Quantification of *Campylobacter* carriage in pigs using a real-time PCR assay


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**Abstract**

*Campylobacter* species are the major agent of bacterial gastroenteritis. *C. jejuni* and *C. coli* together are responsible for more than 95% of all cases of *Campylobacter* induced diarrheal disease in developed countries. Risk analysis shows consumption of foods of animal origin to be a major source of human infection. Pigs are known to be frequently infected with *Campylobacter* and to exhibit high counts of this pathogen in their faeces.

The study describes a rapid, sensitive, and specific real-time polymerase chain reaction (PCR) assay capable of detecting and quantifying *Campylobacter sp.* , *C. jejuni* and *C. coli* directly from faecal samples. The description of excretion of *Campylobacter* was carried out by inoculating pigs with three different strains of *Campylobacter* ; one *C. coli* of porcine origin, one *C. coli* and one *C. jejuni* of poultry origin, alone or in a mix. The number of *Campylobacter* excreted in faeces was determined by numeration on Karmali plates and by the real time PCR assay.

The quantitative PCR results were consistent with data obtained by the bacteriological method. Two days after the inoculation, the inoculated pigs excreted from $10^4$ to $10^5$ CFU of *Campylobacter* per gramme of faeces. These levels of excretion were similar to those observed in the fattening pigs after spontaneous infection. Moreover, the real time PCR allowed species-specific detection of *Campylobacter*. Indeed, when pigs were infected with a mix of the three strains, the PCR showed that *C. coli* was predominant.

**Introduction**

Thermophilic *Campylobacter* are the major cause of bacterial gastroenteritis in humans in industrialized countries (Megraud et al., 2004). The high incidence of clinical disease associated with this organism and its potentially serious complications confirm its importance as a significant public health hazard (Tauxe, 2002). The species involved is mainly *C. jejuni* (80%), followed by *C. coli* (10 to 15%) and *C. fetus* (5 to 10%). *Campylobacter* lives in the intestinal tract of a wide range of birds and mammals, including domestic animals used for food production without causing clinical signs. The dominance of *C. jejuni* is found in most healthy carrier animals, especially broiler chickens (Nielsen et al., 1997). In contrast, pigs are known to be frequently infected with *C. coli* and to excrete high counts of this pathogen in their faeces (Weijtens et al., 1999).

As food safety has become an increasing concern for consumers, there is a growing need for fast and sensitive methods for specific detection and identification of zoonotic microorganisms. Conventional methods for the detection and confirmation of *Campylobacter* in food or in stools requires 4-5 days and involve selective enrichment followed by isolation from selective agar and confirmation by biochemical and serological tests (On, 1996). While selective media are very efficient for the initial isolation of *Campylobacter*, biochemical methods may give ambiguous results. Therefore, molecular genotype-based methods represent an alternative for the identification of *Campylobacter*. Over the last decade, the Polymerase Chain Reaction (PCR) has become a basic tool for the identification of bacterial pathogens such as *Campylobacter* (On, 1996). The recent development of real-time, closed-tube PCR methods remove the need to manipulate PCR products after amplification, thereby reducing the risk of false positive results caused by cross-contamination between amplicons and test samples. Real-time PCR assays, beyond their rapidity, sensitivity and good reproducibility, allow a precise quantification of the target DNA copy number.
The aim of the present study is to describe real time PCR (rt-PCR) assays able to detect and quantify Campylobacter sp., C. jejuni and C. coli directly from faecal samples and to compare results with conventional microaerobic bacterial cultivation.

Material and Methods

Bacterial strains, faecal samples and bacterial culture
The PCR assays were developed using different strains of C. jejuni (NCTC 11168, wild strains isolated from chicken) and C. coli (ATCC 33559, CIP 7081, wild strains isolated from pigs faeces). The specificity was assessed using C. lari, C. upsaliensis, C. fetus, Salmonella typhimurium, Listeria monocytogenes, Listeria innocua, Escherichia coli and Enterococcus faecalis.

Faecal samples were collected individually from specific-pathogen-free (SPF) pigs either inoculated by oral way with 5.10^7 CFU of C. coli (two different strains) and C. jejuni, alone or mixed, or non inoculated (« control » pigs). Two hundred and fifty milligrams of fresh faecal material were used for rt-PCR and ten grams were cultivated according to the protocol described by Denis et al. (1999). Furthermore, for numeration, 100µL of 10-fold serial dilution (10^{-1} to 10^{-5}) were directly plated on Karmali plates and incubated for 72h.

Isolation of DNA from faecal samples and bacterial cultures
Double stranded DNA of all bacteria from the bacterial culture and from faecal samples (250mg) was extracted using the Nucleospin® Tissue mini-kit (Macherey Nagel, Hoerdt, France) according to the manufacturer's instructions. The DNA preparations were stored at -20°C prior to use.

PCR primers and probes
To detect Campylobacter sp., we have used the primer-probe set described by Lund et al. (2004), which is based on the 16S rRNA sequence. The other primers and probes are based on the single-copy hipO and glyA genes for C jejuni and C coli respectively. Primers and probes were selected with Primer express version 2.0 (Applied Biosystems, Foster city, CA, USA) and their homologies with unrelated sequences were checked with the BLAST program (NCBI).

The Taqman probes, labeled with a fluorescent reporter at the 5' end and conjugated to a minor groove binder (MGB) at the 3' end, and the primers were synthetized by Applied Biosystems. The primers selected for detection of C. jejuni were hipO-F 5'-CTTGCGGTCATGATGGAGCATAC-3' and hipO-R 5'-TTAGCACACCACCAAACACCTCTTCA-3' and the TaqMan probe was hipO-P 5'-VIC-ATTGCTTTGTGCAAAGT-MGB-3'. For detection of C. coli, the primers selected were glyA-F 5'-AAACCAAAGCTTATCGTGTGC-3', glyA-R 5'-AGTCCAGCAATGTGTC-3' and the TaqMan probe was glyA-P 5'-FAM-CAACTTCATCCGCAAT-MGB-3'. For detection of Campylobacter sp., we used the primer-probe set described by Lund et al. (2004) with a VIC signal for the probe.

Evaluation of analytical performance of the real-time PCR assays
The analytical specificity of each rt-PCR assay was assessed with purified genomic DNA preparations of different bacterial strains (see above). To evaluate the analytical sensitivity of the rt-PCR, two standard curves were prepared: one with purified genomic DNA from C. jejuni NCTC 11168 and C. coli ATCC 33559 and the other with Campylobacter-negative faecal samples spiked with serial 10-fold dilutions from a broth of the two reference strains.

Quantitative real-time PCR amplification
The rt-PCR was performed in a ABI PRISM® 7300 Sequence Detection System (Applied Biosystems) and the data were analyzed with the appropriate sequence detector software. The 25µL PCR mixture for one reaction contained 12.5µL of 1X Taqman Universal PCR Mastermix (containing AmpliTaq Gold™ DNA polymerase, dNTPs, Passive reference 1 (ROX) and optimised buffer components including 5mM MgCl2), 400nM of each primers, 100nM probe and 5µL of template DNA. The thermal cycle protocol used was the following: activation of the Taq DNA polymerase at 95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 1 min. All reactions were carried out alongside a non template control containing sterile water and a positive control containing DNA from reference strains C. jejuni and C. coli.
Statistical analysis
A total of 68 samples were evaluated in parallel using the rt-PCR assays and the microbiological method. Sensitivity and specificity of rt-PCR were determined comparatively to the microaerobic cultivation and the agreement between the methods was measured using the kappa-statistic (Fleiss, 1981).

Results

Specificity and sensitivity of PCR primers and TaqMan probes
The specificity of the three primer-probes sets was optimized and tested against different strains of other Campylobacter sp. as well as several bacteria, genetically related or not, all of which were found to be negative. We also observed that quantification of DNA from each Campylobacter target was not affected when DNA from a variety of other species was present in the PCR mixture.

The standard curves spanned six to eight orders of magnitude and showed linearity over the entire quantitation range, providing an accurate measurement over a large variety of starting target amounts (R² values were all equal or above to 0.99). The detection limits of the rt-PCRs were of 1 genome copies/reaction PCR and 100 CFU/g of faeces and was similar to the bacteriological method.

Analysis of the faecal samples of pigs experimentally infected with Campylobacter
Two days after the inoculation, pigs infected with C. coli alone or C. coli and C. jejuni excreted from 10⁴ to 10⁶ CFU of Campylobacter per gramme of faeces. The excretion of pigs inoculated with C. jejuni alone was lower (up to 10³ CFU/g of faeces).

Validation of the real time PCR assays for analysis of faecal samples
The numbers of positive and negative samples determined by both detection methods for each assay are summarized in table 1.

<table>
<thead>
<tr>
<th>Microaerophilic culture</th>
<th>C. coli</th>
<th>C. jejuni</th>
<th>C. sp.</th>
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<tr>
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<td>10</td>
<td>14</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>11</td>
<td>0</td>
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<tr>
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<td>11</td>
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<table>
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<th>r-PCR</th>
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<tr>
<td>Total</td>
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</tr>
</tbody>
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C. coli: Sensitivity Se=100%, Specificity Sp=83%, Kappa K=0.89 ; C. jejuni: Se=91%, Sp=86%, K=0.77 ; C. sp.: Se=93%, Sp=100%, K=0.82

There was an excellent correlation between all positive and negative results by both techniques. Indeed for C. coli, all the culture-positive samples appeared to be positive by the rt-PCR assay (Se=100%). However, one culture-negative sample was positive by rt-PCR leading to a specificity of 83%. For C. sp., results were similar: only one culture-positive sample was negative by rt-PCR. The k values equalled to 0.89 and 0.83, respectively, indicated an excellent agreement between the two methods. Finally, for C. jejuni, even if 2 out of 18 tested samples differed between the two methods, the k value (0.77) underlines a good agreement. The amount of CFU of Campylobacter in each faecal sample determined by the rt-PCR was calculated and compared to the results obtained by quantitative bacteriological method. Among the PCR-culture positive samples, 67% of
the samples had a difference in cell number of less than 1 log, 27% less than 2 logs and 6% less than 3 logs.

Discussion

We found a high correlation between the positive and negative results obtained by rt-PCR and culture for all samples. Interestingly, in two samples culture didn't detect any Campylobacter cells, even after an enrichment step, while the rt-PCR detected them (10^5 CFU/g). This difference may be attributable to the presence of viable but not culturable (VNC) or dead Campylobacter sp. Two samples were negative by rt-PCR and positive by culture: few colonies were observed after enrichment for the first one and 10^5 CFU/g of faeces for the second one. Different hypothesis can explain this discrepant result. First, the enrichment of C. jejuni in pig faecal samples can be difficult due to overgrowth by the more numerous C. coli and a high background flora (Madden et al., 2000). Secondly, PCR inhibitors may occur in faeces that provide false-negative results with PCR methods (Wilson, 1997).

Moreover, in our trial, there is a good correlation between the techniques at the quantitative level and the discrepancies between the concentrations found by both methods might be due to dilution factors used for quantitative culture, to an insufficient homogenization of the samples, factors affecting the growth of different isolates, or possibly, the presence of antagonistic bacterial species.

Consequently, the use of PCR-based detection methods is very attractive because the Campylobacter specific rt-PCR assays allow (i) identification and discrimination of C. coli and C. jejuni without need of isolation, enrichment and/or biochemical tests, often difficult to interpret, (ii) a direct quantification of each target even if several species are present in the sample.

In conclusion, the rt-PCR developed in this study provide new tools to study the epidemiology of Campylobacter. Indeed, it could be applied to further epidemiological surveys to investigate the carriage and the excretion of Campylobacter by conventional pigs.

References


