Open Journal of Medical Microbiology, 2014, 4, 29-37
Published Online March 2014 in SciRes. http://www.scirp.org/journal/ojmm
http://dx.doi.org/10.4236/ojmm.2014.41005

Scientific Research

Development and Evaluation of a Multiplex PCR for the Detection of *Campylobacter concisus* and Other *Campylobacter* spp. from Gastroenteritis Cases

Mohsina Huq[1], Gena Gonis[2], Taghrid Istivan[1*]

[1]Biotechnology and Environmental Biology, School of Applied Sciences, RMIT University, Melbourne, Australia
[2]Bacteriology Laboratory, Microbiology, Department of Laboratory Services, The Royal Children's Hospital, Melbourne, Australia
Email: mohsina.huq@rmit.edu.au, gena.gonis@rch.org.au, *taghrid.istivan@rmit.edu.au

Received 12 December 2013; revised 13 January 2014; accepted 20 January 2014

Abstract

We developed and evaluated a multiplex PCR (m-PCR) for application in routine diagnostic laboratories to detect *Campylobacter* spp. in stool samples including *C. concisus*, *C. jejuni*, and *C. coli*. When this m-PCR was applied on spiked faecal samples, *C. concisus*, *C. jejuni*, and *C. coli* were specifically identified at 10^5 cells/gm of faeces. To compare the sensitivity of the m-PCR with conventional culture techniques, the same spiked stool samples were cultured on an antibiotic free Columbia blood agar using the filtration technique. The detection limit of conventional culture method was 10^5 cells/gm of stool for *C. concisus* and 10^6 cells/gm of stool for *C. jejuni* and *C. coli*. The m-PCR was applied to test 127 faecal samples from children with gastroenteritis and the results were compared with the conventional bacterial cultures data. By this m-PCR technique, *C. jejuni* was detected in 7 samples, *C. coli* in 2 samples, and *C. concisus* in 7 samples. However, the conventional culture results for these samples were 6 for *C. jejuni*, 2 for *C. coli* and only one sample was positive for *C. concisus*. In total, 19 samples were positive for *Campylobacter* spp. by m-PCR while only 9 samples were positive for *Campylobacter* spp. by culture. In conclusion, m-PCR is more sensitive than the culture technique to detect *C. concisus* and other fastidious campylobacters in faeces.

Keywords

***Campylobacter concisus*; *Campylobacter* spp.; Gastroenteritis; Faecal Spiking; Multiplex PCR**

*Corresponding author.

How to cite this paper: Huq, M., Gonis, G. and Istivan, T. (2014) Development and Evaluation of a Multiplex PCR for the Detection of *Campylobacter concisus* and Other *Campylobacter* spp. from Gastroenteritis Cases. *Open Journal of Medical Microbiology*, **4**, 29-37. http://dx.doi.org/10.4236/ojmm.2014.41005

1. Introduction

The incidence of campylobacteriosis is gradually increasing, and *Campylobacter* spp. are now considered to be the leading cause of bacterial gastroenteritis worldwide [1] [2]. *Campylobacter* spp. colonise different parts of the human body and may cause many extra-intestinal diseases and severe long-term complications [3]. Although *C. jejuni* and *C. coli* are the most common etiological agents of campylobacteriosis [4], there is evidence suggesting that other *Campylobacter* spp. such as *C. concisus*, *C. upsaliensis*, *C. hyointestinalis*, and *C. fetus* may also cause disease in humans [5]-[7].

Campylobacter spp. are fastidious organisms and require a microaerophilic environment to grow. Most laboratory procedures are optimized to isolate the more common species: *C. jejuni* and *C. coli*. However, the other *Campylobacter* spp. may have additional growth requirements. For example, *C. concisus* is an emerging pathogen which requires hydrogen for growth and is a slow growing organism commonly found in the human oral cavity and was first described in 1981 [8]. It is usually associated with healthy than with diseased periodontal sites and in healthy sites it is usually found in shallow rather than in deeper sites [9]. Past studies have associated this species with gingivitis [10], periodontitis [11], and gastritis since 1989 [12]. More recently, it was isolated from foot ulcers [13], other abscesses from different parts of the body [14] and intestinal biopsies and feacal samples of children with Crohn's disease and patients with inflammatory bowel diseases (IBD) [15] [16]. An early molecular study reported that *C. concisus* produced cytotonic-like effects on CHO cells and induced intracytoplasmic vacuole formation that is similar to *H. pylori* [17]. Also a recent study in the United Kingdom showed a significantly higher incidence of *C. concisus* DNA throughout intestinal biopsies of adults with ulcerative colitis compared to those of healthy controls [18]. *C. concisus* requires a hydrogen enriched atmosphere for growth such as that recommended by the Cape Town Protocol [19]. If optimum growth conditions are not adhered to, it may result in a reduced isolation rate [20]. In a study in Japan, metagenomic analysis detected DNA sequences of the *Campylobacter* spp. genome including *C. concisus*, whilst the standard culture methods were negative [21]. In a recent study in The Netherlands, the detection frequency (DF) of the emerging pathogen *C. concisus* was found to be at least similar to the DF of *C. jejuni* when the species was confirmed by PCR product sequencing [22]. In a more recent study in Chile, a significant difference was reported between conventional culture methods and molecular methods used for the detection of *Campylobacter* spp. and *Arcobacter* spp. in faecal samples of patients with diarrhea and of controls. Emerging pathogens like *C. concisus* and *C. ureolyticus* were only detected by the molecular method used in the study [23].

The true incidence of these *Campylobacter* spp. as causative agents of disease cannot be accurately determined using standard culture techniques. Consequently, there is a demand for a rapid, sensitive and specific method for detecting these organisms. Therefore, the objective of our study was to optimize a four-gene multiplex PCR (m-PCR) for the detection of *C. jejuni*, *C. coli* and *C. concisus* from faecal samples from children with gastroenteritis and to evaluate the PCR results with conventional culture results from the Bacteriology Laboratory at The Royal Children's Hospital (RCH), Melbourne, Australia.

2. Materials and Methods

The study was approved by the Human Research Ethics Committee of RMIT University, Melbourne (Approval number: 15/08) and the Royal Children's Hospital Human Research Ethics Committee (Approval number: CA28021).

2.1. Bacterial Strains and DNA Preparation

A panel of 15 *Campylobacter* spp. strains and a non-Campylobacter strain were included as control strains in the study (**Table 1**). *Campylobacter* spp. strains were grown on Columbia agar base (Oxoid, Australia) supplemented with 5% defibrinated horse blood, and incubated at 35˚C for 3 - 5 days under microaerophilic conditions (6% O_2, 10% CO_2, 10% H2, and balanced N2). For PCR, bacterial DNA was extracted by the Wizard Genomic DNA purification kit (Promega BioSciences, USA) following the manufacturer's instructions.

Cell densities of liquid cultures were estimated by measuring optical density using a spectrophotometer (Eppendorf, Australia) and were also confirmed by viable counts of 10-fold serially diluted cultures (in Brucella broth, Oxoid, Australia) plated on Columbia horse blood agar plates.

Table 1. List of strains used for selectivity testing[a].

Bacterial strains	Origin
Campylobacter spp.	
C. jejuni	NCTC 11168
C. jejuni	NCTC 11828
C. jejuni (n = 4)	Clinical isolate
C. coli	NCTC 11366
C. coli	Clinical isolate
C. concisus	ATCC 51561
C. concisus	ATCC 51562
C. mucosalis	ATCC 43264
C. hyointestinalis	Clinical isolate
C. lari	Clinical isolate
C. upsaliensis	Clinical isolate
C. sputorum subsp. *bubulus*	Clinical isolate
Non-campylobacter	
Escherichia coli	ATCC 25922

[a]ATCC, American Type Culture Collection, Manassas, VA; NCTC, National Collection of Type Cultures, London, United Kingdom.

2.2. Spiking of Faecal Samples

Three blood-free, campylobacter-culture negative faecal samples were selected for the spiking experiment. Liquid campylobacter cultures were added to the faecal samples, resulting in final campylobacter concentrations of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cells/gm of faeces of *C. coli*, *C. jejuni* and *C. concisus*. Faecal DNA was extracted by QIAamp DNA Stool Kit (Qiagen, Australia), according to the manufacturer's instructions. One hundred microliters of the spiked faecal samples were placed on a 0.65 micron filter paper (Sartorius Stedim, Australia) and left to adsorb for 20 minutes then the filter was removed, and the plates were streaked with a loop and incubated in appropriate conditions for 2 - 5 days following The Cape Town Protocol [19]. Any visible growth of campylobacters was identified by biochemical tests including oxidase, catalase, sodium hippurate hydrolysis, indoxyl acetate hydrolysis and light microscopy.

2.3. Collection of Clinical Faecal Samples and m-PCR

To evaluate culture and molecular methods, a total of 140 faecal samples were collected from children with gastroenteritis, aged between 1 month to 18 years at the Royal Children's Hospital (RCH), Melbourne from July to December, 2009. All samples were routinely cultured and identified at the RCH Bacteriology Laboratory on Columbia horse blood agar using the Cape Town Protocol [19] and on blood free Campylobacter media (mCCDA) (Modified Charcoal Cefoperazone Deoxycholate Agar) [24]. Plates were checked on days 3 and 5 for typical small colonies of *Campylobacter* spp. and were identified by biochemical characteristics as described earlier. Samples were also subjected to an in house Rota EIA and cell culture for Adenovirus, both of which were performed at RCH. Then a proportion of each faecal sample was tested by the m-PCR method developed for this study. DNA was extracted from the samples using QIAamp DNA Stool mini kit (Qiagen, Germany) according to the manufacturer's instruction. The extracted DNA was stored at −20˚C until used in the m-PCR to amplify coding regions of three *Campylobacter* spp.; *C. jejuni*, *C. coli* and *C. concisus* and a universal coding region for *Campylobacter* spp. Primers selected for this m-PCR are from the hippuricase gene (*hipO*) of *C. jeju-*

ni [25], the aspartokinase gene of *C. coli* [26], the *gyr*B gene of *C. concisus* [27] and a universal 16S rRNA gene sequence of *Campylobacter* spp. [5] serving as an internal positive control for the PCR (**Table 2**). The primers used in the present study were carefully selected so that the sizes of PCR products, produced by each of the primer sets, are distinguishable on a single gel. The primers' sequences were checked by the Clone Manager suite of analysis tools (Sci Ed Central) to confirm that they do not form primer dimers, hair-pin loops and for cross-reactivity.

The m-PCR system was optimized by serially diluting the *Campylobacter* spp. DNA templates and by gradient temperature cycles using a G-storm thermocycler (Gene works Pty Lt, Australia). All PCR reactions were performed in a total reaction volume of 25 µl containing 1X PCR buffer [10 mM Tris-HCl, 50 mM KCl; pH 8.3], 0.16 mM $MgCl_2$, 0.2 mM dNTP each (Applied Biosystems, Australia), 1.25U recombinant *Taq* Polymerase (Roche Diagnostics, Australia), 0.1 µM of each primer, and 2.5 µl the DNA template. Thermocycler conditions were 94˚C for 2 min, followed by 30 cycles of 94˚C for 15 sec, 54˚C for 30 sec and 68˚C for 2 min, and a final extension of 7 min at 72˚C. PCR products were visualized by UV following electrophoresis on 1.5% agarose gel.

3. Results and Discussion

In our newly developed m-PCR method, PCR products with the expected size of 816 bp, 500 bp, 406 bp and 344 bp were detected for *Campylobacter* spp., *C. coli*, *C. concisus* and *C. jejuni* respectively using a mixture of the four sets of primers (**Table 2**) with DNA samples from the control strains (**Figure 1**). Blood-free campylobacter-negative faecal samples were spiked with 10-fold serial dilutions of our selected three campylobacter strains namely *C. jejuni*, *C. coli* or *C. concisus* cultures resulting in final concentrations of 10^2 - 10^7 cells/gm of, faeces to evaluate the detection limit of the m-PCR method in comparison with conventional culture techniques. Different amounts (40 - 200 ng/µl) of extracted DNA from the spiked faecal samples were tested by the m-PCR method for highest sensitivity and the optimal template volumes were found to be 100 ng/µl per reaction for m-PCR. *C. jejuni*, *C. coli* or *C. concisus* specific primers could produce an amplicon when it had a minimum concentration of 10^5 cells/gm in the spiked faecal sample (**Figure 2**). The detection limit of *C. jejuni* and *C. coli* in a spiked faecal sample was also reported to be at 10^5 cells per ml by Persson *et al.* when the researchers evaluated their developed multiplex PCR method [25].

When the same spiked faecal samples were plated on Columbia horse blood agar plates using the filtration method following the Cape Town Protocol [19] before extracting DNA, the detection limit was 10^5 cells/gm of faeces for *C. concisus* and 10^6 cells/gm of faeces for *C. jejuni*, and *C. coli*. The motility and physical properties such as size and shape of *C. concisus* might be the explanation to be detected at a 10-fold lower concentration than *C. jejuni* and *C. coli*. The use of the filter paper to selectively allow *Campylobacter* spp. to pass through is considerably effective due to the complex bacterial and chemical nature of faeces. However, the the successful isolation from faecal samples depends on the viability of *campylobacter* spp. in the sample during processing. Campylobacters are microaerophilic in nature, and they tend to have a low survival rate if exposed to room temperature and atmospheric air [28]. Long transport time from sample collection to sample analysis reduces the viability of the bacteria in clinical samples. In addition, the progressive decrease in oxygen tension when gas-

Table 2. Primer sets used in m-PCR for *Campylobacter* genus, *C. jejuni*, *C. coli* and *C. concisus*.

Name of Bacteria	Name of primer	Sequence	Tm˚C	Product size (bp)	Target gene	Reference
Campylobacter sp.	C412F	GGATGACACTTTTCGGAGC	51	816	16sRNA	[5]
	C1288R	CATTGTAGCACGTGTGTC	48			
C. jejuni	HipO-F	GACTTCGTGCAGATATGGATGCTT	56	500	Hippuricase	[25]
	HipO-R	GCTATAACTATCCGAAGAAGCCATCA	56			
C. coli	CC18F	GGTATGATTTCTACAAAGCGAG	51	416	Aspartokinase	[26]
	CC519R	ATAAAAGACTATCGTCGCGTG	50			
C. concisus	Pcisus5-F	AGCAGCATCTATATCACGTT	48	344	gyrB	[27]
	Pcisus6-R	CCCGTTTGATAGGCGATAG	51			

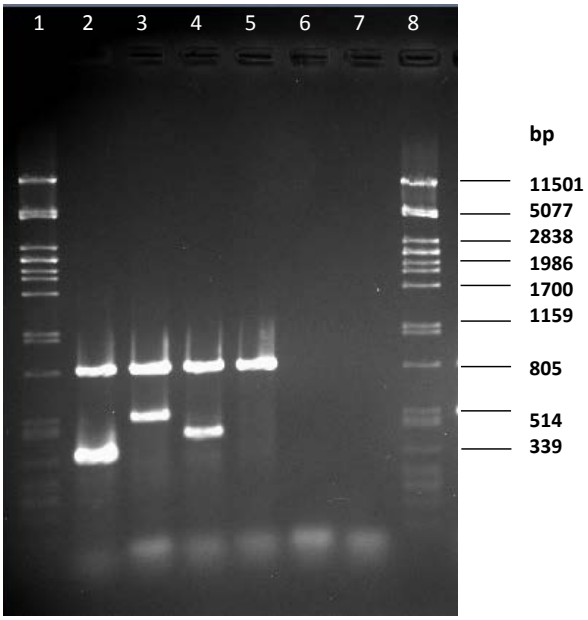

Figure 1. Multiplex PCR for *Campylobacter* spp. Lane 1 and 8: *pst*I digested λDNA marker; lane 2 *C. jejuni* DNA; lane 3 *C. coli* DNA; lane 4 *C. concisus* DNA; lane 5 *C. mucosalis* DNA; lane 6 *E. coli*; lane 7 reagent blank.

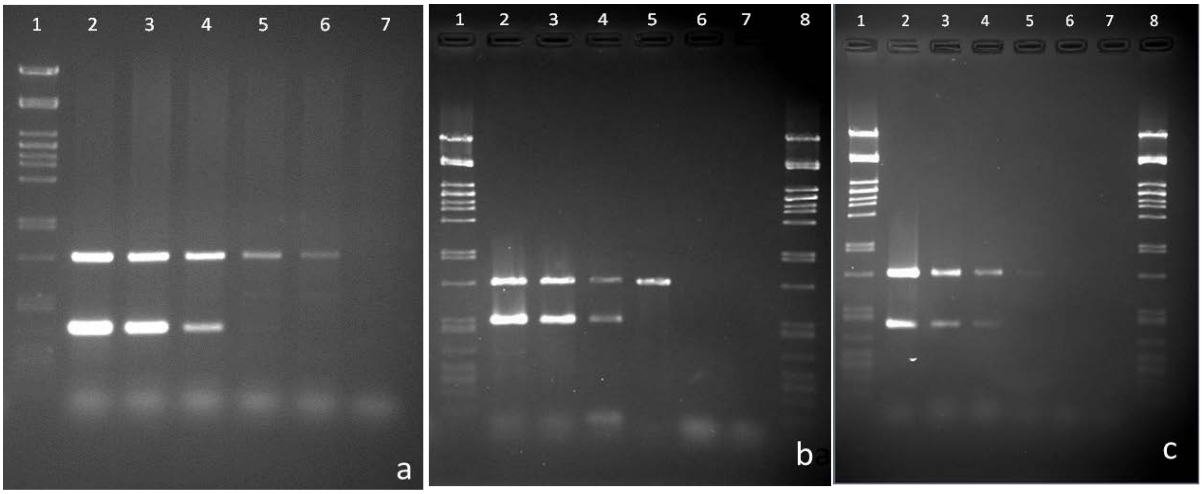

Figure 2. Sensitivity evaluation of the m-PCR method using 10-fold dilutions of bacterial DNA extracted from spiked faecal samples with *C. jejuni* in (a), *C. coli* in (b), and *C. concisus* in (c). Lanes 1&8: *pst*I digested *λ*DNA marker. Bacterial cell concentrations (cells/gm) used: lane 2, 10^7; lane 3, 10^6; lane 4, 10^5; lane 5, 10^4; lane 6, 10^3; lane 7, 10^2.

generating kits are used may not favour adequate growth especially for the stressed bacteria in the sample. Furthermore the use of antibiotic mixtures in common selective media may inhibit the growth of certain *Campylobacter* spp. [29].

To further test the routine diagnostic applicability of our m-PCR, it was compared to the conventional culturing system of 127 faecal samples at the Royal Children Hospital in Melbourne. A total of 140 faecal samples were collected and among these 13 samples were very small in volume and did not contain adequate DNA to run a PCR. Therefore, we excluded these 13 samples from the analysis. Results for positive samples by PCR or culture technique in addition to clinical symptoms and diagnosis are listed in **Table 3**. Nineteen samples were positive for *Campylobacter* spp. By m-PCR while only nine samples were positive for *Campylobacter* spp.

Table 3. Conventional culture and m-PCR results with clinical symptoms of positive faecal samples.

Sample No.	Clinical symptom	Rota EIA[a]/Adeno virus (enzyme immunoassay)	Culture	PCR result			
				Campylobacter spp.	*C. concisus*	*C. coli*	*C. jejuni*
RCH 30	Gastroenteritis	−/−	−	+	−	−	−
RCH 36	Dysentery	−/−	*C. coli*	+	−	+	−
RCH 47	Gastroenteritis	−/−	*C. jejuni*	+	−	−	+
RCH 60	Crohn's disease	ND	*C. concisus*	+	+	−	−
RCH 80	Diarrhoea	+/−	−	+	+	−	−
RCH 85	Recurrent diarrhea Petechial rash on trunk	−/−	*C. coli*	+	−	+	+
RCH 93	Diarrhoae	ND	*C. jejuni*	+	−	−	+
RCH 95	Diarrhoea	−/−	−	+	+	−	−
RCH 98	Bloody diarrhoea, inflammatory bowel disease	−/−	−	+	−	−	−
RCH 102	Diarrhoea	−/−	−	+	+	−	−
RCH 109	Diarrhoea	−/−	−	+	−	−	−
RCH 112	Diarrhoea and vomiting, petechial rash and fever	−/+	−	+	−	−	−
RCH 137	Diarrhoea	−/−	*C. jejuni*	+	−	−	+
RCH 138	Diarrhoea	ND	*C. jejuni*	+	−	−	+
RCH 142	Bloody diarrhoea	+/−	*C. jejuni*	+	−	−	+
RCH 144	Diarrhoea and fever	−/−	*C. jejuni*	+	−	−	+
RCH 147	Bloody diarrhoea	−/−	−	+	+	−	−
RCH 153	Bloody diarrhoea	−/+	−	+	+	−	−
RCH 166[*]	Diarrhoea and vomiting	−/−	−	+	+	−	−

[a]Rota EIA, Rotavirus Enzyme Immunoabsorbent Assay; [*]Giardia cysts were also detected; ND, Not done.

by culture. For *C. jejuni* seven samples were positive by m-PCR while six samples were positive by culture method. It is worth mentioning that *C. jejuni* was isolated in the hospital from three other samples by culture; however these faecal samples did not have adequate DNA when measured by the spectrophotometer, following DNA extraction, and were excluded from the study as mentioned above. For *C. coli*, two samples were positive by both m-PCR and culture methods. Seven samples were positive for *C. concisus* by m-PCR while only one was detected by culture. In the m-PCR, all samples which produced PCR products for *C. jejuni*, *C. coli* and *C. concisus*, also produced a PCR product with the genus specific *Campylobacter* spp. Primers.

By applying the m-PCR in this study we were able to detect a co-infection of *C. jejuni* with *C. coli* in one of the tested samples, while only *C. coli* was detected in this sample by the conventional culture method. This could be due to overgrowth of *C. coli*, or that only one *Campylobacter* spp. representative colony was picked to perform biochemical tests. Co-infections with *Campylobacter* spp. have been reported previously [30] [31] and mixed infection of *Campylobacter* species were previously identified from stool samples by PCR and PCR-enzyme-linked immunosorbent assay [30]. Likewise, co-infections of different subspecies of *C. jejuni* in stool samples were previously identified by Pulsed-field gel electrophoresis and by flagellin gene typing [31]. Linton *et al*. (1997) also reported co-infection with *C. jejuni* and *C. hyointestinalis* by PCR method directly from stool samples [26].

Interestingly in our study *C. concisus* DNA was detected in seven faecal samples while only one was isolated

by the conventional culture method. As *C. concisus* is a fastidious hydrogen-requiring bacterium its isolation is difficult. Yet, only a small amount of its DNA can be detected by the more sensitive PCR method. Culture methods were previously compared to molecular techniques for detection of *C. concisus* and other fastidious campylobacters and were found to be less sensitive, therefore PCR would be the method of choice for the detection of these campylobacters [6] [29]. Furthermore, *Giardia* cysts were found in one of our samples that was positive for *C. concisus* by PCR only. There could be a co-infection of *Giardia* and *C. concisus* leading to gastroenteritis in this patient. *Giardia lamblia* is a prevalent enteric pathogen causing both asymptomatic carriage and diarrheal illness among children worldwide and there are some reports of co-infection of *Campylobacter* spp. with helminths and protozoans [32].

It is worth noting that the faecal sample which was positive for *C. concisus* by both culture and PCR was from a patient previously diagnosed with Crohn's disease. There are reports that *C. concisus* could be related to Crohn's disease in children [33] and adults [34]. Several virulence markers have been previously identified in *C. concisus* such as secreted and cell-associated hemolytic activities and flagellum-mediated attachment [35] [36]. Furthermore, in this study four faecal samples which were negative by culture method produced a *Campylobacter* spp. genus specific PCR product by m-PCR. However, no specific PCR product was detected for any of *C. coli*, *C. jejuni* or *C. concisus*, indicating the presence of another *Campylobacter* spp. Yet, we could not determine the species because specific primers for other *Campylobacter* spp. were not included in the m-PCR, which is intended to be done in the future. This suggests that PCR is more sensitive than conventional culture method for detecting unusual *Campylobacter* spp. [6] [29]. It is also worth mentioning that Adenovirus was detected by conventional culture technique in one of these four samples, but no other infectious agents were detected in the other three samples. Molecular methods for detection of *Campylobacter* spp. are known to be more sensitive than culture [22] [23] [37]. Molecular tests are particularly advantageous for detection of non-cultivable organisms or those difficult to grow by conventional cultures. Detection of these organisms will help to identify them as potential causative agents of disease.

4. Conclusion

In conclusion, the present method offers a fast and robust identification of *Campylobacter* spp. in particular *C. concisus*, *C. coli* and *C. jejuni* present in faecal samples. The use of genus-specific primers also serves as an additional control in this method. Diagnostic PCR on template DNA extracted directly from the primary clinical source offers attractive advantages including reduced identification time and detection of non-viable and non-cultivable bacteria contained in the sample. This m-PCR method with respect to sensitivity and specificity makes it suitable for application in diagnostic laboratories for the detection of *Campylobacter* spp. in clinical specimens.

Acknowledgements

This study was funded by an ECR grant from RMIT University to Dr. Istivan. We thank RMIT University, Microbiology Teaching Unit for providing us the clinical isolates of *Campylobacter* spp. to use as controls. We also thank staff members in Bacteriology Laboratory at The Royal Children's Hospital, Melbourne, for their assistance in sample collection.

Conflict of Interest Statement

None of the authors has any conflicts of interest associated with this study.

References

[1] Anonymous (2012) Monitoring the Incidence and Causes of Diseases Potentially Transmitted by Food in Australia: Annual Report of the OzFoodNet Network, 2010. *Communicable Diseases Intelligence*, **36**, E213-E241.

[2] Eurosurveillance Editorial Team (2012) The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks in 2010. *Euro Surveillance*, **17**, 21. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20113

[3] Man, S.M. (2011) The Clinical Importance of Emerging *Campylobacter* Species. *Nature Reviews Gastroenterology &*

Hepatology, **8**, 669-685. http://dx.doi.org/10.1038/nrgastro.2011.191

[4] Skirrow, M.B. (1994) Diseases Due to *Campylobacter*, *Helicobacter* and Related Bacteria. *Journal of Comparative Pathology*, **111**, 113-149. http://dx.doi.org/10.1016/S0021-9975(05)80046-5

[5] Linton, D., Owen, R.J. and Stanley, J. (1996) Rapid Identification by PCR of the Genus *Campylobacter* and of Five *Campylobacter* Species Enteropathogenic for Man and Animals. *Research in Microbiology*, **147**, 707-718. http://dx.doi.org/10.1016/S0923-2508(97)85118-2

[6] Kulkarni, S.P., Lever, S., Logan, J.M., Lawson, A.J., Stanley, J. and Shafi, M.S. (2002) Detection of *Campylobacter* Species: A Comparison of Culture and Polymerase Chain Reaction Based Methods. *Journal of Clinical Pathology*, **55**, 749-753. http://dx.doi.org/10.1136/jcp.55.10.749

[7] Samie, A., Obi, C.L., Barrett, L.J., Powell, S.M. and Guerrant, R.L. (2007) Prevalence of *Campylobacter* Species, *Helicobacter pylori* and Arcobacter Species in Stool Samples from the Venda Region, Limpopo, South Africa: Studies Using Molecular Diagnostic Methods. *Journal of Infection*, **54**, 558-566. http://dx.doi.org/10.1016/j.jinf.2006.10.047

[8] Tanner, A.C.R., Lai, C.H., Listgarten, M.A., Visconti, R.A. and Socransky, S.S. (1981) *Wolinella* gen. nov., *Wolinella succinogenes* (*Vibrio succinogenes Wolin et al*.) comb. nov., and Description of *Bateroides gracilis* sp.nov., *Wolinella recta* sp. nov., *Camylobacter concisus* sp. nov., and *Eikenella corrodens* from Humans with Periodontal Disease. *International Journal of Systematic Bacteriology*, **31**, 432-445. http://dx.doi.org/10.1099/00207713-31-4-432

[9] Macuch, P.J. and Tanner, A.C. (2000) *Campylobacter* Species in Health, Gingivitis, and Periodontitis. *Journal of Dental Research*, **79**, 785-792. http://dx.doi.org/10.1177/00220345000790021301

[10] Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C. and Kent Jr., R.L. (1998) Microbial Complexes in Subgingival Plaque. *Journal of Clinical Periodontology*, **25**, 134-144. http://dx.doi.org/10.1111/j.1600-051X.1998.tb02419.x

[11] Kamma, J.J., Nakou, M. and Manti, F.A. (1994) Microbiota of Rapidly Progressive Periodontitis Lesions in Association with Clinical Parameters. *Journal of Periodontology*, **65**, 1073-1078. http://dx.doi.org/10.1902/jop.1994.65.11.1073

[12] Vandamme, P., Falsen, E., Pot, B., Hoste, B., Kersters, K. and De Ley, J. (1989) Identification of EF Group 22 Campylobacters from Gastroenteritis Cases as *Campylobacter concisus. Journal of Clinical Microbiology*, **27**, 1775-1781.

[13] Johnson, C.C. and Finegold, S.M. (1987) Uncommonly Encountered, Motile, Anaerobic Gram-Negative Bacilli Associated with Infection. *Reviews of Infectious Diseases*, **9**, 1150-1162. http://dx.doi.org/10.1093/clinids/9.6.1150

[14] De Vries, J.J., Arents, N.L. and Manson, W.L. (2008) *Campylobacter* Species Isolated from Extra-Oro-Intestinal Abscesses: A Report of Four Cases and Literature Review. *European Journal of Clinical Microbiology & Infectious Diseases*, **27**, 1119-1123. http://dx.doi.org/10.1007/s10096-008-0550-2

[15] Zhang, L., Man, S.M., Day, A.S., Leach, S.T., Lemberg, D.A., Dutt, S., Stormon, M., Otley, A., O'Loughlin, E.V., Magoffin, A., Ng, P.H. and Mitchell, H. (2009) Detection and Isolation of *Campylobacter* Species Other than *C. jejuni* from Children with Crohn's Disease. *Journal of Clinical Microbiology*, **47**, 453-455. http://dx.doi.org/10.1128/JCM.01949-08

[16] Kalischuk, L.D. and Inglis, G.D. (2011) Comparative Genotypic and Pathogenic Examination of *Campylobacter concisus* Isolates from Diarrheic and Non-Diarrheic Humans. *BMC Microbiology*, **11**, 53. http://dx.doi.org/10.1186/1471-2180-11-53

[17] Musmanno, R.A., Russi, M., Figura, N., Guglielmetti, P., Zanchi, A., Signori, R. and Rossolini, A. (1998) Unusual Species of Campylobacters Isolated in the Siena Tuscany Area, Italy. *New Microbiology*, **21**, 15-22.

[18] Mukhopadhya, I., Thomson, J.M., Hansen, R., Berry, S.H., El-Omar, E.M. and Hold, G.L. (2011) Detection of *Campylobacter concisus* and Other *Campylobacter* Species in Colonic Biopsies from Adults with Ulcerative Colitis. *PLoS One*, **6**, e21490. http://dx.doi.org/10.1371/journal.pone.0021490

[19] Le Roux, E. and Lastovica, A.J. (1998) The Cape Town Protocol: How to Isolate the Most Campylobacters for Your Dollar, Pound, Franc, Yen, etc. In: Lastovica, A.J., Newell, D.G. and Lastovica, E.E., Eds., *International Workshop on Campylobacter, Helicobacter and Related Organisms*, Institute of Child Heath, Cape Town, 30-33.

[20] Bessede, E., Delcamp, A., Sifre, E., Buissonniere, A. and Megraud, F. (2011) New Methods for Detection of Campylobacters in Stool Samples in Comparison to Culture. *Journal of Clinical Microbiology*, **49**, 941-944. http://dx.doi.org/10.1128/JCM.01489-10

[21] Nakamura, S., Maeda, N., Miron, I.M., Yoh, M., Izutsu, K., Kataoka, C., Honda, T., Yasunaga, T., Nakaya, T., Kawai, J., Hayashizaki, Y., Horii T. and Iida, T. (2008) Metagenomic Diagnosis of Bacterial Infections. *Emerging Infectious Diseases*, **14**, 1784-1786. http://dx.doi.org/10.3201/eid1411.080589

[22] De Boer, R.F., Ott, A., Guren, P., van Zanten, E., van Belkum, A. and Kooistra-Smid, A.M. (2013) Detection of Campylobacter Species and *Arcobacter butzleri* in Stool Samples by Use of Real-Time Multiplex PCR. *Journal of Clinical Microbiology*, **51**, 253-259. http://dx.doi.org/10.1128/JCM.01716-12

[23] Collado, L., Gutierrez, M., Gonzalez, M. and Fernandez, H. (2013) Assessment of the Prevalence and Diversity of

Emergent Campylobacteria in Human Stool Samples Using a Combination of Traditional and Molecular Methods. *Diagnostic Microbiology and Infectious Disease*, **75**, 434-436. http://dx.doi.org/10.1016/j.diagmicrobio.2012.12.006

[24] Lopez, L., Castillo, F.J., Clavel, A. and Rubio, M.C. (1998) Use of a Selective Medium and a Membrane Filter Method for Isolation of *Campylobacter* Species from Spanish Paediatric Patients. *European Journal of Clinical Microbiology & Infectious Diseases*, **17**, 489-492. http://dx.doi.org/10.1007/BF01691131

[25] Persson, S. and Olsen, K.E. (2005) Multiplex PCR for Identification of *Campylobacter coli* and *Campylobacter jejuni* from Pure Cultures and Directly on Stool Samples. *Journal of Medical Microbiology*, **54**, 1043-1047.

[26] Linton, D., Lawson, A.J., Owen, R.J. and Stanley, J. (1997) PCR Detection, Identification to Species Level, and Fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* Direct from Diarrheic Samples. *Journal of Clinical Microbiology*, **35**, 2568-2572.

[27] Matsheka, M.I., Lastovica, A.J. and Elisha, B.G. (2001) Molecular Identification of *Campylobacter concisus*. *Journal of Clinical Microbiology*, **39**, 3684-3689. http://dx.doi.org/10.1128/JCM.39.10.3684-3689.2001

[28] Holler, C., Witthuhn, D. and Janzen-Blunck, B. (1998) Effect of Low Temperatures on Growth, Structure, and Metabolism of *Campylobacter coli* SP10. *Applied and Environmental Microbiology*, **64**, 581-587.

[29] Lawson, A.J., Shafi, M.S., Pathak, K. and Stanley, J. (1998) Detection of Campylobacter in Gastroenteritis: Comparison of Direct PCR Assay of Faecal Samples with Selective Culture. *Epidemiology & Infection*, **121**, 547-553. http://dx.doi.org/10.1017/S0950268898001630

[30] Lawson, A.J., Logan, J.M., O'Neill, L., Desai, G.M. and Stanley, J. (1999) Large-Scale Survey of *Campylobacter* Species in Human Gastroenteritis by PCR and PCR-Enzyme-Linked Immunosorbent Assay. *Journal of Clinical Microbiology*, **37**, 3860-3864.

[31] Richardson, J.F., Frost, J.A., Kramer, J.M., Thwaites, R.T., Bolton, F.J., Wareing, D.R. and Gordon, J.A. (2001) Coinfection with *Campylobacter* Species: An Epidemiological Problem? *Journal of Applied Microbiology*, **91**, 206-211. http://dx.doi.org/10.1046/j.1365-2672.2001.01377.x

[32] Jensen, L.A., Marlin, J.W., Dyck, D.D. and Laubach, H.E. (2009) Prevalence of Multi-Gastrointestinal Infections with Helminth, Protozoan and *Campylobacter* spp. in Guatemalan Children. *The Journal of Infection in Developing Countries*, **3**, 229-234.

[33] Man, S.M., Zhang, L., Day, A.S., Leach, S.T., Lemberg, D.A. and Mitchell, H. (2010) *Campylobacter concisus* and Other *Campylobacter* Species in Children with Newly Diagnosed Crohn's Disease. *Inflammatory Bowel Diseases*, **16**, 1008-1016. http://dx.doi.org/10.1002/ibd.21157

[34] Mahendran, V., Riordan, S.M., Grimm, M.C., Tran, T.A., Major, J., Kaakoush, N.O., Mitchell, H. and Zhang, L. (2011) Prevalence of *Campylobacter* Species in Adult Crohn's Disease and the Preferential Colonization Sites of *Campylobacter* Species in the Human Intestine. *PLoS One*, **6**, e25417. http://dx.doi.org/10.1371/journal.pone.0025417

[35] Istivan, T.S., Smith, S.C., Fry, B.N. and Coloe, P.J. (2008) Characterization of *Campylobacter concisus* Hemolysins. *FEMS Immunology and Medical Microbiology*, **54**, 224-235. http://dx.doi.org/10.1111/j.1574-695X.2008.00467.x

[36] Kaakoush, N.O., Deshpande, N.P., Wilkins, M.R.T., Burgos-Portugal, J.A., Raftery, M.J., Day, A.S., Lemberg, D. A. and Mitchell, H. (2011) The Pathogenic Potential of *Campylobacter concisus* Strains Associated with Chronic Intestinal Diseases. *PLoS One*, **6**, e29045. http://dx.doi.org/10.1371/journal.pone.0029045

[37] Shiramaru, S., Asakura, M., Inoue, H., Nagita, A., Matsuhisa, A. and Yamasaki, S. (2012) A Cytolethal Distending Toxin Gene-Based Multiplex PCR Assay for Detection of *Campylobacter* spp. in Stool Specimens and Comparison with Culture Method. *Journal of Veterinary Medical Science*, **74**, 857-862. http://dx.doi.org/10.1292/jvms.11-0574