

APPLICATION OF POLYMERASE CHAIN REACTION AND  
DENATURING GRADIENT GEL ELECTROPHORESIS ASSAY  
OF THE FLAGELLIN GENE FOR DIRECT DETECTION AND  
SUBTYPING OF *CAMPYLOBACTER JEJUNI* AND  
*CAMPYLOBACTER COLI* IN AVIAN FAECAL SAMPLES

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**Summary**

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The present report investigated the potential of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis assay of the flagellin gene (*fla*-DGGE) for direct detection, identification and typing of *Campylobacter* spp., and pathogenic *Campylobacter jejuni* and *Campylobacter coli* in samples without cultivation. Faecal samples obtained from synanthropic bird species inhabiting areas around broiler chickens fattening farms, and wild-type *C. jejuni* strains isolated from a poultry slaughterhouse were assayed. The PCR assay for *Campylobacter* spp. and the specific PCR assays for *C. jejuni* and *C. coli* generated fragments sized 816, 735 and 500 bp, respectively. Out of the 34 faecal samples studied, 13 were positive for *Campylobacter* spp. *C. jejuni* was detected in 10 (76.9%) and *C. coli* – only in one (7.69%) out of *Campylobacter* positive samples. The employed *fla*-DGGE was applicable in faecal DNA concentrations over 150 ng/mL. Common genotypes with similar *fla*-DGGE profiles were found out in both faecal samples from synanthropic birds and broiler chicken isolates.

**Key words:** broiler chickens, *Campylobacter coli*, *Campylobacter jejuni*, synanthropic birds

INTRODUCTION

The analysis of molecular biological traits of microorganisms, also known as genotyping, is used for both direct identification and epidemiological investigations of the chain of infection (Jackson *et al.*, 1996). Polymerase chain reaction (PCR) is a method in molecular biology readily applicable for rapid detection and differentiation of *Campylobacter* spp. (Wegmuller *et al.*, 1993; Chuma *et al.*, 1997).

So far, numerous phenotype and genotype methods have been used in the epidemiological research of *Campylobacter* spp. (Lior *et al.*, 1982; Uyttendaele *et al.*, 1995). Wong *et al.* (2007) investigated the prevalence of *Campylobacter jejuni* and *Campylobacter coli*. The estimated rates were 89.1% in chicken, 9.1% in pork, 6.9% in lamb and mutton, 3.5% in beef and 10% in veal. *C. jejuni* constituted the

greatest proportion of positive samples (94.9%). By means of serotyping and PFGE genome macrorestriction analysis, 17 serotypes and 56 electrophoretic profiles were established in 247 isolates.

The methods for *Campylobacter* typing in the region of the *fla-A* gene showed better results than serotyping techniques (Nachamkin *et al.*, 1993; Studer *et al.*, 1998). The analysis of the flagellin coding gene is an appropriate marker for epidemiological research purposes (Taylor *et al.*, 1992; Wassenaar *et al.*, 1993), whereas flagellin restriction fragment length polymorphism (*fla*-RFLP) appears to be a good alternative for detection and differentiation of *Campylobacter* spp. (Nachamkin *et al.*, 1993). The 5' and 3' ends of flagellin genes *flaA* and *flaB* are highly conservative in the different *Campylobacter* species, while the intergenic region between them is extremely variable. The utilization of primer sequences from these regions guarantees the high specificity of the PCR assay and allows for the reliable typing in epidemiological and clinical investigations (Wegmuller *et al.*, 1993).

At present, various molecular typing techniques are used in studying the epidemiology of campylobacteriosis (Bull *et al.*, 2006). Denaturing gradient gel electrophoresis (DGGE) is a relatively new, rapid and reliable method allowing for the differentiation of DNA fragments of equal length but different nucleotide sequences. The method was successfully used to establish the microbial diversity in microbial communities and samples of various origins, without preliminary enrichment and cultivation, and furthermore, it is potentially useful in subtyping various strains from the pathogenic *C. jejuni* and *C. coli* species (Hein *et al.*, 2003).

Recent results evidence that the rapid and reliable detection and differentiation of *Campylobacter* is still facing numerous problems. The degrees of resemblance within a given taxonomic unit sometimes lead to confusing results. The precise distinction between *C. jejuni* and *C. coli* is still a challenge. It is therefore recommended to prove the identity of bacterial strains, using more than one method (On, 1996; Wassenaar *et al.*, 1998).

The purpose of this study was to investigate the application of PCR and *fla*-DGGE techniques for direct detection, identification and typing of *Campylobacter* spp., *C. jejuni* and *C. coli* in faecal samples from birds without enrichment and cultivation.

## MATERIALS AND METHODS

### *Samples*

In this study, 34 faecal samples from synanthropic birds inhabiting the regions around broiler chicken farms, and 13 wild-type *Campylobacter* spp. strains obtained from broiler chicken batches in a slaughterhouse, were analysed. Faecal samples were collected from shot pigeons, sparrows, quails, collared doves, and turtle doves. The thoracic and abdominal cavities of birds were aseptically incised and 0.4 g of intestinal content (faeces) was collected for extraction of bacterial DNA. The slaughterhouse bacterial isolates were *C. jejuni* strains, identified phenotypically and biochemically, which included 3 hippurate-negative strains. The hippurate activity is a primary biochemical test to distinguish *C. jejuni* from *C. coli*. Reference *C. jejuni* ILVO and *C. coli* ILVO strains were used as positive controls (provided by the Institute for Agricultural and Fisheries Research, Melle, Belgium).

*DNA isolation from faecal samples and bacterial cultures*

DNA isolation from faecal samples was performed by the method of Yu & Morrison (2004), using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden Germany). The quality of isolated DNA was determined by electrophoresis in 2% LSI LE agarose gel at 100 V for 30 min. Bacterial culture DNA was isolated from colonies using a routine laboratory method (Flamm *et al.*, 1984), and the yield was spectrophotometrically determined. For samples with high and low DNA concentration, Quant-iT™ Assay Kit (InvitroGen, USA) variants Br (Broad range) and Hs (High sensitivity) were respectively used.

*PCR identification and PCR fla-DGGE analysis*

The conventional PCR used the universal primers C412 and C1288 for *Campylobacter* spp. (Brown *et al.*, 2004), as well as species-specific primers JEJ3.3 and JEJ4.4 for *C. jejuni*, and COL3.3 and COL4.4 for *C. coli* (Linton *et al.*, 1997) (Table 1). PCR for *fla*-DGGE was performed with the primers CF02 and CF03 (Hein *et al.*, 2003, Wegmuller *et al.*,

1993), appropriate for subtyping of *C. jejuni*. These primer sequences restrict an intergenic region, separating tandemly oriented *flaA* and *flaB* genes. The forward primer was modified by attaching a GC base pair to the 5' end.

The PCR was performed in a 25 µL reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 0.25 mM of each dNTP (Amersham Pharmacia Biotech), 1 mM MgCl<sub>2</sub> (Perkin Elmer, Cetus, Norwalk, CT, USA), 6.25 pmol of each primer, 1.6 U AmpliTaq polymerase (Applied Biosystems) and 12.5 ng DNA. The used thermocycler programme (GeneAmp PCR system 9700 Gold; Applied Biosystem) consisted of initial denaturation at 94 °C for 2 min, followed by 28 cycles (94 °C/1 min; 56 °C/1 min; 72 °C/1 min), and a final extension for 7 min at 72 °C. Amplification products were checked electrophoretically before each *fla*-DGGE analysis. DNA fragments in the electrophoresis gel were visualized by 20-min staining with 1:10 000 SYBR Green I (Molecular Probes, Leiden, The Netherlands), and photo documented by GelDoc 2000.

*Fla*-DGGE analysis was performed according to the protocol described by Najdenski *et al.* (2008). The total reaction

**Table 1.** Primer pairs used in the study

Target gene	Primer pair	Primer sequence (5'–3')	Reference
16S RNA	C412	GGA-TGA-CAC-TTT-TCG-GAG-C	Brown <i>et al.</i> , 2004
	C1288	CAT-TGT-AGC-ACG-TGT-GTC	
<i>hipO</i>	JEJ3.3	GAA-GAG-GGT-TTG-GGT-GGT-G	Linton <i>et al.</i> , 1997
	JEJ4.4	AGC-TAG-CTT-CGC-ATA-ATA-ACT-TG	
<i>asp</i>	COL3.3	GGT-ATG-ATT-TCT-ACA-AAG-CGA-G	Linton <i>et al.</i> , 1997
	COL4.4	ATA-AAA-GAC-TATCGT-CGC-GTG	
<i>flaA-flaB</i>	CF02	AAG-CAA-GAA-GTG-TTC-CAA-GTT-T	Wegmuller <i>et al.</i> , 1993
	CF03	CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCT-CAA-AGT-GGT-TCT-TAT-GCN-ATG-G	

**Table 2.** DNA concentrations and results from PCR for direct detection and species identification of *Campylobacter* spp. in faecal samples and bacterial cultures

Samples	n	DNA concentration	Electrophoretic analysis (n)	PCR		
				<i>Campylobacter</i> spp.	<i>C. jejuni</i>	<i>C. coli</i>
Pigeons	10	1.58–95.8 µg/mL	10	10	7	1
Sparrows	1	13.1 ng/mL	0	0	–	–
Turtle doves	13	9.74–496 ng/mL	5	3	3	0
Collared doves	1	52.9 ng/mL	0	0	–	–
Quails	9	20.4–60.0 ng/mL	0	0	–	–
<i>C. jejuni</i> ssp. <i>jejuni</i>	9	5.81–39.2 µg/mL	9	9	8	1
<i>C. jejuni</i> ssp. <i>doylei</i>	1	29.8 µg/mL	1	1	1	0
Hippurate (–) <i>C. jejuni</i>	3	8.59–191 µg/mL	3	3	3	0

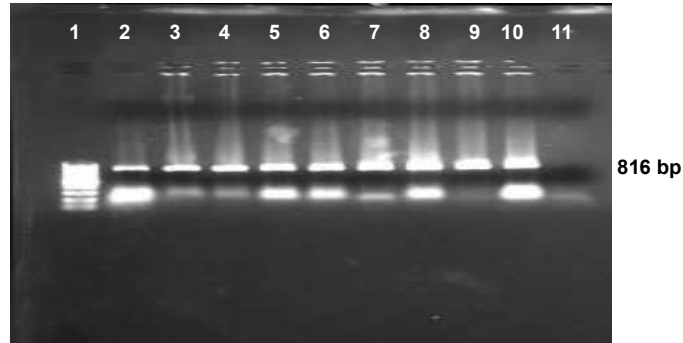
volume was 25 µL including the ReadyToGo® kit, 0.25 pmol/µL of each primer and 2.5 µL of isolated template DNA. The assay conditions consisted of initial denaturation for 2 min at 94 °C, 28 cycles (94 °C/1 min, 56 °C/1 min, 72 °C/1 min) and a final extension at 72 °C for 7 min. Five µL of produced amplification products were used in the *fla*-DGGE analysis (DCode System, Bio-Rad Laboratories, Hercules, CA, USA). *Fla*-DGGE was carried out in 8% polyacrylamide gel with parallel denaturing gradient. The chemical denaturing gradient ranged between 15–45%. The electrophoresis was run in 1× Tris/acetate/EDTA (TAE) buffer at 200 V, 56 °C for 180 min with start tension 20 V for 15 min.

## RESULTS

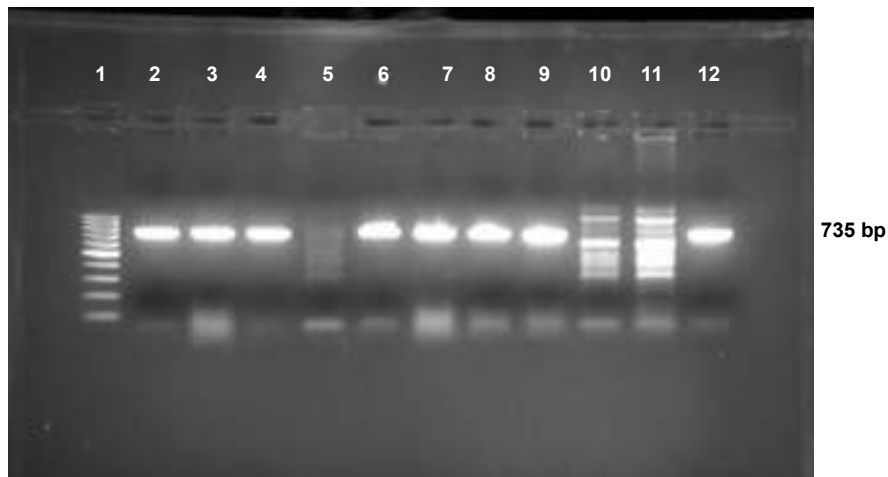
The amount of DNA isolated from faecal samples and bacterial cultures, and positive reactions in the electrophoretic analysis are presented in Table 2. In synanthropic birds, the amount of DNA isolated from faecal samples varied within a broad

range. The highest concentration (95.8 µg/mL) was established in pigeon samples. In the other bird species, concentrations ranged within 9.7–496 ng/mL. The isolated DNA represents the total DNA of all bacteria present in intestinal microflora of birds and DNA of cells released by the intestinal epithelium. That is the reason for the substantial difference in concentrations in the studied bird species. The electrophoretic analysis has shown that despite the relatively high DNA concentration in some of samples, it was not appropriate to conduct a PCR analysis. Such were the samples from sparrows, collared doves, quails and some turtle doves. It could be concluded that only 13 of all 34 faecal samples were suitable for the next PCR step with species-specific primers and PCR *fla*-DGGE.

When DNA was extracted from bacterial colony, the yield varied between 5.8 and 191 µg/mL. The electrophoretic analysis showed that all bacterial samples were suitable for use in species-specific PCR (Table 2).



**Fig. 1.** PCR amplicons for direct detection of *Campylobacter* spp. using the C412 and C1288 primers – lane 1: DNA marker; lane 2: positive control; lane 3: sparrow sample; lanes 4–6: *C. jejuni* ssp. *jejuni*; lanes 7–8: *C. coli*; lanes 9–10: *C. jejuni* ssp. *jejuni*; lane 11: negative control.



**Fig. 2.** PCR amplicons for direct detection of *C. jejuni* using JEJ 3.3 and JEJ 4.4 primers – lanes 1, 10, 11: DNA marker; lanes 2 and 12: positive controls; lanes 3 and 4: *C. jejuni* ssp. *jejuni*; lane 5: turtle dove sample; lanes 6–9: pigeon samples.

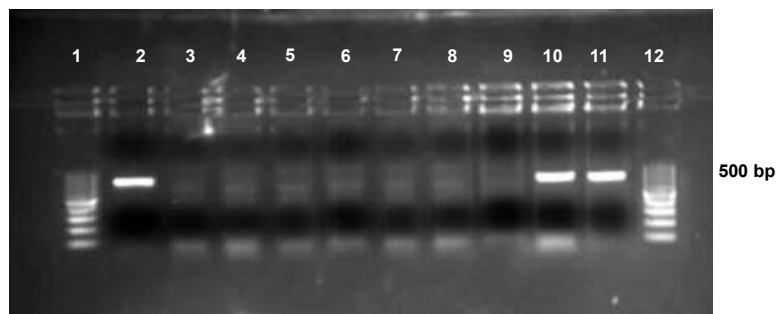
The DNA isolated from faecal samples and bacterial cultures was used in the PCR assay with C412 and C1288 primers that are universal for detection of *Campylobacter* spp. The PCR reaction was positive when an 816 bp amplicon was detected (Fig. 1). Samples from pigeons, turtle doves and all bacterial cultures were *Campylobacter* spp. positive (Table 1). PCR for direct identification with primers specific for *C. jejuni* and *C. coli* was per-

formed only in these positive samples. *C. jejuni* positive samples exhibited a 735 bp amplicon (Fig. 2), whereas the amplicon size in those positive for *C. coli* was 500 bp (Fig. 3). Out of the 26 *Campylobacter* spp. positive samples, 22 exhibited DNA specific for *C. jejuni* and another 2 – for *C. coli*. A simultaneous occurrence of both species was not observed in any of samples. In pigeon samples, *C. jejuni* was predominating while only 1 sample was *C.*

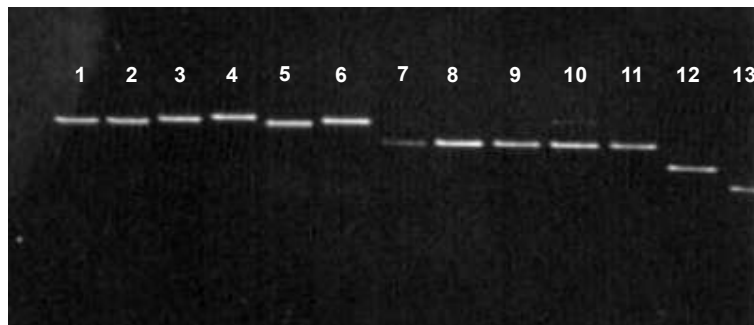
*coli* positive. On the basis of its DNA, one of the tested *C. jejuni* ssp. *jejuni* bacterial strains was classified as belonging to *C. coli*. Different bacterial species often present common biochemical characteristics and therefore, a small percent of cultures are misclassified on the basis of their phenotypic characteristics. The molecular biology techniques are far more correct for identification or differentiation. All tested hippurate-negative strains belonged to the *C. jejuni* as shown by the PCR. Many of routinely used diagnostic tests distinguish *C. coli* from *C. jejuni* by the negative hippurate reaction. Sometimes, it

results in inappropriate classification of bacterial isolates, particularly on the background of resistance to diagnostic antibiotics nalidixic acid and cefalotin, which are also used in tests for *Campylobacter* spp. differentiation.

For PCR-*flaA* performed with the primers CF02/CF03, only DNA positive for *C. jejuni* and *C. coli* was used. The obtained amplification products sized 200 bp served for DGGE typing. The analysis of amplification profiles showed four different genotypes of campylobacteria (Fig. 4). The first genotype (lanes 1 to 6) included *C. jejuni* strains, one of them de-



**Fig. 3.** PCR amplicons for direct detection of *C. coli* using the COL3.3 and COL4.4 primers – lanes 1 and 12: DNA marker; lane 2 – positive control; lanes 3, 6–7, 9–11: pigeons; lanes 4–5: turtle doves; lane 8: sparrow.



**Fig. 4.** DGGE migratory patterns of PCR products using the primers CF02 and CF03; applied on faecal samples and bacterial cultures: lanes 1–4, 6–9: slaughterhouse strains; lane 5: turtle doves; lanes 10, 11, 12: pigeons; lane 13: *C. jejuni* strain ILVO Belgium positive control).

tected directly in a turtle dove faecal sample without preliminary isolation. The second genotype (lanes 7-11) included 4 *C. jejuni* isolates as well as 1 *C. coli* strain which was also directly detected in pigeon faeces. Lanes 12 and 13 present *C. coli* and *C. jejuni* strains, respectively, possessing unique genotypes – the first one isolated from a Bulgarian poultry slaughterhouse, and the second one – from Belgium.

## DISCUSSION

In this study, a PCR *fla*-DGGE analysis of faecal samples from synanthropic birds as well as of wild-type *C. jejuni* and *C. coli* strains has been carried out. The used method for DNA isolation (Yu & Morrison, 2004) allowed obtaining a DNA template with good yield and quality and eliminating inhibitory components, a prerequisite for improvement of *fla*-DGGE analysis. According to Hein *et al.* (2003) the theoretical denaturing profiles of the *flaA* sequence of *Campylobacter* were more variable when a GC base pair is attached to the forward primer and on the contrary, only one sequence is observed when the GC base pair is attached to the reverse primer. Regardless of this fact, we have found out a better separation of fragments after attaching the GC base pair to the forward primer simultaneously with using a modified gradient of the chemical denaturing agent (Najdenski *et al.* 2008). Thus optimized, the method has proved its specificity for both *C. jejuni* and *C. coli*. As already reported (Nielsen *et al.*, 2000; Hein *et al.*, 2003) the *fla*-DGGE method appeared to be less discriminating than *fla*-RFLP. In general, strains of the same *fla*-DGGE type could belong to different *fla*-RFLP types. Usually, *fla*-RFLP analyzes large sequences from different parts

of the *flaA* gene while the used *fla*-DGGE covers only the 3' end of *flaA*, as well as a part of the intergenic sequence between *flaA* and *flaB*. The interest towards *fla*-DGGE was mainly raised by the possibility for typing without preliminary cultivation of samples that could compromise some of existing types. This is especially important due to the fact that multiple genotypes of the same bacterial species could be present in the gastrointestinal tract of animals.

The results from the present study allowed assuming that *fla*-DGGE was able to detect the presence of *C. jejuni* and *C. coli* in faecal samples of birds directly, without enrichments and cultivation. By means of *fla*-DGGE we have detected *Campylobacter* positive faecal samples, which yielded DNA at concentrations >150 ng/mL. The utilization of species-specific primers increased the accuracy and speed of identification of *C. jejuni* and *C. coli*. Common *fla*-DGGE genotypes were discovered in faecal samples from synanthropic birds and slaughtered broiler chickens. Such a similarity suggests a potential for transfer and exchange of campylobacteria among synanthropic birds and broilers fattened at poultry farms, which are then conveyed to the slaughterhouse. In similar studies with broiler chickens, Whyte *et al.* (2001) observed pre-transportation contamination rates of 6.04–6.61 log and 6.83–7.32 log after the transportation to the slaughterhouse. The authors also emphasized the advantage of PCR with primers universal for *Campylobacter* and agarose gel electrophoresis due to the relatively higher sensitivity of the reaction (3.55–3.96 log CFU/mL).

In conclusion, the applied *fla*-DGGE analysis exhibited a number of benefits, including the omission of the enrichment

and cultivation steps, the potential for rapid subtyping of *Campylobacter* spp. and pathogenic *C. jejuni* and *C. coli* in bird faecal samples. The method could be used for detection of various genotypes in bird samples, which could be then analysed by other epidemiological typing techniques after cultivation and isolation of campylobacteria.

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