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PCR revisited: a case for revalidation of PCR assays for microorganisms using identification of *Campylobacter* species as an exemplar

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Abstract

We re-examined the sensitivity and specificity of 31 PCR assays (including four commercially available and developed in-house methods) for the identification of *Campylobacter* species, with particular reference to taxa described since 2004, which are closely related to *C. jejuni* and *C. coli*, the pathogenic species of most interest. Each of the assays was used by at least one of the participating nine laboratories in eight countries. The sensitivity and specificity of these PCR assays examined varied considerably and ranged from 100% to 0% for sensitivity and 100% to 55% for specificity. None of the three assays examined for *C. lari* were successful in detecting all strains of this species, possibly reflecting its complex taxonomy. A number of assays for *C. jejuni, C. coli*, and a subgroup of enteropathogenic campylobacters, were found to yield false positive results for *Campylobacter* species described since PCR tests were reported, including *C. cuniculorum, C. subantarcticus, C. peloridis* and *C. volucris.* Our study supports the need for attention to detail in initial PCR assay design and evaluation, and also for on-going revalidation of laboratory assays to ensure that diagnoses are correct. Recommendations to guide the revalidation process are presented.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, identification, PCR, revalidation

1. Introduction

The accurate detection and identification of microorganisms is an essential pre-requisite for food assurance and many clinical, ecological and epidemiological studies. Results can enable exports, determine therapeutic regimes, or help to identify the source of a contamination or outbreak event. For many microorganisms, classical taxonomic approaches using phenotypic tests are effective but time-consuming. For others, such approaches are ineffectual. For these reasons, the use of PCR to detect specific sequences of genomic DNA has gained increasing use in microbial diagnostics for the detection and identification of an extensive range of microorganisms (Abubakar *et al.*, 2007; Kehl and Kumar, 2009).

Campylobacteriosis is a leading cause of foodborne disease worldwide. It is the most frequently reported notifiable gastrointestinal illness in New Zealand and, despite reductions in cases in recent years, the reported rate still remains high compared to other developed countries (ESR, 2011) (Figure 1). The *Campylobacter* species *C. jejuni* subspecies *jejuni* (hereafter *C. jejuni*) and *C. coli* are established as frequently isolated bacterial pathogens from human diarrhoea (On *et al.*, 2008). They are widely distributed among animals. Hence their predominant route of human infection is through ingestion of contaminated food, milk and water. The accurate identification of these taxa is important for epidemiological and clinical reasons, especially since a greater proportion of *C. coli* strains are resistant to erythromycin, normally the antibiotic of first choice for treatment of *Campylobacter* infections (On and Jordan, 2003).

The relative biochemical inactivity and complex taxonomy of *Campylobacter* has presented significant challenges to those needing effective and rapid identification methods and has driven many researchers to develop PCR-based assays that are claimed to be sensitive and specific (On, 2005; On and Jordan, 2003). As a result, many PCR assays have been described that aim to detect and identify *C. jejuni, C. coli, Campylobacter* spp., or a group often referred to as the thermophilic campylobacters, namely *C. jejuni, C. coli, C. lari, C. upsaliensis* and *C. helveticus*, all of which share the ability or propensity to grow under microaerobic conditions at an elevated temperature of 42 °C. A robust assessment of assay specificity can be made through testing a comprehensive number of strains that are representative of intra- and infra-specific diversity. It has been shown previously that individual assessments of an assay's specificity and sensitivity have differed markedly, and that care should be taken in designing the PCR assay evaluation regime (Debruyne *et al.*, 2008; On and Jordan, 2003). Moreover, continued taxonomic developments suggest that the specificity of PCR assays should be regularly revalidated. For example, a number of novel *Campylobacter* spp. have been described since 2004 that show a close phylogenetic relationship to the thermophilic campylobacters above, including *C. insulaenigrae, C. volucris, C. peloridis, C. cuniculorum* and *C. subantarcticus* (Debruyne *et al.*, 2009, 2010a,b; Foster *et al.*, 2004; Zanoni *et al.*, 2009), but we have not found any evidence that existing PCR tests for *C. jejuni* or *C. coli* have been revalidated to ensure their taxonomic range remains satisfactory. Anecdotally, we believe that laboratories that establish an assay for detecting a given taxon seldom revalidate the specificity of the assay afterwards. Failure to revalidate a test may, in principle, mean that strains of a closely related, but non-target species, could be giving false positive results in that test.

The EC 6th Framework 'MoniQA' Network of Excellence project focused on harmonisation and standardisation of detection methods for foodborne hazards. In this study, organised and coordinated by the working group of microbiological contaminants, the need for revalidation of PCR tests used routinely for the identification of important *Campylobacter* species was examined.

2. Materials and methods

Bacterial strains and DNA extraction

The strains used are listed in Table 1. Cultures were grown for 48 h in microaerobic conditions. DNA was extracted by suspending bacterial growth in phosphate buffered saline to an optical density of 0.4-0.5 at 600 nm and subsequent application of the DNeasy Blood and Tissue kit (Qiagen QIA69504; Qiagen, Valencia, CA, USA), following

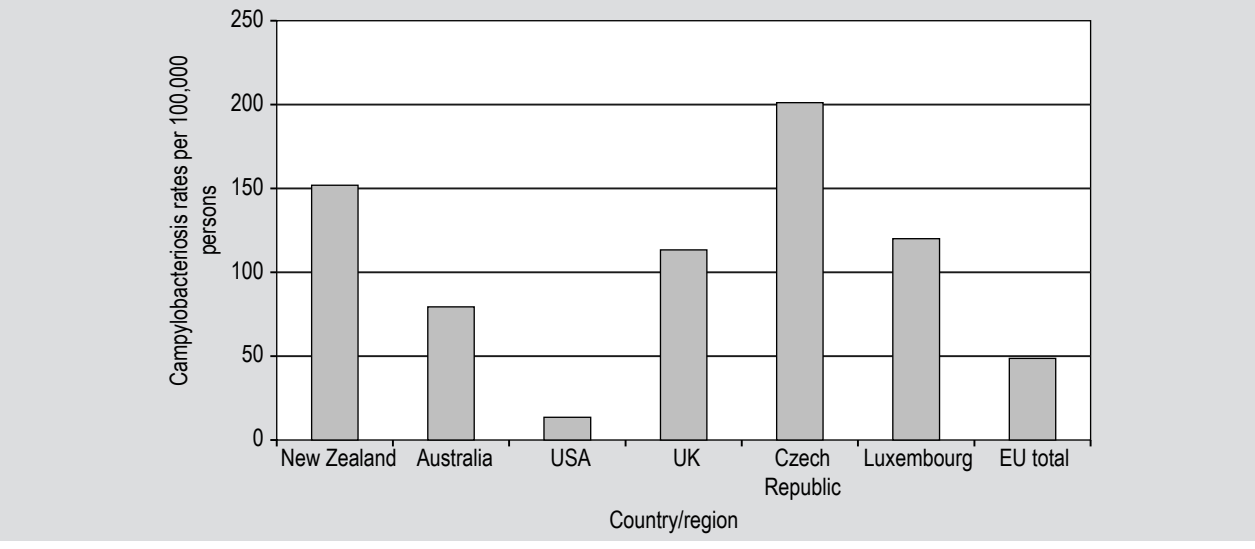

Figure 1. Campylobacteriosis rates per 100,000 population from select countries/regions during the year 2010.

Table 1. Bacterial strains examined.

Campylobacter species	Strain number
C. lari subsp. *lari*	CCUG 23947^T
C. lari subsp. *lari*	CCUG 15035
C. upsaliensis	RM 3195
C. lari subsp. *concheus*	LMG 21009^T
C. lari subsp. *concheus*	LMG 11760
C. lari UPTC	CCUG 20707
C. lari UPTC	CCUG 22396
C. lari UPTC	Lancaster 21/12/OC3
C. insulaenigrae	LMG 22716^T
C. volucris	99H 139 ^{T}
C. volucris	99H 157
C. volucris	99H 161
C. jejuni subsp. *doylei*	CCUG 24567^T
C. coli	RM 2228
C. helveticus	CCUG 34016
C. fetus	CCUG 6823^T
C. canadensis	CCUG 54429^T
C. cuniculorum	CCUG 56289^T
C. subantarticus	CCUG 38513^T
C. subantarticus	CCUG 38507
C. subantarticus	CCUG 38510
C. peloridis	LMG 17564
C. peloridis	LMG 23910^T
C. jejuni subsp. *jejuni*	NCTC 11351^T
C. jejuni subsp. *jejuni*	NCTC 11168

^{T} Type strain.
Abbreviations used: UPTC = urease-positive thermophilic *Campylobacter*; CCUG = Culture Collection, University of Gothenberg, Sweden; RM = Robert Mandrell collection at the US Department of Agriculture, USA; LMG = Laboratorium voor Microbiologie Ghent culture collection, Belgium; NCTC = National Collection of Type Cultures, Lancaster University, UK.

manufacturer's instructions. DNA quality and quantity, from each sample, was assessed spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies Inc., Wilmington, DE, USA). Samples were diluted to 100 ng/µl and 30 µl volumes were aliquoted into 0.5 ml volume Eppendorf vials. All DNA samples were prepared at the Institute of Environmental Science and Research (ESR) from one batch per strain, their identities were validated by comparative 16S rRNA analysis and dispatched to participants in chilled conditions in individually Parafilm-sealed 0.5 µl plastic eppendorf tubes. Samples were sent blinded to avoid investigator bias.

PCR assays examined

Participants volunteered to take part in the study and agreed to use PCR methods routinely employed within their laboratory. Exceptions were assays described for *C. jejuni* and *C. coli* (Eyers *et al.*, 1993; Linton *et al.*, 1996, 1997; Vandamme *et al.*, 1997) that were established at ESR solely for the purpose of this study using methodological parameters described previously (On and Jordan, 2003). A total of 31 different PCR assays were examined, on two separate occasions, including two commercially available assays (manufactured by PrimerDesign, Bulgaria and Bio-Rad, Germany) and two developed in-house (by Gaiker, Spain and CNR-ISPA, France). Details of the genetic targets (where known), original source reference, and species at which the assays were aimed are given in Table 2. Calculation of the sensitivity and specificity of each assay were made using the following formulae, expressed as a percentage value (also presented in Table 2):

- sensitivity = number of true positives / (true positives + false negatives); and
- specificity = number of true negatives / (true negatives + false positives).

Calculations considered the assay specificity for taxa as claimed in the original paper or by individual manufacturers to determine the number of true positives. In the case of multiplex assays, calculations for each assay target were made individually. Details of the PCR primer sequences, amplification and detection conditions as used by each participating laboratory are given in Tables 3-7.

3. Results and discussion

The sensitivity and specificity of the assays examined ranged from 0% to 100% and from 55% to 100%, respectively (Table 2). The poorest result for sensitivity represents results from one laboratory on one occasion for one species (*C. helveticus*) represented by one strain. The positive result from the same laboratory using this test on another occasion resulted in 100% sensitivity, thus indicating a low repeatability of this specific PCR. Moreover, given that the main focus of this study concerned taxa with close phylogenetic relationships with the established human foodborne pathogens *C. jejuni* and *C. coli*, it is not pertinent to comment in detail on the sensitivity and specificity of assays described by Kawasaki *et al.* (2008) directed towards *C. fetus, C. showae, C. hyointestinalis, C. mucosalis, C. curvus, C. concisus* or *C. sputorum*, but it is reassuring to see that no false positive results were observed.

Remarks regarding the *C. helveticus* assay notwithstanding, the sensitivities of several assays were less than complete. In some cases, *C. jejuni* assays failed in detecting strains of *C. jejuni* subsp. *doylei,* a non-thermophilic, genetically distinct variant with no known animal host

Table 2. Summary of results obtained with each of 25 *Campylobacter* strains representing 15 taxa examined with assays established in individual laboratories for various *Campylobacter* spp. The tests used (and their taxonomic range and original description, where relevant) in each laboratory is given. Sensitivity and specificity values for each test were calculated with respect to each assays taxonomic range.

Lab no.	Reference	Assay target	Taxon	Sensitivity (%)	Specificity (%)
1	Eyers *et al.* (1993)	23S rRNA	*C. jejuni*	100	85
	Linton *et al.* (1997)	hipO	*C. jejuni*	100	100
	Van Camp *et al.* (1993)	16S rRNA	*C. jejuni/coli/lari*	100	55
	Vandamme *et al.* (1997), multiplex PCR	Random	*C. jejuni*	66	96
			C. coli	100	100
	Eyers *et al.* (1993)	23S rRNA	*C. coli*	100	63
	Wong *et al.* (2004), multiplex PCR	lpxA	*C. jejuni*	100	100
		ceuE	*C. coli*	100	100
		23S rRNA	Thermophilic *Campylobacter*	100	100
2	Kawasaki *et al.* (2008)	gyrB	*C. jejuni*	66	100
		gyrB	*C. coli*	100	100
		gyrB	*C. lari*	78	86
		gyrB	*C. fetus*	100	100
		gyrB	*C. upsaliensis*	100	100
		gyrB	*C. helveticus*	0 (100[c])	100 (0[c])
		gyrB	*Campylobacter* spp.+[b]	NA	No FPs detected
3	Wang *et al.* (2002)	hipO	*C. jejuni*	100	100
		glyA	*C. coli*	100	89
		sapB2	*C. fetus*	100	100
		glyA	*C. upsaliensis*	100	100
		glyA	*C. lari*	58	100
		23S rRNA	*Campylobacter* spp.	100	100
4	Vandamme *et al.* (1997), multiplex PCR	Random	*C. jejuni*	66	100
		Random	*C. coli*	100	100
	Linton *et al.* (1996)	16S rRNA	*C. lari*	58	100
		16S rRNA	*Campylobacter* spp.	96	100
5	Developed in-house assay (Gaiker)	16S rRNA	Campylobacter spp.	100	100
	Developed in-house assay (Gaiker), RT PCR	hipO	*C. jejuni*	100	100
		ORFA	*C. coli*	100	100
6	Developed in-house assay. Menard *et al.* (2005)	gyrA	*C. jejuni*	100	91
		gyrA	*C. coli*	100	83
7[a]	Stucki *et al.* (1995)	mapA	*C. jejuni*	100	92
	Linton *et al.* (1996)	16S rRNA	*Campylobacter* spp.	100	No FNs
8	Englen and Fedorka-Cray (2002)	hipO	*C. jejuni*	100	100
	Commercial assay (PrimerDesign), RT PCR	cadF	*C. jejuni*	100	100
9	IQ-Check (Bio-Rad)	Unknown (proprietary)	*C.jejuni, C. coli, C. lari*	100	65

[a] All results were derived from the use of DNA at a concentration of 100 ng/µl with the exception of results from lab 7 where a DNA concentration of 50 ng/µl was used throughout.

[b] *Campylobacter* spp. + = *C. showae, C. hyointestinalis, C. mucosalis, C. curvus, C. concisus, C. sputorum*.

[c] Only one set of results (out of two repeats) positive.

and reported infrequently from human disease (Man, 2011). This is in stark contrast with *C. jejuni* subsp. *jejuni* that is thermotolerant, ubiquitously distributed among animals and the most frequent bacterial cause of human gastroenteritis worldwide (On *et al.*, 2008), thus the major target of the PCR tests. Similar results from PCR test evaluations have been described before (Debruyne *et al.*, 2008; On and Jordan, 2003) and for the majority of clinical, food and environmental laboratories, the inability of a PCR test to detect *C. jejuni* subsp. *doylei* will not be alarming.

Table 3. Oligonucleotides used in assays not previously described (where available).

Lab no.	Oligo name	Sequence	Target gene	Product size (bp)	Target *Campylobacter* species	Reference
8		*C. jejuni* commercial kit used for detection (PrimerDesign)	*CadF*			
5	16S1	ATCTAATGGCTTAACCATTAAACT	16SrRNA	856	*C. coli* *C. jejuni*	CCUG11283 CCUG11284
	16S2	GGACGGTAACTAGTTTAGTATT			*C. lari* *C. fetus* *C. upsaliensis*	CCUG23947 CCUG6823 CCUG14913
	HIPO-F HIPO-R HIPO-TM	TGGTGCTAAGGCAATGATAGAAGA TGACCACCTCTTCCAATAACTTCA TETAACTATCCGAAGAAGCCATCATCGCACCTT-BHQ-1	*Gen hipO*	170	*C. jejuni*	CCUG11284
	ORFA-F ORFA-R ORFA-TM	GCACTCATCCAATACTTACAAGA CATTATGGTGTATTCCGCCCA FAMAGTTCCATCTGACGCTGAAGCTACTCAAG-BHQ-1	ORFA-F	105	*C. coli*	CCUG11283
3	CJF CJR	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	*C. jejuni hipO*	323	*C. jejuni*	Wang *et al*. (2002)
	CCF CCR	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGGCAATG	*C. coli glyA*	126	*C. coli*	Wang *et al*. (2002)
	CLF CLR	TAGAGAGATAGCAAAAGAGA TACACATAATAATCCCACCC	*C. lari glyA*	251	*C. lari*	Wang *et al*. (2002)
	CUF CUR	AATTGAAACTCTTGCTATCC TCATACATTTTACCCGAGCT	*C. upsaliensis glyA*	204	*C. upsaliensis*	Wang *et al*. (2002)
	CFF CRR	GCAAATATAAATGTAAGCGGAGAG TGCAGCGGCCCCACCTAT	*C. fetus sapB2*	435	*C. fetus*	Wang *et al*. (2002)
	23SF 23SR	TATACCGGTAAGGAGTGCTGGAG ATCAATTAACCTTCGAGCACCG	*C. jejuni* 23S rRNA	650	*Campylobacter*	Wang et *al*. (2002)
6	F3-*gyrA*-CJ-CC	GTACTTTTGGTGTATTATG	*gyrA*		*C. coli* and *C. jejuni*	Eurofins (Kraainem, Belgium)
	R4-*gyrA*-CJ-CC	TTATCTCTTTTAATTCATCGCG	*gyrA*	443	*C. coli* and *C. jejuni*	Eurofins
	Sensor	Red640-GTTCGTCTGATAATCACTGTTTTTCTATG-p	*gyrA*		*C. coli*, *C. fetus* and *C. jejuni*	Sigma-Aldrich (St. Louis, MO, USA)
	Anchor	GCTCTTGCCTCTTGCTTTTTGAAGTTCAA-F	*gyrA*		*C. coli*, *C. fetus* and *C. jejuni*	Sigma-Aldrich

Of more concern are the results for assays aimed at identification and detection of *C. lari*, a species found in several avian species, cats, and cases of human disease (Debruyne *et al.*, 2009; Man, 2011; Petersen *et al.*, 2007). None of the three assays examined here were successful in detecting all strains included, regardless of the different target gene they used (16S rRNA, *glyA* and *gyrB*). Previous studies have identified this species to be genetically diverse (Debruyne *et al.*, 2009; Duim *et al.*, 2004; Miller *et al.*, 2005) and the separation into two distinct subspecies (*C. lari* subsp. *lari* and *C. lari* subsp. *concheus*) reflects this (Debruyne *et al.*, 2009). Moreover, a number of strains previously described as '*C. lari*-like' have now been reclassified as distinct species (*C. peloridis, C. subantarcticus, C. volucris* and *C. insulaenigrae*) and the close genetic relationship among these taxa likely accounts for the poor specificity observed for one test (Wang *et al.*, 2002). The complex genetics and taxonomy of *C. lari*, and closely related taxa, is likely to present challenges for the development of single PCR assays with appropriate specificity and sensitivity. Nonetheless, their genomic distinctiveness as displayed by other molecular methods (Duim *et al.*, 2004; Miller *et al.*, 2005) suggests that the task is not insurmountable.

As with previous examinations of sensitivity and specificity of PCR assays (Debruyne *et al.*, 2008; On and Jordan, 2003), the performance of tests directed at *C. jejuni* and *C. coli* varied considerably. The poor specificity of the 23S rRNA gene for anything other than clade- or genus level was confirmed here and the performance of the *mapA* based test (Stucki *et al.*, 1995) was similar (92% specific) to that

Table 4. PCR reaction reagents and amounts used.

Lab no.	Reagent	Working concentration	Amount per reaction	Final concentration	Manufacturer[1]
1	***C. jejuni* 23S**				
	0.3	10x	5 µl	1x	2
	dNTP	25 mM each	0.5 µl	250 µM each	7
	$MgCl_2$	25 mM	5 µl	2.5 mM	2
	C. jejuni 23S rRNA forward	13 µM	0.5 µl	130 nM	7
	C. jejuni 23S rRNA reverse 1	13 µM	0.5 µl	130 nM	7
	C. jejuni 23S rRNA reverse 2	13 µM	0.5 µl	130 nM	7
	Amplitaq	5 U/µl	0.1 µl	0.5 U	2
	DNA		1 µl		
	H_2O		36.9 µl		
1	***C. jejuni hipO***				
	PCR buffer	10x	5 µl	1x	2
	dNTP	25 mM each	0.5 µl	250 µM each	7
	$MgCl_2$	25 mM	5 µl	2.5 mM	2
	C. jejuni hipO forward	13 µM	0.5 µl	130 nM	7
	C. jejuni hipO reverse	13 µM	0.5 µl	130 nM	7
	Amplitaq	5 U/µl	0.1 µl	0.5 U	2
	DNA		1 µl		
	H_2O		37.4 µl		
1	***C. jejuni* 16S**				
	PCR buffer	10x	5 µl	1x	2
	dNTP	25 mM each	0.4 µl	200 µM each	7
	$MgCl_2$	25 mM	7 µl	3.5 mM	2
	6-1 forward	10 µM	2.5 µl	500 nM	7
	18-1 reverse	10 µM	2.5 µl	500 nM	7
	Amplitaq	5 U/µl	0.25 µl	1.25 U	2
	DNA		1 µl		
	H_2O		31.35 µl		
1	***C. jejuni* and *C. coli* multiplex (Vandamme *et al.*, 1997)**				
	PCR buffer	10x	5 µl	1x	2
	dNTP	25 mM each	0.5 µl	250 µM each	7
	$MgCl_2$	25 mM	4 µl	2 mM	2
	C. jejuni random (771) forward	13 µM	0.5 µl	130 nM	7
	C. jejuni random (771) reverse	13 µM	0.5 µl	130 nM	7
	C. coli random (364) forward	13 µM	0.5 µl	130 nM	7
	C. coli random (364) reverse	13 µM	0.5 µl	130 nM	7
	Amplitaq	5 U/µl	0.1 µl	0.5 U	2
	DNA		1 µl		
	H_2O		37.4 µl		
1	***C. coli* 23S**				
	PCR buffer	10x	5 µl	1x	2
	dNTP	25 mM each	0.5 µl	250 µM each	7
	$MgCl_2$	25 mM	5 µl	2.5 mM	2
	C. coli 23S rRNA forward	13 µM	0.5 µl	130 nM	7
	C. coli 23S rRNA reverse 1	13 µM	0.5 µl	130 nM	7
	Amplitaq	5 U/µl	0.1 µl	0.5 U	2
	DNA		1 µl		
	H_2O		37.4 µl		

Table 4. Continued.

Lab no.	Reagent	Working concentration	Amount per reaction	Final concentration	Manufacturer[1]
1	***C. jejuni* and *C. coli* (Wong *et al.*, 2004)**				
	PCR buffer	10x	5 µl	1x	2
	dNTP	25 mM each	0.5 µl	250 µM each	7
	$MgCl_2$	25 mM	8 µl	4 mM	2
	Therm 1M forward	0.1 µM	0.5 µl	1 nM	7
	Therm 1M reverse	0.1 µM	0.5 µl	1 nM	7
	LpxA forward	0.1 µM	0.5 µl	1 nM	7
	LpxA reverse	0.1 µM	0.5 µl	1 nM	7
	CeuE forward	0.4 µM	2 µl	160 nM	7
	CeuE reverse	0.4 µM	2 µl	160 nM	
	Amplitaq	5 U/µl	0.25 µl	1.25 U	7
	BSA	2 mg per ml	5 µl	1.25 U	2
	DNA		5 µl		
	H_2O		20.25 µl		
2	**species-specific PCRs for campylobacters (Kawasaki *et al.*, 2008)**				
	Forward primer[2]	10 µM	0.5 µl	0.2 µM	12
	Reverse primer[2]	10 µM	0.5 µl	0.2 µM	12
	Geneamp PCR Gold Buffer	10x	2.5 µl	1x	2
	dNTP	10 mM	0.5 µl	200 µM	2
	Ampli*Taq* Gold	5 U/µl	0.5µl	0.5 unit	2
	DNA		1 µl		
	Deionized sterile H_2O		19.5µl		10
3	Reaction buffer	10x	2.5 µl	1x	5
	dNTP mix	10 mM	0.5 µl	200 µM	5
	$MgCl_2$	25 mM	2 µl	2.0 mM	5
	Primers CJF, CJR	10 µM	1.25 µl	0.5 µM	8
	Primers CCF, CCR	10 µM	2.5 µl	1 µM	8
	Primers CLF, CLR	10 µM	1.25 µl	0.5 µM	8
	Primers CUF, CUR	10 µM	5.0 µl	2 µM	8
	Primers CFF, CFR	10 µM	2.5 µl	1 µM	8
	Primers 23SF, 23SR	10 µM	0.5 µl	0.2 µM	8
	Taq DNA polymerase	5 U	0.25 µl	1.25 U	5
	DNA		2.5 µl		
	H_2O		4.25 µl		
4	***C. jejuni/C. coli* Multiplex PCR**				
	PCR buffer	5x	5.0 µl	1x	9
	dNTP	10 mM	0.75 µl	0.3 mM	7[a]
	$MgCl_2$	25 mM	1.5 µl	1.5 mM	9
	Primers	100 pmol	0.2 µl	20 pmol	7
	Taq DNA polymerase	5 U/µl	0.25 µl	1.25 U	9
	DNA		1 µl		
	H_2O		17.5 µl		12[a]
4	***Campylobacter* genus PCR (only differences with multiplex PCR stated)**				
	dNTP	10 mM	1.25 µl	0.5 mM	7[a]
	$MgCl_2$	25 mM	2.0 µl	2.0 mM	9
	Primer C412F	40900 pmol/tube 1 µg/µl dilution	0.25 µl	42.83 pmol	7
	Primer C1288R	47800 pmol/tube 1 µg/µl dilution	0.25 µl	45.38 pmol	7
	Taq DNA polymerase	5 U/µl	0.2 µl	1.0 U	9
	DNA		1.5 µl		
	H_2O		14.55 µl		12[a]

Table 4. Continued.

Lab no.	Reagent	Working concentration	Amount per reaction	Final concentration	Manufacturer[1]
4	***Campylobacter lari* species PCR (only differences with multiplex PCR stated)**				
	PCR buffer	5x	2.5 µl	0.5x	9
	dNTP	10 mM	0.5 µl	0.5 mM	7[a]
	Primers	100 pmol	0.25 µl	25 pmol	7
	Taq DNA polymerase	5 U/µl	0.2 µl	1.0	9
	DNA		1.0 µl		
	H_2O		18.8 µl		12[a]
5	**Conventional PCR**				
	iQ Sybr Green Super mix	2x	12.5 µl	1x	3
	Primer 16S1	10 µM	0.5 µl	0.2 µM	6
	Primer 16S2	10 µM	0.5 µl	0.2 µM	6
	DNA	100 ng/µl	1 µl	4 ng/µl	
	DNAa	1 ng/µl	1 µl	0.04 ng/µl	
	DNA controls	10 ng/µl	1 µl	0.4 ng/µl	
	Water (molecular grade)		10.5 µl		12
5	**Real-time PCR**				
	QuantiTect Probe mix	2x	10 µl	1x	10
	Primer-F	10 µM	0.5 µl	0.2 µM	6
	Primer-R	10 µM	0.5 µl	0.2 µM	6
	Probe-TM	10 µM	1 µl	0.2 µM	6
	DNA	100 ng/µl	1 µl	5 ng/µl	
	DNAa	1 ng/µl	1 µl	0.05 ng/µl	
	DNA controls	10 ng/µl		0.5 ng/µl	
	Water (molecular grade)				12
6	FastStart DNA hyb probe mix	10x	1 µl	1x	11
	Probes	10 µM	0.2 µl	0.2 µM	12
	$MgCl_2$	25 mM	0.8 µl	3 mM	11
	Forward and reverse primers	10 µM	0.72 µl	0.72 µM	4
	Template DNA		1 µl		
	H_2O		Qsp 10 µl		11
7	*mapA* **–** F	2 pmol/µl	2 µl	0.2 pmol/µl	7
	mapA – R	2 pmol/µl	2 µl	0.2 pmol/µl	7
	Buffer	10x	2 µl	1x	7
	dNTP	2 mM	1 µl	100 µM	7
	$MGCl_2$	50 mM	0.6 µl	1.5 mM	7
	Platinum *Taq*	5 U/µl	0.2 µl	1 unit	7
	DNA		2 µl		
	H_2O		10.2 µl		
7	C412-F	2 pmol/µl	2 µl	0.2 pmol/µl	7
	C1288 – *R*	2 pmol/µl	2 µl	0.2 pmol/µl	7
	Buffer	10x	2 µl	1x	7
	dNTP	2 mM	1 µl	100 µM	7
	$MGCl_2$	50 mM	0.6 µl	1.5mM	7
	Platinum *Taq*	5 U/µl	0.2 µl	1 unit	7
	DNA		1 µl		
	H_2O		10.8 µl		

Table 4. Continued.

Lab no.	Reagent	Working concentration	Amount per reaction	Final concentration	Manufacturer[1]
8	R buffer	10x	2.5 µl		5
	dNTP-100 mM	10 mM	4 x 0.4 µl	1x	5
	$MgCl_2$	25 mM	3 µl	400 µm	5
	hipO forward		1 µl	400 nM	
	hipO reverse		1 µl	400 nM	
	Taq DNA polymerase	5 U/µl	0.3 µl	1.5 U	5
	DNA		1 µl		
	H_2O (DNAse and nuclease free)		14.6 µl		
9	Probes (Proprietary)	5 µl			3
	Amplification mixture (proprietary)	40 µl			3
	Sample template	5 µl			3

[1] 2 = Applied Biosystems (Carlsbad, CA, USA); Bio-Rad (Hercules, CA, USA); 4 = Eurofins (Kraainem, Belgium) 5 = Fermentas (Vilnius, Lithuania); 6 = Thermo Fisher Scientific (Vilnius, Lithuania); 7 = Invitrogenl (Carlsbad, CA, USA); 7a = Invitrogen, 100 mM dNTP set cat. 1029018; 8 = OLIGO (Cascade, CO, USA); 9 = Promega (Fitchburg, WI, USA), GoTaQ Flexi DNA polymerase cat. M8305; 10 = Qiagen (Valencia, CA, USA); 11 = Roche-Diagnostics (Basel, Switzerland); 12 = Sigma-Aldrich (St. Louis, MO, USA); 12a = Sigma, cat. W4502.
[2] For each of 10 individual assays for *C. jejuni, C. coli, C. curvus, C. concisus, C. showae, C. mucosalis, C. fetus, C. hyointestinalis, C. sputorum, C. helveticus, C. upsaliensis, C. lari (cf.* Kawasaki *et al.*, 2008 for details).

described previously (90%, On and Jordan 2003). The current study also confirmed the specificity of the *hipO* gene (Englen and Fedorka-Cray, 2002; Linton *et al.*, 1997; Gaiker, developed in-house) and fragment-based (Vandamme *et al.*, 1997) assays for *C. jejuni* and *C. coli.* A number of other assays (Van Camp *et al.*, 1993; Wang *et al.*, 2002; in-house assay based on Menard *et al.*, 2005; Bio-Rad commercial, unpublished), not previously independently examined to our knowledge, were found to yield false positive results for *Campylobacter* species described since the description of the aforementioned PCR tests, including *C. cuniculorum, C. subantarcticus, C. peloridis* and *C. volucris* (Supplementary Online Material Table 1). In the case of the 'IQ-Check' assay from Bio-Rad, the false positive result with *C. fetus* may be due to the use of this test's reporter probe from the 23S rRNA gene derived from this species (manufacturer's product description). Other PCRs (Kawasaki *et al.*, 2008; Wong *et al.*, 2004; PrimerDesign commercial, unpublished) proved satisfactory with both sensitivity and specificity values found to be optimal in our study.

4. Conclusions and recommendations

As in previous comparative studies (Debruyne *et al.*, 2008; On and Jordan, 2003), our studies indicate that careful attention to detail in PCR assay design and evaluation are necessary to ensure that tests perform with optimal sensitivity and specificity. The use of 'a strain collection that adequately reflects the diversity and taxonomy of the target species to validate PCR assays' (On and Jordan, 2003) seems well justified. That such a strain collection should reflect the contemporaneous, validated taxonomic status of the microbial group in question should be self-evident. Consequently, there is a need to maintain an awareness of taxonomic changes (notably, description of novel closely related taxa) to a group of interest, and to revalidating laboratory assays where prudent, to ensure that diagnoses are correct and that subsequent clinical, epidemiological and/or environmental decisions are appropriate.

Given the focus of the EC 6th Framework MoniQA Network of Excellence project on harmonisation and standardisation of detection methods for foodborne hazards, we suggest the following rationale to laboratories that wish to assure their appropriately validated tests continue to perform to an acceptable degree of accuracy:

- Periodically check (at least annually) for any valid, internationally accepted taxonomic changes to the target genus in question at http://www.bacterio.cict.fr/?CFID=1044459&CFTOKEN=26350761.
- Assess the phylogenetic relationships of any new taxa to those of relevance to the participating laboratory.
- Obtain at least the type strains of taxa with a close phylogenetic relationship to species of interest to the laboratory from a reputable national culture collection (e.g. LMG, CCUG, NCTC, etc.).
- Test the reaction of novel taxa in applicable PCR assays used within the laboratory.

The taxonomy of *Campylobacter* and related organisms (i.e. the class *Epsilonproteobacteria*) has evolved every year since 1988 and now contains over 100 taxa. For this

Table 5. Thermal cycler conditions used by participants.

Lab no. / assay	Denaturation		Amplification cycles					Final extension (temperature/time)	Hold
	Temperature/time	Temperature transition rate	Number	Denaturation (temperature/time)	Annealing (temperature/time)	Extension (temperature/time)	Temperature transition rate		
1 *C. jejuni* 23S rRNA	94 °C/5 min	N/A	27	94 °C/60 s	58 °C/60 s	72 °C/60 s	N/A	72 °C/5 min	4 °C
1 *C. jejuni hipO*	94 °C/5 min	N/A	25	94 °C/60 s	66 °C/60 s	72 °C/60 s	N/A	72 °C/5 min	4 °C
1 *C. jejuni* 16S rRNA	94 °C/5 min	N/A	25	94 °C/60 s	65 °C/60 s	72 °C/30 s	N/A	72 °C/5 min	4 °C
1 *C. jejuni* and *C. coli* multiplex	94 °C/5 min	N/A	10	94 °C/60 s	64 °C/60 s (decrease 1 °C per cycle to 54 °C)	72 °C/60 s	N/A	72 °C/5 min	4 °C
			30	94 °C/60 s	54 °C/60 s	72 °C/60 s		72 °C/5 min	
1 *C. coli* 23S rRNA	94 °C/5 min	N/A	27	94 °C/60 s	54 °C/60 s	72 °C/60 s	N/A	72 °C/5 min	4 °C
1 *C. jejuni* and *C. coli* (ESR)	94 °C/3 min	N/A	40	94 °C/60 s	60 °C/60 s	74 °C/60 s	N/A	72 °C/8 min	4 °C
2 *C. jejuni, C. coli, C. concisus, C. curvus, C. showae, C. fetus, C. helveticus, C. lari*	95 °C/10 min		30	95 °C/20s	69 °C/ s	72 °C/7 s	N/A	72 °C/7 min	
2 *C. mucosalis, C. hyointestinalis, C. upsaliensis*	95 °C/10 min		30	95 °C/20s	68 °C/ s	72 °C/7 s		72 °C/7 min	
2 *C. sputorum*	95 °C/10 min		30	95 °C/20s	65 °C/ s	72 °C/7 s		72 °C/7 min	
3	95 °C/6 min	N/A	30	95 °C/30 s	59 °C/30 s	72 °C/30 s	N/A	72 °C/7 min	N/S
4 *C. jejuni/C. coli* Multiplex PCR	94 °C/5 min	N/A	30	94 °C/60 s, 64 °C/60 s, 72 °C 2x 60 s, 94 °C/60 s, 62 °C/60 s, 72 °C 2x 60 s, 94 °C/60 s, 60 °C/60 s, 72 °C 2x 60 s, 94 °C/60 s, 58 °C/60 s, 72 °C 2x 60 s, 94 °C/60 s, 56 °C/60 s, 72 °C 2x 60 s, 94 °C/60 s, 54 °C/60 s.			N/A	72 °C/10 min	4 °C
4 *Campylobacter* genus PCR	94 °C/60 s	N/A	30	94 °C/15 s	55 °C/15 s	72 °C/30 s	N/A	72 °C/8 min	4 °C
4 *Campylobacter lari* species PCR	94 °C/60 s	N/A	30	94 °C/15 s	62 °C/15 s	72 °C/30 s	N/A	72 °C/8 min	4 °C
5 Conventional PCR	95 °C/3 min	N/A	40	94 °C/30 s	61 °C/90 s	72 °C/60 s	N/A	72 °C/10 min	12 °C
5 Real-time PCR	95 °C/15 min	N/A	40	94 °C/30 s	55 °C/60 s	N/S	N/A	N/S	12 °C
6	95 °C/10 min	20 °C/s	50	95 °C/6 s	54 °C/12 s	75 °C/25 s	20 °C/s[a]	Melting programme: 95 °C/60 s, 38 °C/50 s, 80 °C/0 s (hold) – continuous monitoring of fluorescence	Cooling at 20 °C/ s to 40 °C with a 30 s hold
7 *C. jejuni* (MapA) PCR	95 °C/120 s	N/A	40	94 °C/15 s	60 °C/20 s	72 °C/30 s	N/A	72 °C/2 min	
7 *Campylobacter* genus (Pan) PCR	95 °C/120 s	N/A	40	94 °C/30 s	56 °C/30 s	72 °C/30 s	N/A	72 °C/2 min	
8	95 °C/5 min	N/A	28	95 °C/30 s	66 °C/30 s	72 °C/30 s	N/A	72 °C/4 min	N/S
9	Based on the kit, so automatically set by the manufacturers (Bio-Rad)								

Abbreviations used: N/A = not applicable; N/S = not stated.
[a] Fluorescence measured after each cycle at 640 nm.

Table 6. Detection of conventional PCR products using agarose gel (AGE) or capilliary (CE) electrophoresis.

Lab no.	AGE / CE	% agarose	Agarose manufacturer	Type of buffer (concentration)	Lab made/ purchased	Detection of DNA	Detection equipment	Amount of sample analysed	Molecular weight ladder: type, manufacturer
1	CE	2	SeaKem LE (Madrid, Spain)	TBE (1x)	Laboratory made	Ethidium bromide	Bio-Rad GelDoc (Hercules, CA, USA)	10 µl	1 Kb Plus DNA ladder, Invitrogen (Carlsbad, CA, USA)
2	AGE	2	Sigma (St. Louis, MO, USA)	TAE (1x)	Qiagen	Ethidium bromide	UV transilluminator; Canon camera with Photo Doc-IT imaging system (Tokyo, Japan)	25 µl	GelPilot 50 bp Plus Ladder, Qiagen (Valencia, CA, USA)
3	AGE	1.5	ABO (Gdańsk, Poland)	TAE (1x)	Laboratory made	Midori GreenDNA stain. NIPPON genetics, GmbH	Thermocycler Whatman (Biometra, Kiev, Ukrane) T Gradient. Electrophoresis power supply consort EV 231 (2003)	NS	MassRuler TM DNA Ladder Low range, Fermentas (Vilnius, Lithuania)
4	AGE	1	Invitrogen ultra-pure (Carlsbad, CA, USA)	TBE (1x)	Laboratory made	Ethidium bromide	MicroDoc and Kodak camera (Cleaver Scientific, Rugby, UK). Quantity One software (Bio-Rad)	1 and 1.5 µl per reaction	Ready load 100 bp ladder, Invitrogen (cat. 1-380-012)
5	AGE	2	Biotools agarose (Madrid, Spain)	TAE (1x)	Purchased	Sybr SAFE	Wide Mini-Sub Cell GT System (Bio-Rad). Voltage 75V. Gel analysis with GelDoc XR (Bio-Rad)	20 µl	GelPilot 100 bp Plus Ladder (100), Qiagen
7	AGE	1	Axygen Scientific Agarose LE (Union City, CA, USA)	TBE (0.5x)	Purchased	Ethidium bromide	GelDoc, Quantity One software (Bio-Rad)	3 µl	1 Kb Plus DNA Ladder, Invitrogen (Carlsbad, CA, USA)
8	AGE	1	Duchefa Biochemie B.V. Agarose SPI (Haarlem, Netherlands)	TBE (1x)	Purchased	SyberGreen	MiniBis Pro, bioimaging systems (DNR, Jerusalem, Israel)	10 µl	GelPilot 1 kb Plus Ladder, Qiagen

Table 7. Detection of PCR products using real-time PCR.

Lab no	Real-time thermal cycler model	Real-time thermal cycler manufacturer	Filters/ channels used for detection/ analysis	Software	Calibration details	Colour compensation required (Yes/ No)	Qualitative or quantitative analysis	If qualitative, describe minimum criteria for samples to be considered positive				
3	CFX96TM	Bio-Rad (Hercules, CA, USA)	ROX, Cy5, FAM, HEX	CFX manager version 1.6.541.1028	Factory calibrated (2008)	No	Qualitative	**Thermotolerant *Campylobacter* sp. Channel FAM**	**Internal control channel VICHEX**	***C. jejnui* Channel ROX**	***C. coli* Channel Cy5**	**Result interpretation**
								+ve	+ve or -ve	-ve	-ve	+ve
								+ve	+ve or -ve	+ve	-ve	+ve (*C. jejuni*)
								+ve	+ve or -ve	-ve	+ve	+ve (*C. coli*)
								+ve	+ve or -ve	+ve	+ve	+ve (*C. jejuni* or *C. coli*)
								-ve	+ve	-ve	-ve	-ve
								-ve	-ve	-ve	-ve	invalid
5	iQ5 Multicolour Real-time PCR detection system		Excitation – 475-645 nm range: emission – 515 – 700 nm range. Fluophores: FAM (495-520) and TET (521-536)	iQ5 Optical system	Annually by an external agent. Test performed against a standardised temperature protocol and the MTAS thermal cycler validation procedure	No	Qualitative	CT value				
6	LightCycler 1.5	Roche Applied Science (Penzberg, Germany)	F1 and F2	LightCycler3 Run version 5.32	No calibration	No	Qualitative	CT <35. Melting curve for *C. jejuni*: TM = 48.8 °C				
8	ABI 7300	Applied Biosystems (ABS)(Carlsbad, CA, USA)	N/S	SDS V 3.1	Background, ROI and pure dye calibration (Real time PCR systems spectral calibration kit); TaqMan RN-ase P96 well Instrument verification plate (ABS)	No	Qualitative	Dynamic range of test less than 100 copies of target template. All positive-appearing samples coming out later than the lowest standard (100 copies per µl) are considered 'under the limit of detection' (ULD) of the test.				
9	MiniOpticon MJ	Bio-Rad	N/S	Opticon Monitor v. 3.2	Calibrated and maintained by Bio-Rad agents (2011)	No	Qualitative	CT ≥10				

Abbreviations used: +ve = positive; -ve = negative; CT = cycle threshold; TM = melting temperature.

and other reasons (On, 2005), the accurate identification of strains has proven especially challenging. However, as publications in journals such as the International Journal of Systematic and Evolutionary Microbiology demonstrate (in which hundreds of new taxa are described every year), *Campylobacter* is far from the only group in which change occurs. With the increasing reliance of laboratories on PCR assay results for microbial identifications, it is hoped that the recommendations above from MoniQA project partners will prove useful to laboratories and their clients and stakeholders, who rely upon accurate results for effective and appropriate actions.

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