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Fla-DGGE analysis of *Campylobacter jejuni* and *Campylobacter coli* in cecal samples of broilers without cultivation

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Abstract

In a commercial broiler flock during rearing multiple genotypes of *Campylobacter jejuni* may be present as well as in gastrointestinal tracts of individual birds. The aim of this study was to optimize and apply a denaturing gradient gel electrophoresis assay of the flagellin gene (*fla*-DGGE) for analysis of *Campylobacter jejuni* and *Campylobacter coli* in cecal samples of broilers without prior cultivation. One *C. coli* and 21 *C. jejuni* strains isolated from broiler flocks, of which 14 typed as unique by restriction fragment length polymorphism of *flaA* and two undefined strains, were clustered into 9 groups when applying *fla*-DGGE. Spiking of cecal samples revealed that *fla*-DGGE is able to detect at

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least 4.55-5.96 log CFU *Campylobacter* per ml of cecal material. The presence of 3 strains spiked in cecal material was demonstrated by *fla*-DGGE as the corresponding bands were visible on the DGGE gel. Naturally contaminated cecal samples were shown to contain different types of *C. jejuni* and *C. coli*. *Fla*-DGGE has some potential as a cultivation-independent fast primary subtyping method for *C. jejuni* and *C. coli* in cecal samples of broilers.

Key words: *Campylobacter*; broiler flocks; DGGE; subtyping

1. Introduction

Campylobacters are the most commonly reported bacterial cause of acute gastroenteritis in the European Union. Handling and consumption of contaminated poultry meat have been identified as major sources of human campylobacteriosis (Wingstrand et al., 2006). In Belgium, the *Campylobacter* prevalence at broiler flock level was estimated at 67 and 72% (Herman et al., 2003; Rasschaert et al., 2006). The intestines, especially the ceca are the primary sites of colonization for *Campylobacter* (Achen et al., 1998).

The epidemiology of campylobacteriosis has been investigated by several molecular typing methods (Bull et al., 2006). A new and fast method, allowing separation of DNA fragments of equal length but different sequences is DGGE (denaturing gradient gel electrophoresis). DGGE is usually employed to assess the structure of microbial communities in environmental samples without cultivation, but can also be used for subtyping of *Campylobacter jejuni* and *Campylobacter coli* (Hein et al., 2003).

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The goal of this study was to apply *fla*-DGGE for analysis of *C. jejuni* and *C. coli* directly in cecal contents of broilers without relying on a cultivation step which may introduce bias towards the selection of certain types.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Twenty-one *C. jejuni* subsp. *jejuni* (*C. jejuni* further) strains and 1 *C. coli* strain, originating from commercial broiler flocks (Herman *et al.*, 2003), were selected based on unique clustering profiles using restriction fragment length polymorphism of *flaA* (*fla*-RFLP) (Table 1). Isolates were kept as frozen stock cultures at -80 ^{o}C and cultured on modified Charcoal Cefoperazone Desoxycholate Agar (mCCDA, Oxoid, Basingstoke, UK; Herman et al. (2003)) under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2 in an O_2/CO_2 incubator, Thermo Forma, Ohio, USA) at 42 °C.

Campylobacter lari (LMG 8846, LMG 9152), *Campylobacter hyointestinalis* subsp. *hyointestinalis* (LMG 10891, R26768, R26766, LMG 7817), *Campylobacter insulaenigrae* (LMG 22718, LMG 22716), *Campylobacter concisus* (LMG 14003), *Campylobacter lanienae* (R23962), *Campylobacter "lawrenceae"* (R28144), *Campylobacter upsaliensis* (LMG 7915), *Helicobacter pullorum* (R26767, LMG 16318), *Arcobacter butzleri* (LMG 10828), *Arcobacter cryaerophilus* (LMG 9904), and *Arcobacter skirrowii* (LMG 6621) strains were used to check the specificity of the *fla*-DGGE method. Strains were cultured on Mueller Hinton agar (Oxoid) supplemented with

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5% horse blood for 48 h under microaerophilic conditions generated with CampyGen (Oxoid). *Campylobacter* strains were cultured at 37 °C and *Arcobacter* strains at 28 °C.

2.2. Sampling and microbiological analysis

Cecal contents were aseptically collected from chicken ceca of 4 individual broilers from a commercial slaughterhouse in Belgium. One g of each sample was dissolved in 9 ml of buffered peptone water (BPW, Oxoid). Enumeration was done by serial decimal dilution, plating out on mCCDA (Oxoid) and incubation for 24 h under microaerophilic conditions in an O_2/CO_2 incubator at 42 °C (as above). Microscopy of colonies suspected for *Campylobacter* spp. was done. Each sample of cecal contents was stored at -80 °C after dilution with 30% glycerol (1:1 v/v) until DNA preparation. These samples are referred hereafter with the letter B and a number referring to the individual bird.

2.3. Artificial contamination of cecal samples

The detection limit of *Campylobacter* in cecal samples by *fla*-DGGE was established by spiking a negative cecal sample with 10-fold dilutions of a 24 h culture of either *C. jejuni* KC 45.2 and *C. coli* KC 12 in a ratio of 25:1 (v/v). These samples are referred to by the letter D and a number referring to the dilution factor of the pure culture used for inoculation (e.g. 0 for undiluted, 1 for 10^{-1} dilution, 2 for 10^{-2} dilution etc.).

Spiking was also done with a bacterial mix of the strains used for construction of a DGGE-marker (see Results; KC 1, KC 45.2, and MB 1290). Ringer solutions containing each strain (ca. 6.85 log CFU/ml) were mixed at a volume ratio of 1:1:1 before spiking in

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a ratio of 25:1 (v/v) giving bacterial numbers for each strain in the cecal sample between 4.89 and 5.08 log CFU/ml.

2.4. Isolation of DNA from bacterial culture and cecal samples

DNA from bacterial cultures was isolated by the method of Flamm et al. (1984) and its concentration was measured spectrophotometrically (Genesis 10vis, Thermo Electron Corporation, Madison, WI, USA). The method of Yu and Morrison (2004) including QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used for DNA isolation of cecal samples. Isolated DNA was detected by ethidium bromide staining after electrophoresis at 100 V for 30 min in a 2 % LSI LE agarose gel.

2.5. PCR assays and fla-DGGE analysis

PCR was carried out with primers universal for *Campylobacter* spp. (Eurogentec, Seraing, Belgium) as well as primers specific for *C. jejuni* and *C. coli* (both from Isogen Life Science, Maarssen, The Netherlands) (Table 2) as described in Herman et al. (2003). PCR products were detected as described above.

For *fla*-DGGE, the primers were based on the study by Hein *et al.* (2003) applying *fla*-DGGE for subtyping of *C. jejuni* using previously published primers (Wegmüller et al., 1993). The primers (Eurogentec) originated from the 3' end of the *flaA* and the 3' end of the intergenic region, separating the tandemly orientated *flaA* and *flaB* genes. A GC clamp was attached to the 5' end of the forward primer (CF 03). The 25 µl reaction mix contained 10 mM Tris-HCl , 50 mM KCl, 0.25 mM of each dNTP (Amersham Pharmacia Biotech), 1 mM $MgCl_2$ (Perkin-Elmer Cetus, Norwalk, CT, USA), 6.25 pmol of each

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primer, 1.6 U AmpliTaq Polymerase (Applied Biosystems) and 12.5 ng template DNA. The PCR programme was: 2 min at 94 °C (initial step), 28 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C and a final extension of 7 min at 72 °C in a programmable thermocycler (GeneAmp® PCR System 9700 Gold; Applied Biosystems). PCR products were checked before *fla*-DGGE analysis as described above.

Fla-DGGE was carried out as described by Hein et al. (2003) with some modifications. Briefly, the gradient of the chemical denaturant was adjusted to 15-45%. Five µl PCR product was subjected to DGGE analysis using a DCode™ System (Bio-Rad Laboratories, Hercules, CA, USA) by using 8% polyacrylamide gel with a parallel chemical denaturing gradient. The running buffer was 1× Tris-acetate-EDTA (pH 8.0) and the operating conditions were 200 V at 56 °C for 3 h, applying 20 V for 15 min at the start of the DGGE analysis. DNA bands were visualized by 20 min staining in a 1:10,000 SYBR Green I stain (Molecular Probes, Leiden, The Netherlands), photographed and analyzed using a GelDoc™ 2000 system (Bio-Rad Laboratories) and compared with Bionumerics 4.00 (Applied Maths, Sint-Martens-Latem, Belgium).

3. Results

3.1. Fla-DGGE analysis of isolated bacterial DNA

To check the specificity of the *fla*-DGGE PCR, 12 *Campylobacter* strains other than *C. jejuni* and *C. coli*, 2 *H. pullorum* and 3 *Arcobacter* strains were tested. Samples were negative on both agarose and DGGE gel (data not shown). The 21 *C. jejuni* and 1 *C. coli* strains demonstrated positive *fla*-DGGE patterns (Table 1). PCR bands sized

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approximately 200 bp with different intensity. The 14 strains with unique *fla*-RFLP types and 2 undefined strains were grouped into 9 *fla*-DGGE types. Five strains were analyzed in double and duplicates corresponded to the same *fla*-DGGE type. In 2 cases, strains (KC 83.2/KC 101 and KC1/KC131) from the same *fla*-RFLP type belonged to different *fla*-DGGE types. This experiment allowed us to construct a marker consisting of 3 strains (KC 1, KC 45.2, and MB 1290) each with a different banding profile (Fig. 1). This marker was added to 3 lanes of each DGGE gel for normalization.

3.2. Analysis of cecal samples

Table 3 gives the results of both microbiological and PCR analysis of cecal samples. Campylobacters were detected in the naturally contaminated samples at levels near 8 log CFU/ml (B1, B2 and B4) and 3 log CFU/ml (B3). All samples were positive with universal *Campylobacter* PCR. B1 and B2 were positive with *C. jejuni* PCR and *C. coli* PCR, while B4 was positive with *C. coli* PCR. After *fla*-DGGE PCR, B1, B2 and B4 showed up on agarose gel at approximately 200 bp. B3 was negative with *C. jejuni* PCR, *C. coli* PCR and *fla*-DGGE PCR. *C. jejuni* KC 45.2 was spiked at a concentration of 6.96 (D0) to 2.96 log CFU/ml cecal material (D4). On agarose gel, dilutions up to D3 were positive after universal *Campylobacter* PCR while only D0 was positive after *fla*-DGGE PCR. Using *C. coli* KC 12, *Campylobacter* counts ranged from 5.55 (D0) to 2.55 log CFU/ml cecal material (D3). Dilutions up to D2 were positive on agarose gel after universal *Campylobacter* PCR, while only D0 was positive after *fla*-DGGE PCR.

The *fla*-DGGE patterns of cecal samples are depicted in Fig. 2. The cecal sample spiked with KC 45.2 gave a clear band for D0 and D1, at the same place as KC 45.2 analysed

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from pure culture. D2 was negative on the DGGE gel. For KC 12, a clear band was noticeable for D0 while D1 still showed a slight band. The band corresponded to *fla*-DGGE type 3. Using a bacterial mix of the marker strains for spiking, the 3 strains could be distinguished on the DGGE gel at the corresponding places (data not shown). For the naturally contaminated samples B2 and B4, one band was found on DGGE gel corresponding to *fla*-DGGE type 3. For B1, 3 bands were observed, corresponding to *fla*-DGGE types 3, 5 and 6. B3 was negative (data not shown).

4. Discussion

In this study, the *fla*-DGGE method described by Hein et al. (2003) was applied on cecal samples of broilers. The DNA extraction protocol that resulted in the highest DNA yield and quality as described by Yu and Morrison (2004) was used. This protocol gives DNA free of inhibitory substances and with improved DGGE profiles. According to Hein et al. (2003), the theoretical melting profiles for the *Campylobacter fla*A sequence types of the variable fragment showed several domains with different melting profiles when the GC clamp was attached to the forward primer, while a single domain was found with the GC clamp attached to the reverse primer. Nevertheless, in our study we found a better separation of the fragment with the GC clamp attached to the forward primer and with an adjusted gradient of chemical denaturant. The method was shown specific for *C. jejuni* and *C. coli* as no cross-reaction was found in accordance to Wegmüller et al. (1993). As shown before (Hein et al., 2003; Nielsen et al., 2000), *fla*-DGGE is less discriminative compared to *fla*-RFLP. In general, strains from one *fla*-DGGE type belonged to different

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fla-RFLP types. In 2 cases, 2 strains from the same *fla*-RFLP type were grouped into 2 *fla*-DGGE types. This can possibly be explained by the different region of the flagellin sequence that was used in both methods. While *fla*-RFLP covered the entire *fla*A region, *fla*-DGGE covered the 3' end of *fla*A and part of the intergenic sequence between *fla*A and *fla*B. The main interest in DGGE is however that typing without cultivation is possible, which might introduce bias towards the selection of certain types. This is important as the gastrointestinal tracts of individual birds during rearing may contain multiple genotypes of *C. jejuni* (Höök et al., 2005).

Weaker bands are present on the DGGE gel when analyzing pure strains (Fig. 1) (except for KC1 which is due to the already low position of the strong distinct band), while this was only the case for 2 naturally contaminated cecum samples (Fig. 2, lanes 8 and 9). According to our interpretation, the weaker bands are due to technical artifacts caused by the *fla*-PCR on pure samples, while these artifacts were not visible using commonly DNA from contaminated samples. As a consequence, the weaker bands in Fig. 2, lanes 8-9, may be caused by *Campylobacter* types present in low concentration in the samples.

Fla-DGGE was able to detect *C. jejuni* and *C. coli* in cecal contents of broilers with a detection limit of 4.55-5.96 log CFU of *Campylobacter* per ml of cecal material. As colonization levels of *Campylobacter* in ceca are high, especially posttransport, the *fla*-DGGE method has still some potential despite the observed detection limit. In the studies by Stern et al. (1995) and Whyte et al. (2001), levels averaged 6.04 and 6.61 log and 6.83 and 7.32 log CFU per g cecal material before and after transport to the slaughterhouse, respectively. However, for detection purposes, analysis on agarose after universal

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Campylobacter PCR needs to be preferred because of its higher sensitivity (3.55-3.96 log CFU per ml of cecal material).

The naturally contaminated samples were *Campylobacter* positive on the DGGE gel, except for the sample containing only 3 log CFU/ml cecal material. This was possibly due to the detection limit of *fla*-DGGE as analysis on agarose after universal *Campylobacter* PCR was positive. The other samples contained > 7.98 log CFU/ml cecal material. Birds from two samples were colonised with a strain belonging to *fla*-DGGE type 3. By *C. jejuni* PCR and *C. coli* PCR the presence of both species was however found in one of these sample. Probably, both strains have a similar DGGE pattern and totally coincide. Such a result is explicable taking into consideration the similarity between *C. jejuni* and *C. coli* in the *fla*-DGGE pattern and their classification in one and the same DGGE type in this study. In another study (Studer et al., 1998), using the same flaA-flaB intergenic region for RFLP and sequence typing, differentiation between *C. jejuni* and *C. coli* was also not possible. Also, it could be explained by the higher sensitivity of the *C. jejuni* or *C. coli* PCRs. In one sample, 3 bands were distinguished by *fla*-DGGE and thus the presence of at least 3 different *Campylobacter* strains naturally available in the sample could be demonstrated. Moreover, this result was confirmed examining an artificially contaminated cecal sample with a mix of 3 *Campylobacter* strains. It has to be taken into account that multiple strains in a cecal sample will sometimes be undetected by *fla*-DGGE either because of the detection limit or because bands overlap.

In conclusion, *fla*-DGGE has some potential as a cultivation-independent fast primary subtyping method for *C. jejuni* and *C. coli* in cecal samples of broilers. Before

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cultivation, *fla*-DGGE can illustrate different types in a sample, which can then be searched for by other typing techniques after a more directed picking of colonies.

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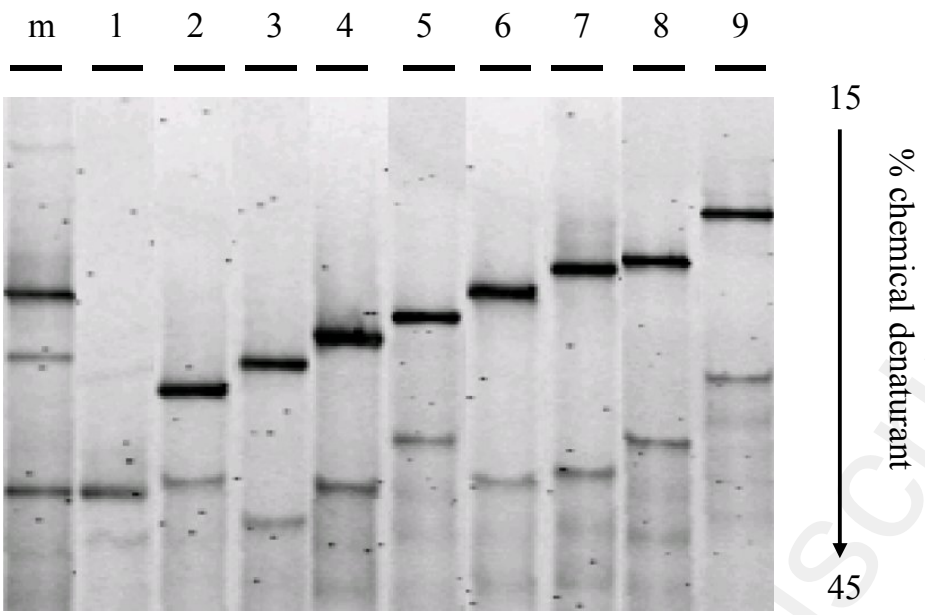

Fig. 1. *Fla*-DGGE types. Lane m, separation pattern of an equimolar amplicon mixture from MB 1290, KC 45.2 and KC 1 (types 6, 3 and 1). The *fla*-DGGE band types 1 to 9 were produced from strains KC 1, KC 64.1, KC 12, KC 59.1, KC 101, KC 22, KC 67.2, KC 131, KC 126.1, respectively.

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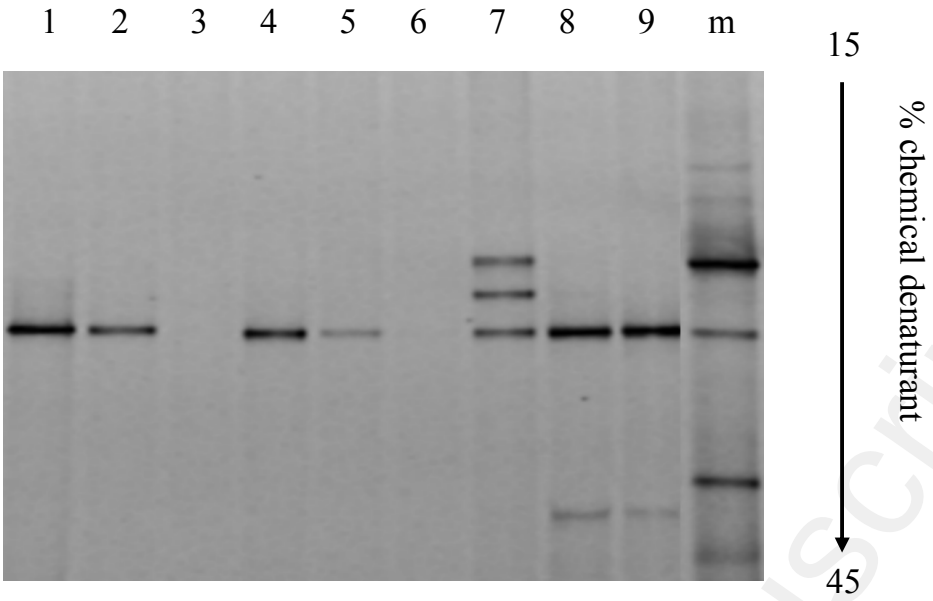

Fig. 2. *Fla*-DGGE analysis of artificially (D0, D1, D2) and naturally (B1, B2, B4) contaminated caecal samples. Lanes 1 to 3, KC 45.2 D0 to D2; lanes 4 to 6, KC 12 D0 to D2; lane 7, B1; lane 8, B2; lane 9, B4; m, separation pattern of an equimolar amplicon mixture from MB 1290, KC 45.2 and KC 1 (types 6, 3 and 1).

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Table 1. Characteristics of *Campylobacter* strains used and their resulting *fla*-DGGE types.

Strain number(flock)[a]	Source	Identi-fication[b]	*fla*-RFLP *Dde*I[c]	*fla*-RFLP *Hin*fI[c]	*fla*-DGGE type
KC 1(2)[M]	cecal drops	J	9	9	1
KC 64.1(9[d]), KC 84.1(11)	cecal drops	J	12	7	2
KC 12(2)	cecal drops	C	1	1	3
KC 41(5), KC 111(13)	faecal material wild animals, cecal drops	J	3	3	3
KC 45.2(7)[M]	cecal drops	J	5	5	3
KC 44(7)	dung hill	J	14	5	4
KC 51(7)	faecal material other poultry house	J	6	6	4
KC 83.2(11)	cecal drops	J	11	7	4
KC 59.1(9), KC 96.1(12)	cecal drops	J	7	7	4
KC 40(6)	dung hill	J	4	4	5
KC 101(11)	farmers footwear[e]	J	11	7	5
KC 22(3), KC 61(10[d])	cecal drops	J	2	2	6
KC 69.1(11)	cecal drops	J	1	7	6
KC 71(11)	farmers footwear[e]	J	10	7	6
MB1290 (= LMG 9880)[M]	chicken	J	NA	NA	6
KC 67.2(9)	cecal drops	J	8	8	7
KC 131(18)	ditch water	J	9	9	8
KC 126.1(16)	cecal drops	J	NA	NA	9

NA: not assessed.

[a] KC strains were obtained from samples taken from commercial broiler flocks as described in Herman et al. (2003); LMG strain is from Laboratory of Microbiology (Ghent, Belgium); M indicates marker strains.

[b] C, *C. coli*; J, *C. jejuni.*

[c] *Fla*-RFLP typing is described in Messens et al. (in preparation)

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1 **Table 2.** Set of primers used for PCR assays.

Name	Oligonucleotide	Sequences (5'-3')[a]	Reference
Universal *Campylobacter* PCR	C412 C1288	GGA TGA CAC TTT TCG GAG C CAT TGT AGC ACG TGT GTC	Brown et al. (2004)
C. jejuni PCR	JEJ3.3 JEJ4.4	GAA GAG GGT TTG GGT GGT G AGC TAG CTT CGC ATA ATA ACT TG	Linton et al. (1997)
C. coli PCR	COL3.3 COL4.4	GGT ATG ATT TCT ACA AAG CGA G ATA AAA GAC TAT CGT CGC GTG	Linton et al. (1997)
Fla-DGGE PCR	CF 02 CF 03	AAGCAAGAAGTGTTCCAAGTTT CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT CAA AGT GGT TCT TAT GCN ATG G	Wegmüller et al. (1993)

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Table 3. PCR analysis of naturally and artificially contaminated cecal samples.

Isolate	Dilution	Counts (log CFU/ml)	Universal *Campylobacter* PCR[a]	*C. jejuni* PCR[a]	*C. coli* PCR[a]	*Fla*-DGGE PCR[a]	*Fla*-DGGE PCR on DGGE gel
B1		8.28	+	+	+	+	+
B2		≥ 8.30	+	+	+	+	+
B3		3.00	+	-	-	-	-
B4		7.98	+	-	+	+	+
KC 45.2	D0	6.96	+	NA	NA	+	+
KC 45.2	D1	5.96	+	NA	NA	-	+
KC 45.2	D2	4.96	+	NA	NA	-	-
KC 45.2	D3	3.96	+	NA	NA	-	-
KC 45.2	D4	2.96	-	NA	NA	-	-
KC 12	D0	5.55	+	NA	NA	+	+
KC 12	D1	4.55	+	NA	NA	-	+
KC 12	D2	3.55	+	NA	NA	-	-
KC 12	D3	2.55	-	NA	NA	-	-

NA: not assessed.

[a] on agarose gel

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