

# Development and evaluation of internal amplification controls for use in a real-time duplex PCR assay for detection of *Campylobacter coli* and *Campylobacter jejuni*

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A common problem of both conventional and real-time PCR assays is failure of DNA amplification due to the presence of inhibitory substances in samples. In view of this, our aim was to develop and evaluate internal amplification controls (IACs) for use with an existing duplex real-time PCR assay for *Campylobacter coli* and *Campylobacter jejuni*. Both competitive and non-competitive IACs were developed and evaluated. The competitive approach involved a DNA fragment of the coding region of the fish viral haemorrhagic septicaemia virus, flanked by the *mapA* PCR primers, whilst the non-competitive approach utilized an extra set of universal 16S rDNA primers. Both IAC-PCR assay types were evaluated using cultures of *Campylobacter* and chicken caecal content samples. Both IACs were sensitive to caecal inhibitors, making them suitable for detecting inhibition which could lead to false-negatives. Results showed that both IACs at optimum concentrations worked well without reducing the overall sensitivity of the PCR assay. Compared to culture, the optimized competitive IAC-PCR assay detected 45/47 positives (sensitivity 93.6 %, specificity 80.1 %); however, it had the advantage over culture in that it could detect mixed infections of *C. coli* and *C. jejuni* and was capable of giving a result for a sample within a day.

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## INTRODUCTION

*Campylobacter* enteritis is the most common cause of acute bacterial diarrhoea worldwide (Linton *et al.*, 1997; Keramas *et al.*, 2004) and poultry and poultry products are considered important sources of human campylobacteriosis (Genigeorgis *et al.*, 1986; Harris *et al.*, 1986; Deming *et al.*, 1987; Evans, 1992; Keramas *et al.*, 2004).

Isolation and identification of *Campylobacter* by conventional culture methods is slow (Keramas *et al.*, 2004) and there is a need for more rapid techniques. There are existing PCR assay protocols for detection of *Campylobacter* in water (Kirk & Rowe, 1994), in some dairy products (Wegmuller *et al.*, 1993), in meat (Giesendorf *et al.*, 1992) and in poultry (Lund *et al.*, 2003), and in human faeces (Linton *et al.*, 1997; Lawson *et al.*, 1999), as well as many papers that describe PCR assay identification of *Campylobacter* from pure culture (Oyofe *et al.*, 1992; Comi *et al.*, 1996; Best *et al.*, 2003). Similarly, there are multiplex PCR assays described for the detection of *Campylobacter coli* and *Campylobacter jejuni* (Denis *et al.*,

1999; Best *et al.*, 2003) and for the detection of *C. coli*, *Campylobacter fetus* subsp. *fetus*, *C. jejuni*, *Campylobacter lari* and *Campylobacter upsaliensis* (Wang *et al.*, 2002).

One of the limitations to the widespread use of PCR is that PCR assays are usually run without internal amplification controls (IACs). Whilst external positive and negative controls may give some assurance that the PCR assay is effective, they do not give assurance of an individual negative result. A negative result may be a true-negative result, or a false-negative due to the presence of inhibitory substances within the sample (Hoorfar *et al.*, 2004). Faecal or caecal samples in particular can contain substances that are inhibitory to PCR assays (Lund & Madsen, 2006). For a PCR assay to be validated through a multicentre collaborative trial, it must contain an IAC (Lund & Madsen, 2006), although the inclusion of IACs in all PCR assays would give assurance of reliable results.

There have been many studies that have looked into the development and use of IACs in PCR assays, as recently reviewed by Hoorfar *et al.* (2004). One such study focused in particular on an IAC for conventional and real-time PCR assay for detection of *Campylobacter* species in

**Abbreviations:** FV-IAC, fish haemorrhagic virus internal amplification control; IAC, internal amplification control.

chicken faecal samples (Lund & Madsen, 2006). However, we were not aware of any study that evaluated different types of IACs in a real-time duplex PCR assay for *C. coli* and *C. jejuni*.

In view of the need to identify the two most important *Campylobacter* species (*C. coli* and *C. jejuni*) rapidly from caecal or faecal samples, in this study we used an existing real-time PCR assay for identifying *C. coli* and *C. jejuni* (Best *et al.*, 2003), but modified it by incorporation of one of two different IACs. We evaluated an existing in-house non-competitive 16S rDNA IAC which incorporated a set of universal 16S rDNA primers and also designed and evaluated a competitive IAC based on a haemorrhagic fish virus sequence flanked by one of the existing primer sets (Best *et al.*, 2003). Both IACs were evaluated with cultures, with spiked caecal samples, with field caecal samples and with PCR assay inhibitor derived from caecal contents.

## METHODS

**Bacterial strains.** *Campylobacter* strains were grown as previously described (Best *et al.*, 2003). The following cultures obtained from the National Collection of Type Cultures (NCTC) were used as positive and negative control strains for PCR optimization: *C. coli* NCTC 12143; *C. jejuni* NCTC 11351<sup>T</sup>; *C. lari* NCTC 11352<sup>T</sup>; *C. fetus* subsp. *fetus* NCTC 10842<sup>T</sup>; *Campylobacter hyointestinalis* NCTC 11608<sup>T</sup>; *Campylobacter sputorum* subsp. *bubulus* NCTC 11367<sup>T</sup>; and *Arcobacter cryaerophilus* NCTC 11885<sup>T</sup>. Crude cell lysates were used to provide the DNA from pure cultures for PCR assay. In brief, crude DNA lysates were prepared by taking growth (one 10 µl loopful) from a non-selective agar plate into 700 µl sterile distilled water in a sterile Eppendorf tube and vortexing to give a suspension. This suspension was placed in a boiling water bath for 10 min, centrifuged for 3 min at 16 200 g and the supernatant was stored at 4 °C (for up to 1 month) prior to use.

**PCR primers and conditions, probe and IAC sequences.** The primer and probe sequences for the *ceuE* gene (which encodes a lipoprotein) of *C. coli* and the *mapA* gene (which encodes an outer-membrane protein) of *C. jejuni* have been described previously (Best *et al.*, 2003). The duplex real-time PCR assay conditions were as described by Best *et al.* (2003) with the following exceptions. Each PCR assay reaction was 25 µl and comprised 12.5 µl Qiagen HotStarTaq PCR assay Master Mix (cat. no. 203445), 3.5 µl 25 mM MgCl<sub>2</sub> solution, 0.5 µl each primer at 20 µM, 0.5 µl each probe at 5 µM, and nuclease-free water to take the volume to 23 µl. Template was 2 µl to make the final volume to 25 µl.

The non-competitive 16S rDNA IAC-PCR assay included an extra set of universal 16S rDNA primers with the sequences 16S-F 5'-ACTACGTGCCAGCAGCC-3' and 16S-R 5'-GGACTACCAGGGTA-TCTAATCC-3', and these were added to the PCR assay Master Mix at the same concentration as the other primers. The non-competitive 16S rDNA IAC-PCR assay Master Mix also included a ROX probe specific for the 16S rDNA amplicon and the sequence of this probe was 5'-ROX-TGTTTGCTCCCCACGCTTTCGCA-BHQ2-3'. For the 16S rDNA IAC-PCR assay, the amount of water in each PCR assay reaction was reduced to allow for addition of extra primers and probe.

The competitive fish haemorrhagic virus IAC (FV-IAC) comprised a 60 bp sequence from the fish haemorrhagic virus sequence (accession no. X66134) flanked by the *mapA* gene PCR primers. The complete

sequence was as follows, with the fish virus sequence in bold underline: 5'-CTGGTGGTTTTGAAGCAAAGATTAGGTCAACTC-AGGTGTCCTCATGAATTTGAAGACATAAACAAGGGACTGGTC-TTATTTATATAAACGCACCTTTAGACACTGGTATTG-3'. The FV-IAC PCR assay Master Mix also included a ROX probe specific for the fish virus sequence amplicon and the sequence of this probe was 5'-ROX-TGA[+G]GA[+C]AC[+C]TGAG[+T]TGACCT-BHQ2-3', where + denotes locked nucleic acids. For the FV-IAC PCR assay, the amount of water in each PCR assay reaction was reduced to allow for addition of the IAC and the probe.

All primers and probes and the FV-IAC were obtained from Sigma-Aldrich. All samples were analysed using a Stratagene MX3000P machine. Cycling conditions were one cycle of 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Fluorescence was read at the 60 °C position.

**Caecal samples and PCR assay inhibitor.** Chicken caeca were collected from broiler flocks at time of slaughter as part of a 3 year national prevalence survey of *Campylobacter* in broilers in the UK. A fresh pooled caecal sample for each flock was produced by suspending 0.02 g caecal contents from each of 10 individual birds in 2 ml 0.1 M PBS (pH 7.2). The campylobacter status of these flocks had been determined by the culture methods specified by the EU (ISO, 2006).

To determine the limit of detection of the PCR assay with and without an IAC, a total of 16 spiked caecal samples were prepared from fresh pooled caecal contents from two flocks. Eighteen further naturally infected caecal samples were also used to compare the PCR assay with and without different IACs. Finally, the chosen PCR-IAC method was assessed on a further 52 pooled caecal samples and the results were compared to those of culture.

DNA from both naturally infected and spiked caecal samples was extracted using an ExtractMaster Fecal DNA Extraction kit (Epicentre Biotechnologies), with 0.25 ml of the caecal contents suspended in 0.75 ml PBS and then DNA extracted according to the manufacturer's instructions. Extraction controls comprising caecal samples known to be positive for *Campylobacter* were run with each batch of DNA extracts from caecal contents.

DNA from both crude culture lysates and caecal extractions was quantified for a representative number of samples using a NanoDrop 1000 (Thermo Fisher Scientific).

Caecal PCR assay inhibitor solution was made by suspending caecal contents in distilled water (50:50, w/v), mixing, centrifuging in a microcentrifuge for 3 min at 13 000 r.p.m. and then storing the supernatant (Caecal PCR assay inhibitor solution) at -20 °C until required for use. In experiments that included Caecal PCR assay inhibitor solution, this was added at 1 µl per reaction and the volume of water in each reaction was reduced commensurately.

**Spiking caecal samples.** Caecal samples from flocks found to be free from *Campylobacter* by culture were spiked with strains *C. jejuni* NCTC 11351<sup>T</sup> and *C. coli* NCTC 12143. Strains were grown from frozen (-80 °C) stocks in 10 % glycerol broth (Oxoid) on 7 % sheep blood agar with 0.1 % actidione (Oxoid) and pure culture was harvested into PBS to McFarland standard 4 to give ~log<sub>10</sub> 9 c.f.u. ml<sup>-1</sup>. *C. coli* and *C. jejuni* in PBS were then mixed 1:1 to give ~log<sub>10</sub> 8 c.f.u. ml<sup>-1</sup> of each, and further diluted to give ~log<sub>10</sub> 6, 4, 2 c.f.u. ml<sup>-1</sup> of each *Campylobacter* strain. Respective dilutions (0.1 ml) were then added to duplicate 0.9 g caecal content sample to give samples spiked with ~log<sub>10</sub> 7, 5, 3 and 1 c.f.u. *Campylobacter* (g caecal sample)<sup>-1</sup>. Duplicate sets of spiked caecal samples were prepared on two different occasions and the DNA was extracted from them, so in all four replicates of spiked samples and four different dilutions were tested, a total of 16 spiked samples.

**Enumeration of *Campylobacter* in spiked caecal samples.** To enumerate the number of *Campylobacter* in the spiked caecal samples, the original PBS suspensions used to create the spiked caecal samples were serially diluted in PBS until a  $10^{-9}$  dilution was created and then 100  $\mu$ l of each dilution was plated onto duplicate modified Charcoal Cefoperazone Deoxycholate agar (Oxoid) plates. Plates were incubated at  $41.5 \pm 1$  °C in a microaerobic atmosphere (84% N<sub>2</sub>; 10% CO<sub>2</sub>; 6% O<sub>2</sub>) for 48 h and the numbers of typical *Campylobacter* colonies were counted. The most dilute pair of plates that contained a mean of between 15 and 200 typical colonies was used to calculate the number of *Campylobacter* c.f.u. (g caecal content)<sup>-1</sup>. A colony from each plate used for calculation was purified and confirmed as *Campylobacter* sp. using the methods previously described (ISO, 2006).

**Optimization of the PCR assay.** IACs are known to have the capacity to reduce the sensitivity of PCR assays, particularly for competitive IACs (Hoorfar *et al.*, 2004). In order to minimize the competitive effect of the FV-IAC, it was tested at dilutions of  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  (from 200 down to 2 pmol) and the strength of the *C. coli* and *C. jejuni* signals was compared to the PCR assay with no IAC. In a similar way, for the non-competitive IAC, the 16S rDNA primers were tested at a standard concentration, as well as at 1/2, 1/4 and 1/10 standard concentrations. This initial optimization was performed using cell lysate preparations derived from the positive and negative control cultures.

**Evaluation of PCR assays.** Once the different PCR assays were optimized for use with pure culture, they were evaluated with *C. coli* and *C. jejuni* cell lysates at doubling dilutions from neat to 1/1024, spiked ( $n=16$ ) and natural ( $n=18$ ) chicken caecal DNA extracts and with 'Caecal PCR assay inhibitor solution'. The PCR assay type that overall gave the most sensitive and specific results was then used to test a further 52 natural chicken caecal DNA extracts and the results were compared to those obtained by culture.

The effect of Caecal PCR assay inhibitor solution for inhibiting the IAC components as well as the *C. coli* and *C. jejuni* components of the PCR assays was determined for 1  $\mu$ l Caecal PCR assay inhibitor solution added to each 25  $\mu$ l PCR assay reaction. The Caecal PCR assay inhibitor solution was tested neat, and at doubling dilutions ranging down to 1/2048.

**Testing natural caecal samples for the presence of *Campylobacter*.** For 52 natural caecal samples, detection of *Campylobacter* by PCR assay was compared to detection by culture. The *Campylobacter* status of these samples was determined by the culture methods specified by the EU baseline survey for monitoring of *Campylobacter* prevalence in broiler flocks (ISO, 2006).

**Adjusting C<sub>t</sub> values to relative values.** The output from the Stratagene PCR assay machine is such that the higher the C<sub>t</sub> value (threshold cycle), the weaker the signal. For PCR assay of cultures, samples with C<sub>t</sub> values above 32 are considered negative (Best *et al.*, 2003). To make graphics more meaningful (e.g. so stronger signals appeared as higher values rather than lower values), C<sub>t</sub> values were adjusted to relative C<sub>t</sub> values so that higher values would correlate to stronger signals. This adjustment was made using the formula  $(40/C_t \text{ value}) - 1$ , where 40 is the highest possible C<sub>t</sub> attainable.

**Calculation of sensitivity and specificity and statistical analysis.** Sensitivity for PCR assay was calculated as the number of test (PCR assay) positives divided by the number of 'gold standard' (culture) positive strains multiplied by 100. Specificity for PCR assay was calculated as the number of test (PCR assay) negatives divided by the number of 'gold standard' (culture) negatives multiplied by 100.

PCR assay was capable of detecting both *C. coli* and *C. jejuni* at the same time, whereas by culture methods only one organism was

detected based on one colony picked and identified. As such, it was not really possible to compare the sensitivity and specificity of PCR assay compared to culture for individual samples. The value for the sensitivity and sensitivity calculations therefore was determined on the basis of a positive or negative result for both or either species by either method.

To determine whether the different PCR assays gave comparable results, the *t*-test function of Microsoft Excel was used for statistical comparisons of C<sub>t</sub> values for both FAM and HEX for different PCR assays of the same samples. FAM and HEX are the reporter dyes used on the probes for detection of *C. jejuni* and *C. coli* (Best *et al.*, 2003). These dyes give rise to fluorescent signals for detection of either or both organisms.

PCR assays with no IAC, with the 16S rDNA IAC and with the FV-IAC were compared for DNA extracts from 18 naturally infected caecal samples.

## RESULTS AND DISCUSSION

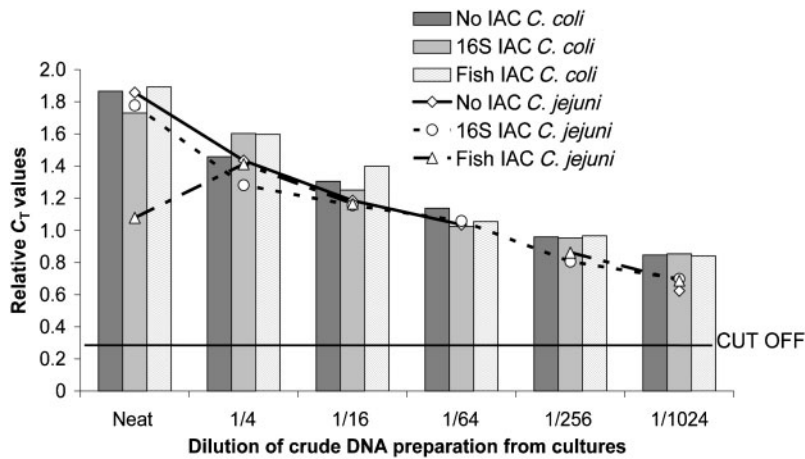
### Initial optimization of IACs

When the non-competitive 16S rDNA IAC-PCR assay was used for pure culture lysates, there was no signal if the 16S rDNA PCR primers were used at less than 1/2 concentration, and the 16S rDNA IAC signal strength was markedly reduced at 1/2 concentration, so these primers were used at previously stated concentrations only. The 16S rDNA IAC signals were generally lost in the presence of signals (unless C<sub>t</sub> values were very high indicating weak signal) for either the *C. coli* or *C. jejuni* components of the PCR assay.

The FV-IAC caused inhibition of the PCR assay at dilutions of  $10^{-5}$  and above (results not shown). At  $10^{-6}$  dilution (20 pmol IAC), there was slight inhibition of weak signals (e.g. where C<sub>t</sub> values were high; results not shown), but at  $10^{-7}$  dilution (2 pmol IAC), there was no inhibition seen for either dilutions of cell lysates from cultures (Fig. 1) or for DNA extracts from 18 natural caecal samples (results not shown). A typical output for a sample positive for *Campylobacter* and for the competitive FV-IAC is shown in Fig. 2.

The effect of different dilutions of the Caecal PCR assay inhibitor solution on the signal from *C. coli* DNA is shown in Fig. 3. The Caecal PCR assay inhibitor solution was shown to be more potent at inhibiting the IAC signals for both the 16S rDNA IAC and the FV-IAC than the signals from the *C. coli* or *C. jejuni* components of the PCR assay.

Our work was based on a duplex real-time PCR assay that had previously been carefully evaluated with a panel of cell lysates from 60 *C. coli* and *C. jejuni* strains, from negative control strains and from a further 5877 clinical isolates (Best *et al.*, 2003). In view of the initial thorough nature of the PCR assay evaluation, our evaluation was on a smaller scale, but did show that both the competitive and non-competitive IACs worked without reducing the sensitivity of the PCR assay. Additionally, both types of IACs were more sensitive to Caecal PCR assay inhibitor solution than the *C. coli* or *C. jejuni* components of the PCR assays, making them suitable for detecting inhibition and



**Fig. 1.** Relative  $C_T$  values for dilutions of *C. coli* and *C. jejuni* cell lysates for duplex real-time PCR with and without IACs at optimum concentrations.

preventing the recording of false-negatives (results not shown).

### **Campylobacter numbers in spiked caecal samples**

The actual counts of *Campylobacter* in caecal samples spiked with both *C. coli* and *C. jejuni* from culture were as expected, i.e. giving  $\sim \log_{10} 7, 5, 3, 1$  c.f.u.  $g^{-1}$  for each *Campylobacter* species.

### **Limit of detection**

The mean (SEM) DNA concentration for culture lysates from the seven positive and negative control cultures was  $131 (17.3) \text{ ng } \mu\text{l}^{-1}$ , whilst the mean (SEM) DNA concentration for 38 chicken caecal DNA extracts was  $17 (4.6) \text{ ng } \mu\text{l}^{-1}$ .

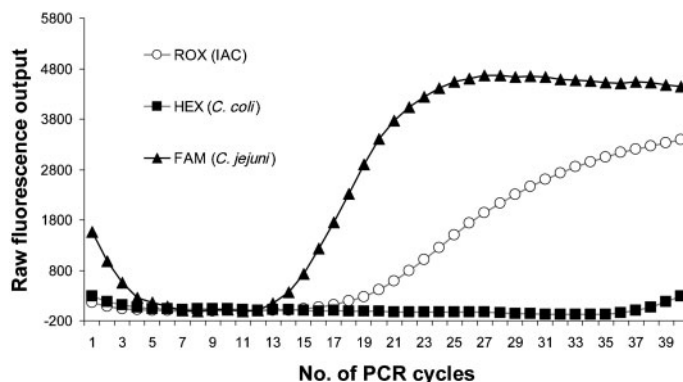
Culture lysates were still positive for *C. coli* and *C. jejuni* by PCR assay with and without the presence of both types of IACs when the DNA samples were diluted 1/1024 (Fig. 1). These results show that these PCR assays with and without IACs are capable of detecting *Campylobacter* DNA in samples at concentrations as low as  $\sim 0.1 \text{ ng } \mu\text{l}^{-1}$  or even

lower still, since at a dilution of 1/1024 the  $C_T$  values for *C. coli* and *C. jejuni* were still in the range 21.6–24.6 (samples  $> C_T 32$  negative).

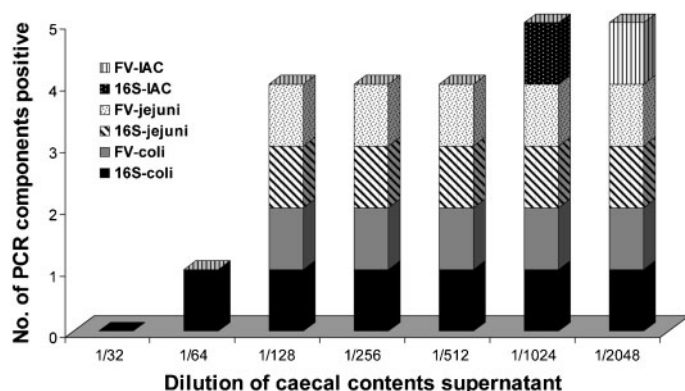
The spiked caecal samples were only positive when *Campylobacter* levels were at least  $\log_{10} 5$  c.f.u.  $(g \text{ faeces})^{-1}$ , and in most cases only positive with samples spiked with  $\sim \log_{10} 7$  c.f.u.  $(g \text{ faeces})^{-1}$ . This detection limit was not altered by the presence or absence of the different IACs.

The sensitivity of the PCR assay to detect *Campylobacter* in spiked chicken caecal samples was disappointing, especially when other workers report sensitivity down to  $10^3$  c.f.u.  $(g \text{ faeces})^{-1}$  (Lin *et al.*, 2008) or 36 c.f.u.  $\text{ml}^{-1}$  (Lund *et al.*, 2003). However, in the method of Lund *et al.* (2003), the DNA extraction method included use of magnetic beads and this may have increased sensitivity over our method.

The primary aim of this work though was to develop an IAC sensitive to caecal inhibitors that would not affect the sensitivity of an existing PCR assay, rather than optimize the detection of *Campylobacter* from chicken faeces or caeca contents, and both IAC-PCR assays could be used with more efficient DNA extraction protocols. The titration of culture DNA extracts down to 1/1024 showed that the addition of both of the IACs to the PCR assay did



**Fig. 2.** Fluorescence readings for a sample positive for *C. jejuni* and for the FV-IAC.



**Fig. 3.** Number of components of the 16S-IAC and FV-IAC PCRs positive for increasing dilutions of caecal contents supernatant (PCR inhibitor).

not reduce the sensitivity of the PCR assay and that the PCR assay was overall able to detect low levels of DNA. These results would suggest that the DNA extraction method rather than the PCR assay itself was the limiting factor in the overall sensitivity.

#### Comparison of PCR with and without IACs for caecal contents

When the PCR assays with no IAC, with the 16S rDNA IAC and with the FV-IAC were compared for DNA extracts from 18 naturally infected caecal samples, there were no statistical differences between the  $C_t$  values for both FAM and HEX between any of the methods (results not shown).

With no IAC, 9/18, 14/18 and 7/18 samples were positive for *C. coli*, *C. jejuni* and both *C. coli* and *C. jejuni*, respectively. With the 16S rDNA IAC, 9/18, 15/18 and 7/18 samples were positive for *C. coli*, *C. jejuni* and both *C. coli* and *C. jejuni*, respectively. With the FV-IAC, 9/18, 16/18 and 9/18 samples were positive for *C. coli*, *C. jejuni* and both *C. coli* and *C. jejuni*, respectively.

Results were similar for the PCR assay with and without the addition of the two different IACs and as such there was no evidence that the IACs were inhibiting detection of *Campylobacter* in chicken caecal samples as compared to the PCR assay without any IAC.

#### Comparisons of FV-IAC PCR assay to culture

Overall, the FV-IAC was considered the best IAC to use, so the FV-IAC PCR assay was used to compare with culture for 52 chicken caecal samples (Table 1).

The PCR identified 45/47 culture-positive samples as positive, with a 93.6 % sensitivity and 80.1 % specificity when comparing isolation or identification of both *C. coli* and *C. jejuni* (Table 1). However, an advantage of the PCR assay over the culture was that it was able to detect both *C. coli* and *C. jejuni* at the same time and within 24 h, whilst only one colony from culture was tested to confirm one *Campylobacter* type and the minimum time for this was 48 h. Thus PCR assay has the potential to be more rapid and, in a sense, more informative than culture. To obtain information on more than one *Campylobacter* species in caecal samples by culture would entail taking several colony picks from plates and this would become very time-consuming.

The competitive FV-IAC was chosen to evaluate with a further 52 chicken caecal DNA extracts, since the signal for this IAC was not lost in the presence of strong signals from *C. coli* or *C. jejuni*, this FV-IAC did not rely on the presence of 16S rDNA to work and since FV-IAC also did not have an extra set of primers which are reported to be able to cause problems by interacting with the target gene primers (Hoorfar *et al.*, 2004). Additionally, previous authors state

**Table 1.** Comparison of culture to PCR with FV-IAC for natural chicken caecal samples ( $n=52$ )

Sample test results	No. of samples		
	<i>C. coli</i>	<i>C. jejuni</i>	<i>C. coli</i> and <i>C. jejuni</i>
Culture-positive	10	38	0
Culture-negative	42	14	0
PCR-positive	22	39	15
PCR-negative	30	13	6
	<b>Organism</b>	<b>% Sensitivity</b>	<b>% Specificity</b>
Culture/PCR*	<i>C. coli</i> or <i>C. jejuni</i>	93.6	80.1

\*Culture was the 'gold standard' with which PCR was compared.

that the non-competitive IACs have the disadvantage that amplification of non-competitive sequences may not accurately reflect amplification of the primary target due to differences in the primary sequences (Hoorfar *et al.*, 2004).

Compared to culture, the PCR assay showed a good sensitivity and specificity for confirming the presence of *Campylobacter* in the 52 naturally infected caecal samples, and had the advantage of being able to identify the presence of both *C. coli* and *C. jejuni* simultaneously, which by culture would have been more laborious and would have required the testing of multiple colonies. A total of 15 samples were found to be positive for both *C. coli* and *C. jejuni* by PCR assay, only two samples confirmed positive by culture were negative by PCR assay and only one sample was PCR assay-positive but culture-negative.

*Campylobacter* concentrations in caecal contents of commercial chickens range from about 5 to 8 log<sub>10</sub> c.f.u. g<sup>-1</sup> and when a flock is colonized birds can carry high numbers in their intestines (Mead *et al.*, 1995; Rosenquist *et al.*, 2006; Sahin *et al.*, 2003; Stern *et al.*, 1995; Stern & Robach, 2003). Experimental studies where chickens were infected with 20 different strains of *C. coli* or *C. jejuni* showed most strains to colonize at levels of log<sub>10</sub> 8–9 c.f.u. g<sup>-1</sup> 2 weeks after infection (Ziprin *et al.*, 2003).

The 52 caecal samples that were tested for the presence of *Campylobacter* by culture compared to PCR assay were obtained at the time of year when numbers of *Campylobacter* shed from chickens are generally high, and this may have contributed to the good sensitivity of the PCR assay compared to culture, despite the apparent low detection rate using spiked samples. Different DNA extraction methods will be investigated to determine whether the detection threshold can be improved.

## Conclusions

We have designed and evaluated both competitive and non-competitive IACs in a duplex real-time PCR assay for *C. coli* and *C. jejuni* and have shown both to work well for DNA from culture or from *Campylobacter*-infected chicken caecal content. The competitive FV-IAC is recommended since it does not rely on the presence of 16S rDNA for a signal, it was not inhibited by *C. coli* or *C. jejuni* signals, it does not involve an extra set of primers that could interfere with the target gene primers and the signal derived from the FV-IAC should be detectable relative to the sample signal.

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