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Development of a real-time fluorescence resonance energy transfer PCR to identify the main pathogenic *Campylobacter* spp.

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ABSTRACT

A simple real-time fluorescence resonance energy transfer (FRET) PCR, targeting the *gyrA* gene outside the quinolone resistance-determining region, was developed to identify *Campylobacter jejuni* and *Campylobacter coli*. These species were distinguished easily, as the corresponding melting points showed a difference of 15°C. A second assay using the same biprobe and PCR conditions, but different PCR primers, was also developed to identify the less frequently encountered *Campylobacter fetus*. These assays were applied to 807 *Campylobacter* isolates from clinical specimens. Compared to phenotypic identification tests, the FRET assay yielded the same results for all except three of the isolates. Analysis by standard PCR and 16S rDNA sequencing demonstrated that two of these isolates were hippurate-negative *C. jejuni* strains, resulting in an erroneous phenotypic identification, while the third was an isolate of *C. coli* that contained a *gyrA* gene typical of *C. jejuni*, resulting in misidentification by the FRET assay. The FRET assay identified more isolates than standard PCR, which failed to yield amplification products with *c.* 10% of isolates. It was concluded that the FRET assays were rapid, reliable, reproducible and relatively cost-efficient, as they require only one biprobe and can be performed directly on boiled isolates.

Keywords *Campylobacter*, FRET, *gyrA*, identification, PCR, real-time PCR

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INTRODUCTION

The incidence of *Campylobacter*-associated food poisoning has gradually increased, and this organism is now considered to be the leading cause of bacterial gastroenteritis worldwide [1]. The species isolated most frequently from man are *Campylobacter jejuni* [1], *Campylobacter coli* and *Campylobacter upsaliensis* [2]. Another important species is *Campylobacter fetus*, a common cause of sepsis or extra-intestinal infections which, in our experience, ranks third among the *Campylobacter* spp. encountered in human disease [3]. Campylobacters have a limited range of phenotypic characteristics that can be used for species identification. While the hippurate hydrolysis test [4] has great value in differentiating *C. jejuni* (positive) from *C. coli* (negative), this assay can be difficult to interpret, and atypical hippurate-negative *C. jejuni* isolates have been reported. Indeed, while hippurate-negative *C. jejuni* isolates constitute a relatively small percentage (1.6%) of total *C. jejuni* isolates, they represent 20% of all hippurate-negative *Campylobacter* isolates [5]. In addition, identification of *C. jejuni* and *C. coli* on the basis of nalidixic acid susceptibility is now impracticable, given the high rate of acquired nalidixic acid resistance in these species [6]. Moreover, the emergence of *Arcobacter* spp., which have phenotypic characteristics close to those of *C. fetus* or *C. coli*, as human pathogens is a further limitation to the use of conventional phenotypic tests.

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The problems associated with the standard phenotypic methods for identification of *Campylobacter* spp. have led to the development of new molecular approaches to assist in the identification of these organisms [7]. Among these methods, real-time PCR using the fluorescence resonance energy transfer (FRET) technology is very promising, since it can detect single nucleotide polymorphisms with only one biprobe. The present study describes the application of this technique to the identification of the most frequent pathogenic *Campylobacter* spp. by targeting the essential *gyrA* gene outside the quinolone resistance-determining region.

MATERIALS AND METHODS

Bacteria

The strains used as positive controls ($n = 22$) were eight *C. jejuni* subsp. *jejuni* strains (comprising strain NCTC 11168 (CIP 107370), strain CCUG 11284 and six clinical isolates: strains 01248, 01252, 01253, 01251, 01197 and 93097), *C. jejuni* subsp. *doylei* strain NCTC 11951 (CIP103751), eight *C. coli* strains (comprising strain NCTC 11366 (CIP 7080) and seven clinical isolates: strains 01260, 88642, 87003, 01271, 01187, 01227 and 00254), and five *C. fetus* strains (comprising strain NCTC 10842 (CIP 53.96) and four clinical isolates: strains 01125, 01185, 95125 and 01190).

Various bacterial species were used to investigate the specificity of the PCR primers, including enteric bacteria such as *Escherichia coli*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, and other species from the family Campylobacteraceae, such as *Campylobacter hyointestinalis*, *Campylobacter sputorum*, *C. upsaliensis* (all clinical isolates), *Campylobacter lari* (CCUG 23947) and *Arcobacter butzleri* (CCUG 10373). Also included were *Flexispira rappini* (CCUG 29176) and *Helicobacter* spp. such as *Helicobacter pylori* (ATCC 700392 and 700824), *Helicobacter felis* (CCUG 28539 T), *Helicobacter bilis* (ATCC 51630), *Helicobacter hepaticus* (ATCC 51448), *Helicobacter muridarum* (ATCC 49282) and *Helicobacter pullorum* (CCUG 33842 and CCUG 33839).

In total, 807 *Campylobacter* isolates from clinical specimens, mostly faeces, obtained throughout France between November 2003 and February 2004 and received successively at the French National *Campylobacter* Reference Centre, were tested in this study. All were identified to the genus level by phenotypic methods, i.e., morphology, motility, growth in a microaerobic atmosphere, and positive oxidase activity. Identification to the species level was achieved by phenotypic methods [8] based on biochemical and physiological characteristics (i.e., growth at 25°C, catalase activity and hippurate hydrolysis test) and susceptibility to antibiotics (nalidixic acid and cephalothin). The first 100 isolates were also examined with a PCR species identification assay for *C. jejuni* and *C. coli* [9,10]. Sequencing of the 16S rRNA gene was used to analyse discordant results (see below). All isolates were stored frozen at −80°C until required for testing, and were then grown at 37°C on trypticase soy medium (bioMérieux, Marcy l'Etoile, France) containing horse blood 5% v/v under microaerobic conditions (N_2 85%, O_2 5%, CO_2 10%, v/v) for 24 h.

DNA preparation

Genomic DNA was isolated from control strains and the bacterial species used to determine PCR specificity with a QIAamp DNA mini kit (Qiagen SA, Courtaboeuf, France). For the other 807 *Campylobacter* isolates tested in this study, the conventional boiling method was used to prepare the DNA template. Purified DNA was stored at −20°C.

Identification of *Campylobacter* spp. by DNA sequencing of the 16S rRNA gene

For isolates that failed to produce an amplicon by the real-time FRET-PCR (see below), a *c.* 1150-bp fragment of the 16S rRNA gene was amplified with the pseudo-universal primers F2-16S-CHPEC (5′-ATCCTGGCTCAGAGTGAACG-3′) and R2-16S-CHPEC (5′-AAGGGCCATGATGACTTGAC-3′) and sequenced on both strands. Amplified primer-less sequences were compared to the GenBank database with the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) [11].

Primer design

All standard PCR primers used in this study were designed using the web Primer3 software (http://www.broad.mit.edu/cgi-bin/primer/primer3_http://www.cgi).

PCR and DNA sequencing of the *gyrA* gene

Apart from sequences for the quinolone resistance-determining region of several *C. coli gyrA* genes [12], the only complete sequences of the *gyrA* gene available were those of two *C. jejuni* strains (strains NCTC 11168 and UA580; GenBank accession numbers AL139077 and L04566) and one *C. fetus* strain (strain UA60; GenBank accession number U25640). These three sequences were aligned and a search was performed to identify conserved regions at the extremities of the gene. Following this alignment, two sets of primers (Table 1) were designed to amplify a 2798-bp or a 2740-bp sequence from *C. jejuni* or *C. fetus* isolates, respectively (F3/R1-*gyrA*-camp), and a *c.* 2390-bp sequence from *C. coli* isolates (F4/R2-*gyrA*-camp).

The *gyrA* gene from 17 clinical *Campylobacter* control isolates (*C. jejuni* strains 01248, 01252, 01253, 01251, 01197 and 93097; *C. coli* strains 01260, 88642, 87003, 01271, 01187, 01227 and 00254; and *C. fetus* strains 01125, 01185, 95125 and 01190) was amplified with the above primers and partially sequenced with PCR primers and internal primers F1-, F6-, F8- and R3-*gyrA*-camp (for *C. jejuni* and *C. coli* strains), and F1-, F6- and R3-*gyrA*-camp (for *C. fetus* strains) (Table 1).

Design of primers and probes for FRET identification of *C. jejuni*, *C. coli* and *C. fetus*

The available *gyrA* sequences from *C. jejuni* and *C. fetus*, together with part of the region of the newly sequenced *gyrA* genes, were aligned, and a search was performed to design probes that could differentiate between *C. jejuni*, *C. coli* and *C. fetus*. LightCycler probe design software v. 1.0 (Roche

Table 1. Sequences of the primers and probes designed for this study

Primer designation	Sequences (5′ to 3′)	Nucleotide positions
gyrA amplification and sequencing		
F3-*gyrA*-camp	GYGTTATWATAGGTCGTGCTTTGC (sense)	89–112
R1-*gyrA*-camp	AATTCCYTCRCCRACTGCTG (antisense)	231–212[a]
F4-*gyrA*-camp	GAGCGTTATTATAGGTCGTG (sense)	87–112
R2-*gyrA*-camp	CGTATTACGCCCTGCTTTTC (antisense)	2472–2453
F1-*gyrA*-camp	GGAATATCTGAAGTAAGAGATGAGAGC (sense)	865–891
F6-*gyrA*-camp	CAAAARGCAAGAGCAAGAGC (sense)	1114–1133
F8-*gyrA*-camp	GGCCGTTTAACAGGACTTG (sense)	1291–1309
R3-*gyrA*-camp	CCTTCAGGAATTTTATAAACTTTAAGC (antisense)	1730–1704
LC-FRET assays		
F3-*gyrA*-CJ-CC	GTACTTTTGGTGTGATTATG (sense)	986–1005
R4-*gyrA*-CJ-CC	TTATCTCTTTTAATTCATCGCG (antisense)	1429–1408
F2-*gyrA*-CF	GATGCTATGAGTGAGATCGT (sense)	934–953[b]
R2-*gyrA*-CF	CAGAGTTTGTATCGGCG (antisense)	1222–1206[b]
Sensor	Red 640-GTTCGTCTGATAATCACTGTTTTTCTATG-p (antisense)	1100–1072
Anchor	GCTCTTGCTCTTGCTTTTTGAAGTTCAA-F (antisense)	1133–1106

The nucleotide positions of primers and probes were compared to the *gyrA* gene numeration of the *Campylobacter jejuni* NCTC 11168 reference strain.
[a]This nucleotide position was compared to the probable lipoprotein *Cj1026c* gene numeration of the NCTC 11168 reference strain.
[b]These nucleotide positions were compared to the *gyrA* gene numeration of the *Campylobacter fetus* UA60 reference strain.

Diagnostics, Neuilly sur Seine, France) and the LC-PDS module with the mutation search module (Roche Applied Science, Mannheim, Germany) were used. The sensor probe, 5′-labelled with LC-Red 640 and 3′-phosphorylated, hybridised precisely with the *C. coli gyrA* sequence, and with three and four nucleotide mismatches, respectively, with the *C. jejuni* and *C. fetus gyrA* sequences (Fig. 1). The anchor probe (T_m = 67.7°C), 3′-labelled with fluorescein, hybridised five bases upstream from the sensor probe (T_m = 61.5°C), with a frequent A/G mismatch at position 1119 of the *gyrA* gene for *C. jejuni* strains (Fig. 1), but this mismatch is in the middle of the probe and was not predicted to result in a significant decrease in T_m. Oligonucleotide primers were also designed, based on the *C. coli gyrA* gene, to identify *C. jejuni* and *C. coli*, and on the *C. fetus gyrA* gene to identify *C. fetus*, according to the same principles. Primer F3/R4-*gyrA*-CJ-CC was selected to identify *C. jejuni* and *C. coli*, and primer F2/R2-*gyrA*-CF to identify *C. fetus* (Table 1). F3-*gyrA*-CJ-CC presents a very rare

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Strain     Nucleotide position
      1048                                                                                                1147
NCTC11168 GAACTTTTAAATCTTTTCTTAACTCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
UA580     GAACTTTTAAATCTTTTCTTAACTCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
CJ-01252  GAACTTTTAAATCTTTTCTTAACCCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
CJ-01253  GAACTTTTAAATCTTTTCTTAACCCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
CJ-01248  GAACTTTTAAATCTTTTCTTAACCCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
CJ-01197  GAACTTTTAAATCTTTTCTTAACCCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
CJ-93097  GAACTTTTAAATCTTTTCTTAACCCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
CJ-01251  GAACTTTTAAATCTTTTCTTAACCCATAGAAAAACAGTTATTATTAGAAGAACGATTTTTGAACTTCAAAAGGCAAGAGCAAGAGCTCATATTTTAGAAG
          -----------------------*--------------M-----M---M----------------------X----------------------------

CC-88642  GAACTTTTAAATCTTTTCTTAAACCATAGAAAAACAGTGATTATCAGACGAACAATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCACATTTTAGAAG
CC-87003  GAACTTTTAAATCTTTTCTTAAACCATAGAAAAACAGTGATTATCAGACGAACAATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCACATTTTAGAAG
CC-01260  GAACTTTTAAATCTTTTCTTAAACCATAGAAAAACAGTGATTATCAGACGAACAATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCACATTTTAGAAG
CC-01187  GAACTTTTAAATCTTTTCTTAAACCATAGAAAAACAGTGATTATCAGACGAACAATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCACATTTTAGAAG
CC-01227  GAACTTTTAAATCTTTTCTTAACCCATAGAAAAACAGTGATTATCAGACGAACAATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCACATTTTAGAAG
CC-01271  GAGCTTTTAAATCTTTTCTTAAATCATAGAAAAACAGTGATTATCAGACGAACGATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCATATTTTAGAAG
CC-00254  GAGCTTTTAAATCTTTTCTTAAATCATAGAAAAACAGTGATTATCAGACGAACGATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCATATTTTAGAAG
          --*-------------------*------------------------------------------------------------------*----------

UA60      GAGCTTTTAAAGCTGTTTTTAAATCATAGAAAAACAGTTATCATCAGGCGTACTATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCATATTTTAGAAG
CF-01185  GAGCTTTTAAAGCTGTTTTTAAATCATAGAAAAACAGTTATCATCAGGCGTACTATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCATATTTTAGAAG
CF-01125  GAGCTTTTAAAGCTGTTTTTAAATCATAGAAAAACAGTTATCATCAGGCGTACTATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCATATTTTAGAAG
CF-95125  GAGCTTTTAAAGCTGTTTTTAAATCATAGAAAAACAGTTATCATCAGGCGTACTATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCATATTTTAGAAG
CF-01190  GAGCTTTTAAAGCTGTTTTTAAATCATAGAAAAACAGTTATCATCAGGCGTACTATTTTTGAACTTCAAAAAGCAAGAGCAAGAGCTCATATTTTAGAAG
          --------------------------------------M--M-----M--M-------------------------------------------------

Consensus GAgCTTTTAAAgCTgTTtTTAAatCATAGAAAAACAGTtATcATcAGgcGtACtATTTTTGAACTTCAAAAaGCAAGAGCAAGAGCTCAtATTTTAGAAG
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Fig. 1. Internal nucleotide sequence of the *gyrA* gene from the three major pathogenic *Campylobacter* spp., showing the location of the probes used for real-time PCR amplification. The sequences are shown from nucleotides 1048 to 1147 as compared to *Campylobacter jejuni* NCTC 11168 reference strain (GenBank accession number AL139077). The sensor probe (boxed) is 5′-labelled with LC-Red 640 (LightCycler-Red 640-N-hydroxysuccinimide ester), and a 3′ terminal phosphate block was added. The anchor probe (underlined) is 3′-labelled with fluorescein. M, mutations on the sensor probe inducing a different melting point (when FRET was used). X, silent anchor probe mutations. *, spontaneous point mutations outside the region of interest. The *C. jejuni* (strains NCTC11168 and UA580; GenBank accession numbers AL139077 and L04566) and *Campylobacter fetus* (strain UA60; GenBank accession number U25640) *gyrA* genes are also included.

mismatch at its 5′-end on the *C. coli* sequence, and R4-*gyrA*-CJ-CC presents a mismatch at its 5′-end on the *C. jejuni* sequences (data not shown). As these mismatches are localised at the 5′-end of the primers, they should not have any influence on the efficiency of the PCR.

Real-time FRET-PCR for the identification of *Campylobacter* spp. with the biprobe

The PCR and hybridisation reactions were performed in glass capillary tubes in a LightCycler thermocycler (Roche Diagnostics). Each 7-μL reaction mixture contained 0.7 μL of FastStart DNA Master Hybridization probe mixture (Roche Diagnostics), 3 mM $MgCl_2$, 0.72 μM forward and reverse primers, 0.2 μM each probe, and 0.7 μL of template DNA. Following initial denaturation at 95°C for 10 min with a temperature transition rate of 20°C/s, 50 amplification cycles (95°C for 6 s, 54°C for 12 s and 72°C for 25 s) were performed with a temperature transition rate of 20°C/s. Fluorescence was measured at 640 nm after each cycle. This was followed by a melting program of 95°C for 60 s, 38°C for 50 s at a temperature transition rate of 20°C/s, and 80°C for 0 s (hold time) at a rate of 0.1°C/s, with continuous monitoring of the fluorescence. The final step consisted of cooling at 20°C/s to 40°C with a 30-s hold.

RESULTS AND DISCUSSION

Development of the assay

Three real-time detection formats are currently available, namely SYBR Green, TaqMan and FRET chemistries. The SYBR Green format was excluded, since no probe is used and the specificity is not optimal. In order to identify the two *Campylobacter* spp. encountered most frequently, the TaqMan chemistry would require a mixture of at least two probes (each labelled twice), with a cost at least double that of FRET with the single nucleotide polymorphism detection format, for which only one biprobe (labelled once) is used. For this reason, FRET was chosen for development in the present study.

The primer set and biprobe were designed to target a gene common to *Campylobacter* spp., but with species-specific sequence divergences to allow differentiation by melting curve analysis. Since the goal of the study was to identify all of the *C. jejuni* and *C. coli* isolates, ribosomal genes were not considered, as the 16S rDNA sequences are highly conserved [13] and do not allow a clear differentiation [14]. Other genes common to both *C. jejuni* and *C. coli* include the *ceuE* gene, encoding a lipoprotein involved in the transport system for the siderophore enterochelin [15,16], and the *gyrA* gene, encoding a subunit of DNA gyrase [17–19], which is also an important tool for bacterial phylogeny [20–22]. Although iron acquisition is a crucial aspect of bacterial infectivity, the *ceuE* gene is not essential for bacterial viability, in contrast to the *gyrA* gene, which was therefore chosen to ensure a common target for PCR amplification.

Alignment of the available *gyrA* sequences from two isolates of *C. jejuni* and one of *C. fetus*, as well as newly sequenced *gyrA* genes (*c.* 711 bp) from six *C. jejuni*, seven *C. coli*, four *C. fetus* strains and one *C. lari* strain, revealed that the *C. jejuni* and *C. coli gyrA* genes were closely related, while the *C. lari* and *C. fetus gyrA* genes were more distant from each other (Fig. 2). In contrast to the recently published dendrogram based on 16S rRNA gene sequence similarity [13], there was a clear distinction between the *C. jejuni* and *C. coli* strains.

Numerous nucleotide sequence variations were observed between the *C. jejuni*, *C. coli* and *C. fetus gyrA* genes. The various constraints of design imposed by the LightCycler probe design software (highly conserved anchor probe and mismatches on the sensor probe, a maximum gap of five bases between the probes, and the need to take into account the melting temperature of the probes) allowed only one region of the *gyrA* gene (nucleotides 1072–1136) to be used to design the sensor and anchor probes. As *C. coli* is clustered phylogenetically between *C. jejuni* and *C. fetus* (Fig. 2), and in order to use the same biprobe for *C. jejuni*, *C. coli* and *C. fetus* identification with a

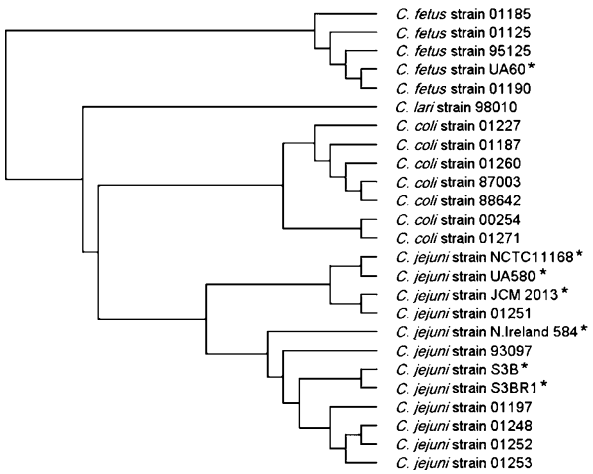

Fig. 2. Dendrogram of *Campylobacter* spp. based on data from 711 bp of the *gyrA* gene. Most sequences grouped into species-specific clusters. Strain sequences obtained from GenBank are indicated by asterisks.

minimum of mismatches between the sensor probe and the target *Campylobacter* DNA, all of the probes used in this study were designed around the *C. coli gyrA* gene sequence. Primers for the identification of *C. jejuni* and *C. coli* targeted the *C. coli gyrA* gene in a region that was highly conserved for these two species. Primers for the identification of *C. fetus* specifically targeted variations found in the *C. fetus gyrA* gene sequence.

Identification of control strains

Melting curve analysis of the 444-bp amplicons from *C. coli* (n = 8) and *C. jejuni* (n = 9) control strains indicated T_m values of *c.* 64.4°C and 48.8°C, respectively (Fig. 3), thereby allowing a clear differentiation between these two species. The rare *C. jejuni* subspecies *doylei* could not be differentiated from the common *C. jejuni* subspecies *jejuni*. A maximum difference of 0.2°C in the melting peak temperatures was observed between different runs, resulting from variations in the temperature profile created by the LightCycler instrument. Using boiled cell lysates, this real-time PCR assay was able to detect one to seven copies of the *C. coli* or *C. jejuni* genomes. No amplification occurred with the various other bacterial species listed in Materials and Methods.

As this assay did not allow the identification of *C. fetus*, the third most frequently encountered *Campylobacter* spp. in France, a second assay was developed with the same biprobe, but with different primers that amplified 289 bp of the *C. fetus gyrA* gene, with all of the reactions being performed under the same conditions and in the same run. Melting curve analysis of amplicons from *C. fetus* control strains (n = 5) exhibited a very low T_m of *c.* 48.4°C (data not shown), caused by four nucleotide mismatches between the sensor probe and the *C. fetus gyrA* gene (Fig. 1). This real-time PCR assay did not amplify the *gyrA* gene from *Campylobacter* spp. other than *C. fetus*, or that from strains of the other bacterial species tested. A difference in T_m of 0.8°C was observed for a few *C. fetus* strains between different runs, although sequencing revealed an identical sequence for these PCR products. This variation may result from an unstable hybridisation caused by nucleotide mismatches at the 3′-end of the sensor probe (Fig. 1).

The close proximity of the T_m for *C. fetus* (48.4°C) and that for *C. jejuni* (48.8°C), and the fact that a few *C. fetus* strains showed slight melting peak variations between different runs, meant that these two species could not be differentiated simultaneously, thereby excluding the possibility of a multiplex PCR.

Identification of *Campylobacter* spp. in clinical specimens

Among the 807 clinical isolates investigated, real-time FRET-PCR with the appropriate primers identified 593 (73%) *C. jejuni*, 151 (19%) *C. coli* and 31 (4%) *C. fetus*, with 32 (4%) isolates failing to produce a signal. Sequencing of the 16S rRNA gene from the non-identified isolates yielded 16 *A. butzleri* strains, 11 *C. lari* strains, three *C. upsaliensis* strains, one *C. hyointestinalis* strain and one *H. pullorum* strain.

With three exceptions where an unexpected T_m was obtained, there was a perfect correlation between the 775 results obtained with the

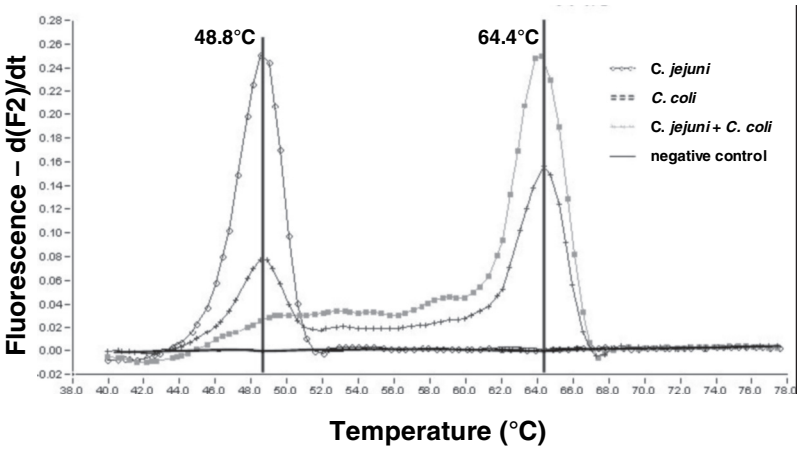

Fig. 3. Melting curve analysis obtained with the real-time PCR assay for the 444-bp amplicon of the *gyrA* gene from *Campylobacter coli* and *Campylobacter jejuni*. A melting curve corresponding to a mixture containing *C. jejuni* and *C. coli* is also shown, and two melting peaks with the corresponding T_m can be observed. Values on the y axis represent the ratio of the first negative derivative of the change in fluorescence in channel 2 (d*F*2) with the variation in temperature.

C. coli/C. jejuni and *C. fetus* real-time PCRs and the phenotypic methods used as a standard. Analysis by standard PCR [9,10] and 16S rDNA sequencing demonstrated that two of the discrepant isolates were hippurate-negative *C. jejuni* strains, resulting in an erroneous phenotypic identification, while the third was an isolate of *C. coli* that contained a *gyrA* gene typical of *C. jejuni*, resulting in misidentification by the FRET assay. This last discrepant result could possibly be explained by horizontal transfer of the *gyrA* gene from *C. jejuni* to *C. coli*. Hakanen *et al.* [23] have reported horizontal gene transfer between *Campylobacter* spp., and described six *C. jejuni* strains that had replaced their *gyrA* gene with a gene received from *C. coli*. This transfer involved the conserved region of the *gyrA* gene associated with resistance to fluoroquinolones and resulted in antibiotic resistance. In the present study, which concerned the conserved region of the *gyrA* gene, this event appeared to be very rare, as it occurred only once (0.13%) among the 775 isolates studied.

Accurate species identification of *Campylobacter* isolates is important because it is the first step in comparing isolates for epidemiological studies; indeed, it is possible that outbreaks have gone unnoticed in the past in the absence of proper identification of *Campylobacter* spp. Compared to phenotypic tests, the assay described in the present study has a higher specificity.

Comparison with standard multiplex PCR

Species identification of the first 100 *C. jejuni* and *C. coli* isolates in the study was also performed by standard multiplex PCR as described previously by Stonnet *et al.* [9,10]. Amplification only occurred with 90 isolates, but there was complete agreement between the standard multiplex and real-time PCR methods for the 90 isolates speciated. However, the real-time FRET-PCR assay amplified more isolates than standard PCR, and the results suggested that the boiling method, which does not always yield PCR products by standard PCR [24], can be applied to all isolates when real-time FRET-PCR is used.

CONCLUSIONS

The present study developed new real-time PCRs for the identification of the three *Campylobacter* spp. encountered most frequently. Amplification was always obtained, as *gyrA* is an essential gene. The method was rapid, and the system of sensor and anchor probes used in the assay was able to discriminate between the two most common *Campylobacter* spp., i.e., *C. jejuni* and *C. coli*, in a single reaction, thereby contributing to the efficiency of the method. Compared to phenotypic tests, the real-time assay had a higher specificity. The assay was shown to be reliable, reproducible and more sensitive than standard PCR. The assay is relatively economical, as it can be performed directly on boiled cell lysates with only one biprobe in a low final volume, and without any need for post-PCR processing. This method can be implemented easily in a setting where a FRET-PCR apparatus is available. Attempts are being made currently to extend this methodology to other campylobacters and related bacteria.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The *C. jejuni*, *C. coli*, *C. fetus* and *C. lari* partial *gyrA* sequences determined in this study have been assigned accession numbers AY575036 to AY575041 (strains 01248, 01252, 01253, 01251, 01197 and 93097), AY575042 to AY575048 (strains 01260, 88642, 87003, 01271, 01187, 01227 and 00254), AY575049 to AY575052 (strains 01125, 01185, 95125 and 01190) and AY575053 (strain 98010), respectively.

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