



Multiplex PCR for direct identification of *Campylobacter* Species in Human Stool

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ABSTRACT

Background

Campylobacter spp. are a major cause of bacterial gastroenteritis worldwide. Is one of the most frequently isolated bacteria from the feces of infants and children in developing countries, Conventional diagnostic methods for the detection and differentiation of *Campylobacter* species are tedious and time consuming, we have developed a multiplex PCR protocol using a novel combination of species-specific and virulence genes, The multiplex PCR protocol was capable of detecting the type strains and clinical isolates from all five species with a high degree of specificity.

Methods: Molecular testing for *Campylobacter Universal* gene was done on 50 Stool specimens using Multiplex PCR technique. Stool specimens were collected from patients suffering from gastroenteritis, and kept at -20 °C till used. DNA Extraction was done by using Vivantis GF-1 Nucleic acid extraction kit (Vivantis, MALAYSIA). The amplification reaction was carried out in thermo cycler with program system consisting of "(Initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), ending with a final extension at 72°C for 7 min)" PCR products were separated in a 1.5% agarose gel, then stained with ethidium bromide and viewed under gel documentation system. A result was considered positive when a band of the appropriate size was visible in the gel. Standard procedures for reducing contamination were strictly followed.

Results: eight samples (16%) out of 50 were positive by Multiplex PCR, while 42 samples (84%) were negative.

Conclusion: The multiplex PCR is very useful for direct detection and diagnosis of campylobacteriosis.

INTRODUCTION

Campylobacter spp. are a major cause of bacterial gastroenteritis worldwide ⁽¹⁾. Is one of the most frequently isolated bacteria from the feces of infants and children in developing countries, with peak isolation rates in children 2 years of age and younger ⁽²⁾. The spectrum of disease may range from mild, self-limiting, non-inflammatory diarrhea to severe, inflammatory, bloody diarrhoea with pyrexia, abdominal cramps, bacteraemia and faecal leukocytes ⁽³⁾. *Campylobacter jejuni* is now recognized as a leading bacterial cause of food-borne disease in both developed and developing countries ⁽⁴⁾. A notable complication of *C. jejuni* infection is the development of Guillain-Barre' syndrome⁽⁵⁾. 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*. These methods for the detection and differentiation of *Campylobacter* species are tedious time consuming and expensive, usually taking five days to

produce a negative result and up to seven days to confirm a positive result .

In recent years, numerous molecular diagnostic approaches for the detection and identification of *Campylobacter spp* have been developed, including various PCR-based assays ⁽⁶⁾. PCR methods have several advantages because they are faster and more sensitive and specific than the cultivation-based procedures. Several workers have investigated the application of multiplex PCR for *Campylobacter* detection and speciation ⁽⁷⁾. Only few reports have described the direct application of a multiplex protocol on stools obtained from patients with enteritis and both used primers targeting the *ceuE* gene.

In our 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 human, A multiplex PCR was developed and optimized to simultaneous by identify the 23S rRNA from *Campylobacter* ; *spp* the *hipO* gene (hippuricase) from *C. jejuni* subsp. *jejuni*; the *glyA* gene (serine hydroxymethyltransferase) from *C. coli* ; *C. lari*, and *C. upsaliensis*; and the *sapB2* gene (surface layer protein) from *C. fetus* subsp. *fetus*. The multiplex PCR protocol was capable of detecting the type strains and clinical isolates from all five species with a high degree of specificity.

METHODS:

Study design :

This is descriptive cross sectional study was done on patients under 5 years with diarrhea. The study conducted in may to June 2014 at Alfoad Specialized Hospital and Modern Medical Center. Fifty Diarrheal specimens were collected in clean, wide neck, leak proof and preservative free containers.

Ethical approval:

This was obtained from Research Ethical Committee-Sudan Ministry of Health. Verbal consent was taken from children mothers. The participants were informed using simple language about the infection, aim and the benefits of the study.

DATA ANALYSIS:

Collected data were analyzed using the statistical package of social science (SPSS) program. Chi-square statistical analysis was used to determine *P* value significance range.

SAMPLES:

The 50 samples were collected and examined for *Campylobacter spp* DNA by using multiplex PCR. The specimens were stored at -20°C until used.

DNA EXTRACTIONFROM STOOL:

Nucleic acid extraction kit (Vivantis, MALAYSIA).The DNA was extracted according to the manufacturer's instructions. First, Stool samples were collected and extracted in plain container, followed by a lysis buffer and proteinase K. Finally, the DNA was eluted in 175 µl elution buffer provided with the kit .

Primers:

Oligonucleotides, ranging from 18- to 24-mers, were selected from the published DNA sequences of the various *Campylobacter* species (Table 1) using Oligo software (version 3.4). Synthesis of oligonucleotides was carried out at the DNA Core Facility in the National Microbiology Laboratory, Winnipeg, Canada. The six pairs of primers were designed to identify the genes *hipO* from *C. jejuni*; *glyA* from *C. coli*, *C. lari*, and *C. upsaliensis*; *sapB2* from *C. fetus* subsp. *fetus*; and the internal control 23S rRNA ⁽⁸⁾ . The primer sequences used in the multiplex PCR are outlined in Table 1.

Table 1: Primer sequences used in the multiplex PCR assay and the expected sizes of the products

Primer	Size (in bp)	Sequence (5_–3_)	GenBank accession no.	Target gene	Gene location (bp)
CJF	323	ACTTCTTTATTGCTTGCTGC	Z36940	<i>C. jejuni</i> <i>hipO</i>	1662–1681
CJR		GCCACAACAAGTAAAGAAGC			1984–1965
CCF	126	GTAACAACCAAGCTTATCGTG	AF136494	<i>C. coli</i> <i>glyA</i>	337–357
CCR		TCCAGCAATGTGTGCAATG			462–444
CLF	251	TAGAGAGATAGCAAAAGAGA	AF136495	<i>C. lari</i> <i>glyA</i>	318–337
CLR		TACACATAATAATCCCACCC			568–549
CUF	204	AATTGAAACTCTTGCTATCC	AF136496	<i>C.upsaliensis</i> <i>glyA</i>	63–82
CUR		TCATACATTTTACCCGAGCT			266–247
CFF	435	GCAAATATAAATGTAAGCGGAGAG	AF048699	<i>C. fetus</i> <i>sapB2</i>	2509–2532
CFR		TGCAGCGGCCACCTAT			2943–2926
23SF	650	TATACCGTAAGGAGTGCTGGAG	Z29326	<i>C. jejuni</i> 23S Rna	3807–3829
23SR		ATCAATTAACCTTCGAGCACCG			4456–4435

Multiplex PCR protocol:

PCR products were separated in a 1.5% agarose gel, INGeNius. (Figure 1,2). then stained with ethidium bromide and viewed under gel documentation system . A result was considered positive when a band of the appropriate size was visible in the gel.

Each multiplex PCR tube contained 200 μM deoxynucleoside triphosphate; 2.5 μl of 10x reaction buffer (500 mM Tris-HCl [pH 8.3], 100 mM KCl, and 50 mM [NH4]2SO4); 20 mM MgCl2; 0.5 mM *C. jejuni* and *C. lari* primers; 1 mM *C. coli* and *C. fetus* primers, 2 mM *C. upsaliensis* primers; 0.2 mM 23S rRNA primer (Table 1); 1.25 U of FastStart Taq DNA polymerase (Roche

Diagnostics, GmbH, Mannheim, Germany), and 2.5 μl of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 μl. DNA amplification was carried out in a Perkin-Elmer thermocycler using an initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), ending with a final extension at 72°C for 7 min⁽⁸⁾. Primers' specificity was experimentally verified with simple horizontal agarose-gel electrophoresis and melt curve analysis performed with SYBR Green fluorescent dye⁽⁸⁾.

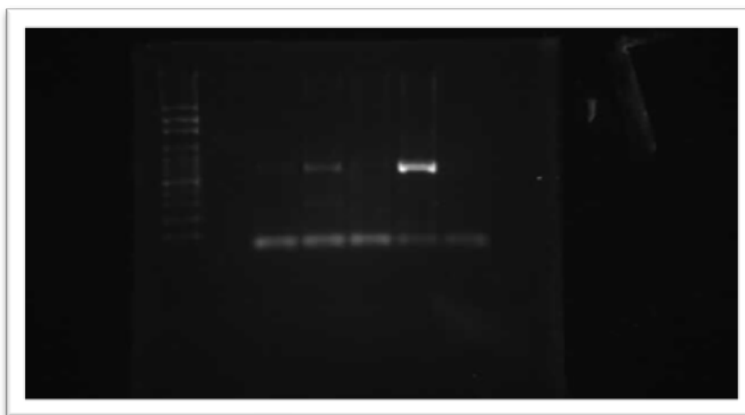


Figure 1: Gel electrophoresis of *Campylobacter Universal gene* DNA PCR product. Lane no. 1 contains 100-bp DNA ladder. indicate for positive Lane no

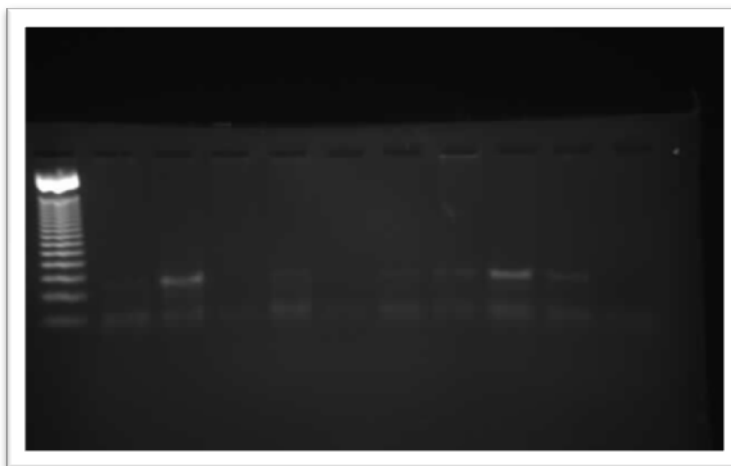


Figure 2: Gel electrophoresis of *Campylobacter jejuni* DNA PCR product. Lane no. 1 contains 100-bp DNA ladder. indicate for positive Lane no

RESULTS :

As shown in table (2) , 8 out of the 50 (16%) examined samples were found positive for *Campylobacter jejuni*.

Table 2: show the results of multiplex pcr

	<i>Campylobacter spp</i>					Total
	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. upsaliensis</i>	<i>C. lari</i>	<i>C. fetus</i>	
Positive	8 (16%)	0	0	0	0	8

Discussion:

For the assessment of the multiplex protocol, DNA extraction was carried out for a total of 50 human stool specimens. The human stool specimens provided by participating centres (Alfoad Specialized Hospitals & Modern Medical Center) were from patients with gastroenteritis, and included both bloody and mucoid specimens. A total of 8 specimens were identified as *Campylobacter* positive, the remaining 42 were *Campylobacter* negative.

Stool represents a heterogeneous specimen consisting of micro-organisms, dietary by-products, bile salts, complex polysaccharides and fat⁽⁹⁾, 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.

We developed a multiplex PCR protocol using a novel combination of genus-specific virulence genes and species specific genes that has already been validated on stool specimens from humans⁽⁹⁾⁽¹¹⁾. (8) positive stool specimens (16%) were detected. Fig. 1 shows a representative electrophoresis gel of samples from a direct application of the multiplex PCR protocol using human stool specimens. Multiplex PCR is particularly attractive as it enables the simultaneous detection and speciation of the micro-organism.

The results obtained in the study weren't near to⁽¹⁰⁾, where the rate of *Campylobacter jejuni* infection was reported as 52%. This variation of results could be attributed to ethnic differences and the small sample size used in our study.

CONCLUSION:

The frequency of *Campylobacter jejuni* among gastroenteritis patients was 16 %, so the multiplex PCR is useful for diagnosis of *Campylobacter* infections directly from stool specimens rather than conational culture techniques.

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