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To cite this version:

Winy Messens, Lieve Herman, Lieven De Zutter, Marc Heyndrickx. Multiple Typing for the Epidemiological Study of Contamination of Broilers with Thermotolerant. Veterinary Microbiology, Elsevier, 2009, 138 (1-2), pp.120. <10.1016/j.vetmic.2009.02.012>. <hal-00490545>

HAL Id: hal-00490545
https://hal.archives-ouvertes.fr/hal-00490545
Submitted on 9 Jun 2010

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Accepted Manuscript

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PII: S0378-1135(09)00086-8
Reference: VETMIC 4365

To appear in: VETMIC

Received date: 18-9-2008
Revised date: 6-2-2009
Accepted date: 6-2-2009

Please cite this article as: Messens, W., Herman, L., De Zutter, L., Heyndrickx, M., Multiple Typing for the Epidemiological Study of Contamination of Broilers with Thermotolerant Campylobacter, Veterinary Microbiology (2008), doi:10.1016/j.vetmic.2009.02.012

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Multiple Typing for the Epidemiological Study of
Contamination of Broilers with Thermotolerant
Campylobacter

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Abstract

This study aims to investigate the genetic diversity of thermotolerant *Campylobacter* in commercial broiler flocks and in the environment of broiler farms in Belgium. Seven out of 18 investigated flocks became colonized during rearing. Fluorescent amplified fragment length polymorphism (FAFLP), pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism of the flagellin A gene (*fla*A-RFLP) and antimicrobial resistance profile (ARP) were used for typing of the isolates. By the combination of FAFLP and PFGE, 22 *Campylobacter* genotypes could be distinguished. Colonization was almost exclusively with *Campylobacter jejuni* and unique genotypes were found in each flock. Multiple genotypes were detected in the broilers of three flocks, either simultaneously or successively. In five flocks, strains that were resistant to at least one antibiotic (mostly tetracycline) were found. The presence of other broiler houses on the farm did not result in a higher probability of colonization. The nipple water was contaminated with the same genotype as the broilers, illustrating its importance for transmission of *Campylobacter*. The same genotype was detected in a water puddle and in the broiler flock during rearing in three flocks. Once, the same genotype was isolated from the ditch water shortly before it was detected in the broilers.

Keywords: *Campylobacter*, broilers, risk factor, molecular epidemiology, transmission
1. Introduction

Campylobacteriosis was the most frequently reported zoonotic disease in humans within the European Union (EU) in 2006. Its overall incidence was 54.6 cases per 100,000 inhabitants in the EU and 64.3 per 100,000 inhabitants in Belgium (EFSA 2007). Its true incidence is uncertain since many unreported infections occur (WHO 2000). Ninety-one percent of human isolates speciated in the EU were identified as *Campylobacter jejuni* subsp. *jejuni* (hereafter *C. jejuni*), while *Campylobacter coli* accounts for most of the remaining cases (EFSA 2007). The majority of infections occur sporadically, with the major vehicle of infection assumed to be contaminated food, and in particular chicken (Friedman et al. 2002; Skirrow 1994). In Belgium, in recent years 72% of the broiler flocks arriving at the slaughterhouse were colonized with campylobacters. After slaughter, 79% of the flocks yielded contaminated carcasses. Isolates were mainly identified as *C. jejuni* (89%) (Rasschaert et al. 2006). It has been estimated that consumption of poultry meat in Belgium causes at least 22,000 campylobacteriosis cases with a cost of illness of €10.9 million (Gellynck et al. 2008). The relative risk reduction by reducing the flock prevalence has a linear (one-to-one) relationship, i.e. 50% reduction in flock prevalence results in a 50% reduction in risk (Messens et al. 2007).

The most significant sources for flock colonization remain unclear. However, a number of epidemiological studies have shown that lack of hygienic barriers, use of non-disinfected drinking water and deficient hygienic measures are significant risk factors for introducing *Campylobacter* in broiler flocks (Johnsen et al. 2006). Other vectors such as litter beetles, house flies and wild birds have also been identified as potential transmission risks (Chuma et al. 2000).
In the present study, 18 Belgian broiler flocks and their environment inside and outside the poultry house were sampled and cultured for the presence of *Campylobacter*. Isolates were typed by fluorescent amplified fragment length polymorphism (FAFLP), pulsed field gel electrophoresis (PFGE), and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the flagellin (*flaA* and *flaB*) gene and their antimicrobial resistance was determined to determine contamination patterns.

2. Materials and methods

2.1. Sampling

Eighteen Belgian commercial broiler flocks from 17 different farms were examined (flocks nos. 6 and 7 were successive flocks in the same broiler house). A detailed description about sample collection can be found in Herman *et al.* (2003) and in the Table. Briefly, before arrival of the one-day-old chicks (day 1), the hygiene inside the broiler houses was checked. During rearing, the farms were visited three times (days 14, 28 and 42) with sampling of cecal drops (mostly 4 pools of 10 cecal drops; detecting a prevalence of 7.3% at 95% confidence level for 10,000 birds), overshoes, farmer’ indoor footwear, feed and drinking water. The environment of the farms was sampled before and during rearing.

2.2. Isolation and confirmation of *Campylobacter*

The isolation protocol included both direct (using modified Charcoal Cefoperazone Desoxycholate Agar (mCCDA, Oxoid, Basingstoke, UK)) and enrichment culturing (using selective Preston broth (Oxoid) and mCCDA plating). Incubation was for 24 to 48 h at 42°C
under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂ in an O₂/CO₂ incubator, Thermo Forma, Ohio, USA). Isolates were stored in brain-heart infusion broth (BHI; Oxoid) supplemented with 5% (vol/vol) lysed, defibrinated horse blood at -80°C using 15% (w/v) glycerol as cryoprotectant. A sample was determined as positive for *Campylobacter* as soon as at least one of the isolation methods yielded a positive result. The *Campylobacter* status of the flock was considered as positive if *Campylobacter* was detected in at least one of the pools of cecal drops.

Identification of the isolates was performed on the *Campylobacter* genus level and as *C. jejuni* or *C. coli* in a multiplex PCR. When no PCR identification could be achieved, the identity of the presumptive *Campylobacter* isolates was determined by sequence analysis of the 16S ribosomal DNA as described in Herman *et al.* (2003).

2.3. *Fluorescent amplified fragment length polymorphism* genotyping

FAFLP was performed on 500 ng of genomic DNA digested in a total volume of 30 µl, which consisted of 5 U of *Hind*III and *Hha*I endonuclease (Promega, Leiden, The Netherlands), 3 µl of buffer C (Promega) and 0.3 µl of 100× bovine serum albumin (BSA) (Promega), for 3 h at 37°C. To the digested DNA, 25 µl of a solution containing 0.5 µl of 2 µM *Hind*III adaptor, 0.5 µl of 20 µM *Hha*I adaptor (both from Eurogentec, Seraing, Belgium), 40 U of T4 DNA-ligase, and 5 µl of 10× T4 ligase buffer (both from Amersham Pharmacia Biotech, Uppsala, Sweden) was added. Ligation was carried out overnight at room temperature. The sequence of the *Hind*III adaptor was 5’-CTC GTA GAC TGC GTA CC, 3’-CTG ACG CAT GGT CGA. The sequence of the *Hha*I adaptor was 5’-GAC GAT GAG TCC TGA TCG, 3’-G CTA CTC AGG ACT A.
Preselective PCRs were carried out in 25-µl volumes containing 5 µl of the two-fold diluted ligation product, 2.2 µl of HindIII primer (5’ GAC TGC GTA CCA GCT T-3’, 1 µM, Eurogentec), 2.2 µl of HhaI primer (5’-GAT GAG TCC TGA TCG C-3’, 5 µM, Eurogentec), and 15.6 µl of amplification core mix (Applied Biosystems). Amplification was performed in a programmable thermocycler (GeneAmp® PCR System 9700; Applied Biosystems) using an initial denaturation step at 94°C (2 min), followed by 20 cycles consisting of denaturation (94°C for 20 s), annealing (56°C for 45 s) and extension (72°C for 2 min). Next, selective PCRs were carried out in 20-µl volumes containing 3 µl of the five-fold diluted PCR product, 3 µl of FAM labeled HindIII+A primer (1 µM), 3 µl of HhaI+A primer (5 µM, Eurogentec), and 11 µl of the amplification core mix. Amplification was performed in the GeneAmp® PCR System 9700 programmable thermocycler using an initial denaturation step at 94°C (2 min), followed by 20 cycles consisting of denaturation (94°C for 20 s), annealing (66°C for 45 s) and extension (72°C for 2 min), and a final extension step (72°C for 10 min). Three microliters of the selective PCR product was mixed with 12 µl of Hi-Di formamide (Applied Biosystems), 1 µl of Gene-Scan-500 size standard labeled with the red fluorophore 6-carboxy-x-rhodamine (ROX) (Applied Biosystems) and heated at 92°C for 2 min. Subsequently the mix was chilled on ice for a few minutes.

Separation of the selective PCR products was generated in an ABI PRISM 310 genetic analyzer (Applied Biosystems). Each run was performed at 60°C for 35 min at 10 mA and 15 kV. After electrophoresis, the banding pattern data were collected with the ABI GeneScan 3.1 software (Applied Biosystems). Each gel track was then imported into the Bionumerics 4.00 software package (Applied Maths, Sint-Martens-Latem, Belgium) with the program ABICON (Applied Maths). Gels were normalized using the internal standard and the levels of genetic similarity between patterns were calculated with the Pearson product-moment correlation coefficient. For cluster analysis the unweighted pair group method using average
linkages (UPGMA) was used at an 0.06% optimisation coefficient. Fragments in the size range of ca 50-500 bp were used in the analysis. The reproducibility of the FAFLP analysis was assessed by performing the whole assay, starting with DNA isolation, three times with three C. jejuni strains as indicated in the Table. Preselective PCRs and selective PCRs were performed in one run (as for all the samples). The similarity between patterns obtained was at least 90% (93.9, 95.8 and 97.4%) and these results correlated perfectly with previously reported values (Duim et al. 2001; On and Harrington 2000).

2.4. Pulsed field gel electrophoresis genotyping

PFGE was performed as described previously (Zorman et al. 2006) using SmaI as restriction enzyme (Boehringer, Mannheim, Germany). The macrorestriction fragments were separated using the Clamped Homogeneous Electric field method on a Chef Mapper (CHEF-DRII, Bio-Rad Laboratories, Richmond, USA) using pulse times from 4 s to 40 s in 22 h. The gels were stained with ethidium bromide, photographed and analyzed using a GelDoc™ 2000 system (Bio-Rad Laboratories). PFGE profiles were clustered with the BioNumerics 4.00 software package (Applied Maths) using the Dice coefficient (1% position tolerance) and UPGMA.

2.5. Restriction fragment length polymorphism genotyping

For flaA-RFLP, the consensus pair of primers for flaA gene amplification was used (Wassenaar and Newell 2000). PCR reagents and conditions were from Nachamkin et al. (1993). For flaA PCR amplicons of the expected 1.7-kb size RFLP analysis was performed using Ddel (New England Biolabs, Hitchin, United Kingdom) and HinfI (New England
Biolabs) as restriction enzymes. PCR amplicons (3-14 µl) were digested according to the manufacturer’s instructions and then analysed by electrophoresis using 3% (w/v) agarose in 0.5× Tris-Acetate-EDTA (TAE) buffer. The gels were stained, photographed and analyzed similarly as the PFGE gels.

2.6. Definition of genotypes

For every typing experiment separately, genotypes were defined within the strain collection. FAFLP genotypes were assigned with a capital letter on the basis of an interpretation of the UPGMA-dendrogram: a cut-off similarity value of 90% was used to delineate genotypes labelled with a capital letter (e.g. A); genotypes clustering at a similarity level between 80 and 90% were classified as subtypes of a genotype, labelled with a capital letter followed by a number (e.g. A₁, A₂); if visual but small band pattern differences were observed within the 90% cut-off range, this was indicated as a variant of a genotype with a quotation mark after the capital genotype letter.

PFGE genotypes were assigned with a number on the basis of any difference in the presence of at least one band (above 50 kb); variants of a genotype with minor polymorphisms, defined as a small shift or density difference of a particular band, were indicated with one or more apostrophes after the PFGE genotype number. FlaA genotypes were assigned with a number on the basis of any difference in the presence of at least one band.

Overall genotypes were defined by a combination of the separately defined genotypes by FAFLP, PFGE, flaA-DdeI and flaA-HinfI in that order.

2.7. Antibiotic resistance profiling (ARP)
Minimal inhibitory concentrations (MICs) were obtained with the Etest (AB Biodisk, Solna, Sweden) according to the manufacturer’s instructions. Isolates were classified as sensitive (S), intermediate (I) or resistant (R) applying the breakpoints of S, I and R (in µg/ml) for ampicillin (Am) ≤8, 16, ≥32, for amoxicillin-clavulanic acid (2/1) (Ac) ≤8, 16, ≥32 for ciprofloxacin (Ci) ≤1, 2, ≥4, for erythromycin (Em) ≤2, 4, ≥8, for gentamycin (Gm) ≤4, 8, ≥16, for nalidixic acid (Na) ≤8, 16, ≥32, and for tetracycline (Tc) ≤4, 8, ≥16. *E. coli* ATCC 25922 was used as reference strain. Mueller Hinton agar plates (BioMerieux, France) supplemented with 5% (vol/vol) sheep blood were used.

3. Results

3.1. Molecular typing of *Campylobacter* isolates

Using FAFLP, 20 genotypes could be distinguished within the 92 *Campylobacter* isolates (Figure). Three FAFLP-genotypes could be further divided in 2 (genotypes P and Q) or 3 (genotype N) subtypes; genotype F contained two variants. Using PFGE, 19 genotypes could be distinguished with genotype 11 showing 3 variants. FAFLP and PFGE results showed to be complementary (Figure). FAFLP-genotype F was divided in PFGE-genotypes 2 and 14 (corresponding with the variant FAFLP-genotypes F and F’), FAFLP-genotype E was divided in PFGE-genotypes 3 and 9, FAFLP-genotype O was divided in PFGE-genotypes 11’ and 12 and FAFLP-genotype I was divided in PFGE-genotype variants 11 and 11”. Non-typeable strains in PFGE could be typed with FAFLP and belonged to FAFLP-genotypes M and P (P₁ and P₂). When FAFLP and PFGE were combined, this resulted in an increased total of 22 genotypes (+ 2 variants) for 92 isolates of 14 flocks where *Campylobacter* was isolated.
Using flaA-RFLP, 13 genotypes could be distinguished using DdeI as restriction enzyme and only 9 using HinfI. FlaA-RFLP was not complementary to the FAFLP-PFGE combination, with one exception where flaA-DdeI enabled the distinction of genotypes 11 and 12 for 2 isolates belonging to PFGE genotype 11 (this also corresponded with a different antibiotic resistance profile (ARP) for these 2 isolates). PFGE-genotype 5 was divided in flaA-DdeI genotypes 5 and 13, which corresponded with the FAFLP results.

3.2. Broiler houses

Campylobacters were not isolated from the samples taken in the broiler house shortly before arrival of the 1-day-old chicks. During rearing, seven of the 18 broiler flocks became colonized with Campylobacter spp. First detection was for three flocks after 14 days (flocks 2, 3 and 11), for two flocks after day 28 (flocks 7 and 9), and at depletion for flocks 12 and 16. The Table shows the isolates with their source of isolation, genotypes and antibiotic resistance profile.

Six flocks were colonized exclusively with C. jejuni and one flock (flock 2) was initially colonized with C. jejuni (day 14) but replaced by C. coli at depletion. The nipple water inside the broiler house of all colonized flocks was initially found positive either at the same sampling time as the flocks (flock 12) or at a next sampling time (flocks 2, 3, 7, 9, 11). In flock 16, the nipple water was not found contaminated during the study because the flock colonization was not found earlier than at 42 days with only 3 of 12 pooled cecal samples positive for Campylobacter.

Broiler flocks were colonized by a variety of unique strains, as a strain with the same genotype was not isolated from more than one flock. The birds of flock 2 were colonized with C. jejuni strains of genotype P_1/P_2,-9,9 at day 14, but these were replaced by a C. coli
strain with genotype S,1,1,1. This latter type was also isolated from the nipple water at day 42. Colonization of flock 3 was detected from 14 days of rearing until depletion onwards with a *C. jejuni* strain of genotype F,2,2,2. The nipple water was also contaminated with this genotype both at 28 d and 42 d of rearing. Only at depletion, birds from flocks 12 and 16 were colonized with *C. jejuni*. For flock 12, the genotype C,16,7,7 was found on samples of broilers, nipple water and indoor footwear; for flock 16, the genotype M,-,-,- was found only on samples of broilers. *C. jejuni* strains with either genotype R₁,5,5,5 or R₂,5,5,5 were found colonizing flock 7 at 28 to 42 days of rearing and contaminating the nipple water and indoor footwear at 42 days of rearing. One of the two other poultry houses on this farm was colonized with a *C. jejuni* strain of another genotype (L,6,6,6). Flock 9 was colonized with two genotypes at 28 days of rearing (H,7,7,7 and N₂,7,7,7). Both genotypes were also isolated from the outdoor footwear at 28 days and genotypes H,7,7,7 and N₁,7,7,7 were also isolated from the nipple water at 42 days. At depletion, this flock was still found colonized with genotype N₁,7,7,7 (no more with genotype H,7,7,7), but also with another genotype B,8,8,8. The indoor footwear was at that moment still found contaminated with genotype H,7,7,7. At depletion, the other two flocks housed at this farm were colonized with *C. jejuni*: one with genotype B,8,8,8 and the other with genotypes G,13,12,7 and N₃,7,7,7. In flock 11, a succession of different genotypes was found. *C. jejuni* strains with genotypes Q₁,15,1,7 or Q₂,15,1,7 were found colonizing flock 11 at 14 days of rearing, but these genotypes were not found later on. At 28 days, the flock was colonized with a strain with genotype I,11,11,7, while the nipple water and the indoor footwear were contaminated with another genotype (A,10,10,7). At depletion, the flock was still colonized with genotypes I,11,11/12,7, but also with genotypes O,11'/12,12,7; nipple water and indoor footwear were then contaminated with genotype I,11/11”11,7.
3.3. Environment of broiler house

Campylobacter was isolated from the environment surrounding the broiler house and/or from other broilers on the same farm in four of the seven colonized flocks but also in four of the eleven negative flocks. On the farms with Campylobacter-positive status, isolation was done most frequently from the faecal material of other poultry houses (2 of 2 flocks sampled or 2/2, number of samples taken N=12) and from the ditch water (1/1, N=2), but also from the water puddles (3/5, N=22), from the footwear used outside the broiler house (1/4, N=5), from the container with dead chickens (1/4, N=12) and from the dung hill (1/6, N=16). All these isolates were identified as C. jejuni and some were of identical genotype as those of the broilers (Table). This was the case for one of the two broilers at the same farm for flock 9, but not for flock 7. All positive samples of the ditch water (flock 7), water puddles (flocks 9, 11 and 12) and footwear used outside the broiler house (flock 9) and from the container with dead chickens (flock 11) harboured the same genotype as those of the broilers. The dung hill of flock 7 harboured another genotype (D,5,13,5) as the ones that colonized the broilers (genotype R₁/R₂,5,5,5). The preceding flock in this broiler house (flock 6) remained negative during rearing, but the dung hill was also found contaminated with a strain with genotype K,4,4,4. These subsequent flocks 6 and 7 on the same farm harboured the same FAFLP-genotype K (in dung hill and wild birds’ faeces, respectively), but this genotype was not isolated from the broilers.

On the farms with Campylobacter-negative status, C. jejuni was isolated from the drains of the broiler house (1 of 2 flocks sampled or 1/2, N=2), from the dung hill (2/5, N=14), from the faecal material from other poultry houses (2/7, N=36), from the ditch water (1/5, N=7) and from the water puddles (1/8, N=23), but not from the footwear used outside the broiler house (0/7, N=14) or from the container with dead chickens (0/7, N=21). Remarkably, in the
environment of flock 18, three genotypes were already at day 1 found contaminating either wild birds’ faeces (-,11,1,7), the dung hill (-,18,1,7) or ditch water (J,19,9,9), but none of them was isolated from the broilers. The same FAFLP-genotype E was isolated from the environment of two flocks (wild birds’ faeces in flock 5 and faecal material other poultry house, drains of poultry house and water puddle in flock 13); this FAFLP-genotype was divided in two PFGE-genotypes according to the flock.

3.4. Animals surrounding the broiler house

Both in *Campylobacter* positive flocks and negative flocks, *Campylobacter* was detected in wild bird’s faeces (1 of 6 positive and 3 of 6 negative flocks sampled, N=25) and cow faeces (1 of 4 positive and 1 of 3 negative flocks sampled, N=16). The wild bird’s faeces always harboured *C. jejuni* but the genotype was different to the one colonizing the broilers (flock 7). The cow faeces on the other hand harboured *C. hyointestinales* in both occasions (flocks 12 and 15).

*Campylobacter* was not isolated from dog faeces (N=16), horse faeces (N=16), sheep faeces (N=5), caterpillars (N=2), insects (N=33), mice or rats (N=3), snails (N=2) or worms (N=8), deer faeces (N=2) or pig faeces (N=1).

3.5. Antibiotic resistance profiles

Overall, 47 of the 87 tested strains (54%) showed resistance to at least one of the antibiotics tested. No resistance to amoxicillin and gentamycin was found. Resistance to ampicillin was found for only one strain isolated from faecal material in another house from flock 9. Ciprofloxacin resistance was found in strains isolated from cecal drops from flocks 3
Isolates with erythromycin resistance were only found within flock 2. Resistance to nalidixic acid was found in strains isolated from cecal drops from flocks 3, 10 and 11. Also an isolate from cow faeces in flock 15 was resistant to nalidixic acid. Resistance to tetracycline was found in strains isolated from cecal drops from flocks 2, 3, 9, 10 (other house) and 11.

The ARP’s found were specific for strains from a certain flock. With two exceptions (sensitive to all antibiotics tested and ampicillin resistance for two strains of another poultry house), the \textit{C. jejuni} isolates from flock 9 were only resistant to Tc. The profile EmTc was found occurring in \textit{C. coli} isolates from flock 2. The profile CiNaTc was found in \textit{C. jejuni} isolates from flock 3, while the profile NaTc was found in \textit{C. jejuni} isolates from another house from flock 10. The profile CiNa was found in isolates from flock 11. In this flock, a succession of ARP’s was found which correlated well with the genotypes found. At day 14, all strains isolated (belonging to genotypes Q_{1/2},15,1,7) were found sensitive. At 28 days, a strain from the nipple water and belonging to genotype A,10,10,7 was found resistant according to the CiNa profile, while a strain from cecal drops belonging to the genotype I,11,11,7 was found sensitive to all antibiotics tested. At 42 days, strains belonging to genotypes I,11/11”,11,7 isolated from the nipple water and clean footwear were found resistant again according to this CiNa profile. However, at day 42, the strains from cecal drops of genotype I,11,11,7 were again sensitive, while other strains from cecal drops belonging to other genotypes were either intermediary resistant to tetracycline (genotypes -,11,12,7; O,12,12,7; O,11’,12,7) or resistant to nalidixic acid (genotype -,11,11,7).

4. Discussion
In this study, 38.9% (N=18) of the broiler flocks became colonized with thermotolerant *Campylobacter* spp. during rearing. Other studies report prevalence at the farm level of 27% in Sweden (Berndtson et al. 1996) to more than 90% in the United Kingdom (Evans and Sayers 2000). Although this study was not designed to evaluate seasonal differences in flock colonization, a trend in seasonal variation as mentioned by others (Berndtson et al. 1996; Jacobs-Reitsma et al. 1994) was found. The prevalence was highest when depletion occurred in the summer (75.0%, N=4) and spring (50.0%, N=4). In the autumn, the prevalence was 28.6% (N=7) and all flocks raised in the winter were considered *Campylobacter* free (N=3).

In contrast to other findings (Katsma et al. 2004), in our study the presence of other broilers on the same farm did not give rise to a more frequent colonization of the flock under investigation (P>0.05). In the absence of other flocks, 4 of 8 flocks became colonized. In the presence of two and three poultry houses on the farm, respectively 0 of 1 and 3 of 6 flocks became colonized. In the farms with each 4, 5 and 8 poultry houses, the flock under investigation was negative for *Campylobacter*. In the farms with 4 and 5 houses, all the other poultry houses were also sampled and were all found negative for *Campylobacter*. It may be that good biosecurity was practiced on these farms, as this can help to either prevent or delay flock colonization as shown before (Gibbens et al. 2001). In flock 10, in total 8 poultry houses were present on the farm. At 28 days, six flocks on this farm were sampled of which two were found colonized by an indistinguishable strain of *Campylobacter*. At 42 days, depletion already occurred for these two flocks. The remaining six flocks tested were all negative for *Campylobacter*.

Multiple typing was helpful for genotyping the 92 *Campylobacter* isolates. Using FAFLP, 20 genotypes could be distinguished and using PFGE, 19 genotypes could be distinguished. FAFLP and PFGE results showed to be complementary as when FAFLP and PFGE were
combined, this resulted in an increased total of 22 genotypes (+ 2 variants). *Fla* A-RFLP was not complementary to the FAFLP-PFGE combination, with one exception.

Multiple types of *Campylobacter*, as determined by FAFLP, PFGE and *fla* A-RFLP, were detected during rearing in three of the seven positive broiler flocks (i.e. 42.9%). This is in accordance with data presented by Shreeve et al. (2002), Hein et al. (2003) and Bull et al. (2006) who found that respectively 40%, 77% and 40% of flocks were colonized with more than one *Campylobacter* genotype. According to Höök et al. (2005), two hypotheses may explain the subsequent addition of genotypes: either subsequent introductions of *Campylobacter* or frequent mutations of the dominant clones. Although no firm conclusions can be drawn from our study, both processes could have taken place in some flocks. Subsequent introduction has occurred in some flocks as genotypes of strains differed greatly for flock 2 (*C. jejuni* genotype P₁/P₂-,9,9 and *C. coli* genotype S,1,1,1), for flock 9 (*C. jejuni* genotypes H/N₁/N₂,7,7,7 and B,8,8,8) and for flock 11 (*C. jejuni* genotypes Q₁/Q₂,15,1,7; I,11,11,7 and O,11'/12,12,7). However, in some of these flocks related genotypes were found, supporting the mutation hypothesis: for flock 9 (*C. jejuni* genotypes H,7,7,7 and N₁,7,7,7) and for flock 11 (*C. jejuni* genotypes O,12,12,7 and O,11’,12,7). In this study, the *Campylobacter* genotype that first colonized the broilers was sometimes replaced by other genotypes. At depletion, multiple types were detected in two broiler flocks. Of the seven positive flocks, in four occasions strains were resistant to at least one antibiotic tested. Mostly resistance was towards tetracycline (four flocks), but also multiresistance (EmTc, CiNaTc) was found in two flocks.

Following colonization, the nipple water of nearly all colonized flocks was contaminated with strains indistinguishable from these isolated from the broilers illustrating the importance of transmission of *Campylobacter* via the nipple water. This has been postulated by others (Evans 1992; Gregory et al. 1997), but has now been confirmed by detailed isolate
characterization. Chaveerach et al (2004) have shown that acidified drinking water can prevent *Campylobacter* spread via drinking water in broiler flocks. It should be remarked that in one flock (flock 11), the nipple water and indoor footwear were contaminated with a *C. jejuni* strain with genotype A,10,10,7. As this strain was not recovered from the cecal drops, it might be unable to infect the broilers.

*Campylobacter* was found in the environment surrounding the broiler house in four of the seven positive flocks. In three cases, isolates from water puddles were indistinguishable from isolates of the broilers. On nearly all occasions, isolation was accomplished at the same sampling time in the flock and water puddles. Once (flock 7) however, the ditch water was contaminated prior to flock colonization with a strain indistinguishable from isolates found later in the broiler flock. The study by Bull et al. (2006) also confirmed that water puddles were most frequently contaminated and they explained this by protection from desiccation, a stress to which campylobacters are particularly sensitive.

Wild birds have, under good management practices, no access to the broiler houses. However, contaminated droppings can be brought into the house, e.g. by footwear. In our study, *C. jejuni* was detected in wild bird’s faeces of 4 of all 12 flocks sampled. Once, also the flock was positive but it harboured another genotype. However, the wild bird’s faeces found at farm 5 (negative flock) harboured a *Campylobacter* strain with a related genotype as these isolated from the broilers at farm 10. Both farms were at a distance of about 21 km. It has been shown by Petersen et al. (2001) that the genotypes of the isolates from wild birds and from broilers are seldom the same, suggesting that the importance of wild birds as a reservoir of infection is limited. The presence of pet animals and livestock, such as pigs, cattle and sheep around the broiler house can also lead to contaminated material brought into the broiler house. In our study, cattle reared on two farms (7 farms sampled, N=16) were *Campylobacter* positive, but harbored *C. hyointestinalis*. In contrast, identical strains have
been found in cattle next to the broiler house and subsequently in the broiler house (Newell and Fearnley 2003). Samples of dog (10 flocks sampled, N=16), horse (3 flocks sampled, N=6), sheep (3 flocks sampled, N=5), deer (1 flock sampled, N=2) or pig faeces (1 flock sampled, N=1) were negative for *Campylobacter* in our study.

The significance of rodents, mice in particular, as vectors and reservoirs of *Campylobacter* has been shown by Meerburg et al. (2006). In our study, *Campylobacter* infected mice or rats were not found (N=3). Anyhow, since most farms apply rodent control programs, rodents are no longer considered as a significant risk factor for introducing *Campylobacter* in broiler houses (Berndtson et al. 1996). More difficult to control are insects. All insects sampled in 12 flocks (N=33) in our study did not carry *Campylobacter*, while other studies reported on this and considered this a considerable risk for infection of broilers with *C. jejuni* and *C. coli* (Hald et al. 2004; Hald et al. 2008). In the study by Hald et al. (2008), the prevalence of *Campylobacter* spp.-positive flies in summer was considerably higher on a farm with pig production compared to these without other livestock, for which the prevalence was below 1.0%. In our study, pig production was apparent in only one farm, in which the flock status was *Campylobacter* negative and sampling occurred at the end of September. At this period, the prevalence in flies dropped to 2.0% for a farm with pig production in the study by Hald et al. (2008). Also, the caterpillars (2 flocks sampled, N=2), snails (1 flock sampled, N=2) and worms (5 flocks sampled, N=8) sampled in our study did not harbour *Campylobacter*.

In conclusion, molecular typing is an important tool to provide knowledge on the transmission of *Campylobacter* at farm level. Our results show that multiple genotypes of *C. jejuni* may be present in a commercial broiler flock during rearing. New types probably appeared either by subsequent introductions of *Campylobacter* or by frequent mutations of the dominant clones. The outdoor environment constitutes a risk for infecting the broilers: the ditch water was found contaminated prior to while the water puddles at the same moment as
the flock colonization. This study also clearly demonstrated that once *Campylobacter* is introduced in the flock, the nipple water is subsequently contaminated leading to a further spread of the organisms throughout the whole flock.

Acknowledgements

This study was financed by the Belgian State, Services of the Prime Minister – Federal Services for Scientific, Technical and Cultural Affairs in the framework of the programme “Scientific support of a prenormative investigation of the food sector in the frame of a durable development” and by the Federal Public Service Health, Food Chain Safety and Environment, project R-04/002-CAMPY. Petra Vanmol, Elly Engels, Veronique Ottoy, Jessy Claeys and Sandra Vangeenberghe are acknowledged for excellent practical assistance.
Figure legend. Dendrogram derived from FAFLP patterns of 88 *C. jejuni* and 4 *C. coli* isolates from broilers, broiler houses and environment. The collection number is given followed by the flock identity, the age of the birds at sampling, the sample type and the genotype (see Table). FAFLP fingerprints were generated from chromosomal DNA digested with the *Hind*III and *Hha*I. The specific primers used were *Hind*III+A and *Hha*I+A. The dendrogram was constructed by using UPGMA. The scale indicates percentages of similarity, as determined with the Pearson coefficient.
References


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# Table.

*Campylobacter jejuni* (unless otherwise stated) genotypes from broilers, broiler houses and environment

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</thead>
<tbody>
<tr>
<td>1 d</td>
<td>n.a.</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>14 d</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>28 d</td>
<td>0/6</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>42 d</td>
<td>0/3</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

**Flock 15 (5)**

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
<th>Se</th>
<th>Flock Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 d</td>
<td>n.a.</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>14 d</td>
<td>0/4</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>28 d</td>
<td>0/4</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>42 d</td>
<td>0/4</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Flock 16 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>1 d</td>
<td>n.a.</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>14 d</td>
<td>0/6</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>28 d</td>
<td>0/9</td>
<td>0/1</td>
<td></td>
</tr>
</tbody>
</table>
| 42 d                      | 3/12    | M,-,,-,- (1) | se | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         | 0/1D, 0/1C, 0/3DC, 0/1DH, 0/1PU
|                           |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |

| Flock 17 (4)              |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
|---------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 1 d                       | n.a.    | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
| 14 d                      | 0/3     | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
| 28 d                      | 0/7     | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
| 42 d                      | 0/5     | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |

| Flock 18 (1)              |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
|---------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 1 d                       | n.a.    | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
| 14 d                      | 0/5     | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
| 28 d                      | 0/4     | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |
| 42 d                      | 0/5     | 0/1     |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |       |

1 n.a., not applicable
2 -, not determined
3 se, sensitive to all tested antibiotics
4 ° Flock number and number of poultry houses on the rearing farm (between brackets); age of the birds at sampling in days (d)
5 Bold type indicates positive results
6 ° Samples of broiler flocks were obtained from cecal material.
7 ° Indoor footwear is footwear by the farmer used inside the broiler house.
8 ° Other symbols in superscript: B, wild birds faeces; C, cow faeces; CP, caterpillar; D, dog faeces; DC, container with dead chickens; DE, faecal material deer; DH, dung hill; DR, drains of poultry house; DW, ditch water; H, horse faeces; I, insects; OH, faecal material other houses; OF, outdoor footwear; M, mouse; P, pig faeces; PU, water puddles; R, rat; SN, snail; SH, sheep faeces; W, worm.
1 number of positive samples per number of samples tested.

2 Genotype determined by a combination of FAFLP, PFGE-SmaI, FlaA-DdeI and FlaA-Hinfl in that order. The number of samples from which each genotype was isolated, is given between brackets. If more than one genotype was obtained from the same sample, the different genotypes are separated by a slash.

3 resistant or intermediate resistance (between brackets) for antibiotics.

4 This strain was used to assess the reproducibility of the FAFLP assay.

5 This strain is a C. coli strain; unless otherwise stated, all other strains were C. jejuni.

6 This strain was lost during storage.

7 This strain is a C. hyointestinalis strain; unless otherwise stated, all other strains were C. jejuni.