, suggesting that the protocol is highly specific. This is in keeping with other reports, which

Fig. 2. Sensitivity of the multiplex PCR protocol. Lanes 1–4 and 5–8, serially diluted DNA from *C. jejuni* and *C. coli*, respectively. Lanes 3 and 7 show the lowest concentrations of DNA that the multiplex protocol was able to detect from *C. jejuni* and *C. coli*, respectively.

have demonstrated the specificity of *hipO* gene for *C. jejuni* and *asp* gene for *C. coli* when tested using a wide range of enteric and non-enteric micro-organisms (Linton *et al.*, 1997). For *cadF*, although there is a single report of false positivity for this gene in *Enterococcus casseliflavus* and *Pasteurella aerogenes* (Nayak *et al.*, 2005), and *Escherichia coli* (ATCC 43889), the authors reported the specificity of their multiplex protocol as 97 %. In our study, *cadF* was not detected in any of the reference bacterial strains tested including *E. coli* (O157:H7) NCTC 12900. Although *Enterococcus casseliflavus* and *P. aerogenes* were not tested in our study, these bacterial strains have little clinical relevance. It should be stated that we have not tested any other species of *Campylobacter* and there has been no testing on arcobacters, which are closely related to campylobacters. However, their limited clinical relevance and the high specificity of the genes targeted, as shown in the literature, suggest that this is a minor limitation.

Comparison of the multiplex protocol and the hippurate hydrolysis test

Although questions remain about the effectiveness of the hippurate test in correctly differentiating the two *Campylobacter* spp. *C. jejuni* and *C. coli*, it is the only biochemical test used in most diagnostic laboratories. Therefore, 74 *Campylobacter* isolates were tested for hippurate biochemical activity and the results compared with findings of multiplex PCR assays applied on the same specimens. A total of 17 isolates (4 from humans and 13 from chicken) showed false-positive hippurate enzymic activity despite the absence of hippuricase gene in the multiplex PCR products. On the other hand, seven isolates (five from human and two from chicken) showed false-negative hippurate enzymic activity despite the presence of hippuricase gene in the multiplex PCR products.

Specimens showing mixed infection by multiplex PCR were identified as *C. jejuni* according to the biochemical test. In light of these findings, the detection of the hippuricase gene by PCR was used as the gold standard in the evaluation of the performance of the multiplex PCR versus the hippurate test (Table 3).

Although we were able to assess only 74 isolates due to loss of viability of some of the isolates during cryopreservation, the findings are still in agreement with other reports describing a discrepancy between hippurate biochemical and PCR-based speciation, with a majority of false positivity (Rautelin *et al.*, 1999; Nayak *et al.*, 2005). Some *C. jejuni* strains harbour the hippuricase (*hipO*) gene but fail to express the enzymic activity (Rautelin *et al.*, 1999), and as shown in this study, such isolates can only be correctly identified using molecular methods. Interestingly, similar to other reports, *C. coli* isolates without a detectable *hipO* gene by PCR but with a positive hippurate enzyme activity were identified in this study (Nayak *et al.*, 2005). We speculate that the presence of another gene (not *hipO*) encoding hippuricase might explain this phenomenon. Alternatively, another enzyme with similar substrate activity resulting in hippurate hydrolysis might exist in *C. coli*, thus interfering with the results of the hippurate test.

The multiplex PCR protocol we have described has the potential to improve the clinical management and epidemiological tracking of *Campylobacter* enteritis. The procedure had a turnaround time of ~6 h (from DNA extraction to gel electrophoresis) with an estimated cost of US\$3.7 per test. The conventional diagnostic method (inclusive of culture and biochemical tests) requires 2–5 days to obtain a result with an estimated cost of

US\$0.92 per test. The availability of the laboratory results (showing speciation) to the clinician on the same day would, therefore, have an impact on clinical management. Additionally, the application of the protocol for chicken stool specimens makes it a useful tool for investigating the epidemiology of *Campylobacter* colonization in poultry. It also is potentially applicable for *Campylobacter* testing in food, and further studies are needed to investigate this. In outbreaks of campylobacteriosis, the application of the multiplex PCR approach can help to rapidly establish the causative *Campylobacter* species, and because the protocol can be applied to frozen stools, stored specimens can be retrospectively analysed, even after an outbreak, to correctly understand patterns of transmission.

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Table 3. Evaluation of the species identification by hippurate hydrolysis biochemical test versus multiplex PCR

Using the PCR as the gold standard, there were 34 and 9 true positives for *C. jejuni* and *C. coli*, respectively (correctly identified by both test methods). Seventeen strains identified as *C. coli* by PCR were wrongly identified as *C. jejuni* by the hippurate test. Seven *C. jejuni* isolates with hippuricase gene were wrongly identified as *C. coli* by the hippurate test. All specimens with mixed *C. jejuni* and *C. coli* isolates were identified as *C. jejuni* by the hippurate test. The positive predictive value for the hippurate test was 83% and the negative predictive value was 35%.

Multiplex PCR*	Hippurate test*		
	<i>C. jejuni</i>	<i>C. coli</i>	Mixed infection (<i>C. jejuni</i> and <i>C. coli</i>)
<i>C. jejuni</i>	34	7	0
<i>C. coli</i>	17	9	0
Mixed	7	0	0

*n=74 isolates for both tests.

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