Epidemiological study of Campylobacter contamination of broiler farms in Belgium by amplified fragment length polymorphism analysis

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Amplified fragment length polymorphism (AFLP) analysis was used for genetic typing of 88 Campylobacter jejuni subsp. jejuni and 4 Campylobacter coli isolates. Restriction endonucleases HindIII and HhaI were used in combination with the selective PCR primers HindIII+A and HhaI+3+A. Selectively amplified PCR products were separated in an ABI 310 genetic analyser. After normalization, pattern similarity was calculated using the Pearson correlation coefficient. Banding patterns exhibiting 89.6 to 92.8% homology were obtained upon assessing the reproducibility of the method. As a consequence, a cut-off similarity value of 90% was used as the cut-off level for identical patterns. A total of 18 broiler flocks, including 16 independent and 2 successive flocks in the same broiler house, were sampled for Campylobacter. A wide range of samples was taken including caecal droppings, nipple water, footwear, animal (e.g. faecal material domestic/wild animals) and non-animal (e.g. compost heap, ditch water, puddle) material in the environment. The infection of the broiler flocks increased during rearing, with a total of seven positive flocks at the end of rearing. Almost exclusively C. jejuni subsp. jejuni was detected during rearing, with the exception of one flock with a mixed C. jejuni subsp. jejuni – C. coli infection. On the farms with Campylobacter-positive status, isolation was done most frequently in the environment from the puddles (3 out of 4 flocks), the faecal material from other poultry houses (2 out of 2 flocks), the ditch water (1 out of 1 flock) and dirty footwear used outside the broiler house (1 out of 2 flocks). Isolates that clustered together always originated from the same farm; while within a single farm only limited diversity (1-4 AFLP types) was found. Isolation of the same AFLP type from animals and environmental sources gave evidence for dispersal and circulation of Campylobacter in the environment. The clustering obtained by AFLP analysis was in very good agreement with the grouping of the isolates based on previously generated pulsed field gel electrophoresis fingerprints. We concluded that AFLP analysis is an attractive tool which can be used for typing large numbers of Campylobacter strains and is extremely useful for epidemiological investigations.

Keywords: Campylobacter; poultry; amplified fragment length polymorphism; molecular epidemiology; genomic fingerprinting

Introduction

The zoonosis campylobacteriosis, which is caused by thermophilic Campylobacter spp., is an important public health problem in most areas of the world. Campylobacter was the most commonly reported gastrointestinal bacterial pathogen in humans in the EU in 2004 (EFSA 2005). In Belgium, the reported incidence of campylobacteriosis was 64.3 per 100,000 inhabitants (Ducoffre 2005). The true incidence of campylobacteriosis in industrialized countries is uncertain since many unreported infections occur for every diagnosed case, but it may exceed one percent per year (WHO 2000). Typically, about 90% of the isolates from human campylobacteriosis cases are identified as
Campylobacter jejuni subsp. jejuni (hereafter C. jejuni), while C. coli accounts for most of the remaining cases (EFSA 2005). Thermophilic Campylobacter spp. are widespread in nature and the principal reservoirs are the alimentary tracts of wild and domesticated birds and mammals. Consequently, isolations are commonly done from water sources, farm animals such as poultry, cattle, pigs and sheep, as well as from cats and dogs (Jones 2001). The bacteria can readily contaminate various foodstuffs, including meat, raw milk and dairy products, and, less frequently, fish and fishery products, mussels and fresh vegetables (EFSA 2005). The majority of human Campylobacter infections occur sporadically, with the major vehicle of infection assumed to be contaminated food, and in particular chicken (Skirrow 1994; Friedman et al. 2004). In Belgium in 2004, surveillance showed a broiler carcass contamination rate of 28% at slaughter and 35% at retail. Mainly C. jejuni was isolated (EFSA 2005).

The use of typing methods, and in particular genotyping methods such as pulsed field gel electrophoresis (PFGE), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the flagella (flaA and flaB) genes and amplified fragment length polymorphism (AFLP), have proven valuable tools for epidemiological investigation of Campylobacter spp. (Wassenaar and Newell 2000). In our study, Campylobacter isolates from 18 individual broiler flocks were typed by multiple genotypic techniques in order to determine contamination routes in the farm environment.

Materials and methods

**Bacterial strains.** All isolates were obtained from samples taken from 18 Belgian commercial broiler flocks, consisting of 16 independent flocks and 2 successive flocks in the same broiler house (nos. 6 and 7). More information about sample collection can be found in Herman et al. (2003). Briefly, before arrival of the one-day-old chicks (day 1), the hygiene inside the broiler houses was checked by sampling feed and water supplies, swabs from feed boxes, ventilation and heating provisions, walls, insects and spiders, and movable material. During the rearing period (6 weeks), the farms were visited three times (days 14, 28 and 42) with sampling of caecal drops, overshoes, feed and drinking water. The environment of the farms was sampled before and during the rearing period (days 1, 14, 28 and 42) by taking faeces from other animals, water from puddles and ditches, the containers with dead animals and the footwear of the farmers. Campylobacter isolates were obtained from these samples using selective enrichment methods. Strains were stored in brain-heart infusion broth (BHI) supplemented with 5% defibrinated horse blood at -80°C using 15% (w/v) glycerol as cryoprotectant. Identification of the isolates as one of the thermotolerant campylobacters, C. jejuni or C. coli, was performed in a multiplex PCR as described by Herman et al. (2003).

**FAFLP.** Strains were cultured for 2 days at 42°C under microaerophilic conditions on blood agar plates supplemented with 7% defibrinated horse blood. Cells were scraped from the plates, DNA was prepared as described by Flamm et al. (1984) and DNA concentrations were standardized at 1 µg/26 µl. DNA integrity was checked by agarose gel electrophoresis, and DNA preparations were stored at –20°C. FAFLP was performed on 500 ng of genomic DNA digested in a total volume of 30 µl, which consisted of 5 U of HindIII endonuclease, 5 U of HhaI endonuclease, 3 µl of a buffer containing 100 mM Tris-HCl, 100 mM MgCl₂, 500 mM NaCl, 10 mM DTT (pH 7.9) and 0.3 µl of 100× bovine serum albumin (BSA), for 3 h at 37°C. To the digested DNA, 25 µl of a solution containing 0.5 µl of 2 µM HindIII adaptor, 0.5 µl of 20 µM HhaI adaptor, 40 U of T4 DNA-ligase, and 5 µl of 10× T4 ligase buffer was added. Ligation was carried out overnight at room temperature. 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 (1 µM), 2.2 µl of HhaI primer (5 µM), and 15.6 µl of amplification core mix. Amplification was performed in a programmable thermocycler using an initial denaturation step at 94°C (2 min), followed by 20 cycles consisting of 94°C for 20 s, 56°C for 45 s and 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), and 11 µl of amplification core mix. Amplification was performed using an initial denaturation step at 94°C (2 min), followed by 20 cycles consisting 94°C for 20 s, 66°C for 45 s and 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,
1 µl of Gene-Scan-500 size standard labeled with ROX 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. 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 4.0 software
package (Applied Maths, Sint-Martens-Latem, Belgium) with the program ABIICON (Applied Maths).
Gels were normalized by using the internal standard that was added to each sample. After
normalization of the gels, the levels of genetic similarity between AFLP patterns were calculated with
the Pearson product-moment correlation coefficient. For cluster analysis of AFLP banding patterns the
unweighted pair group method using average linkages (UPGMA) was used. The best possible match
among profiles was obtained by use of a 0.06% optimisation coefficient. Profiles containing fragments
in the size range of ca 50-500 bp were used in the analysis.

PFGE. Strains were cultured for 2 days at 42°C under microaerophilic conditions on charcoal
cefoperazone desoxycholate agar (CCDA) plates. PFGE was performed as described previously
(Zorman et al. 2006) using SmaI as restriction enzyme. 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 4s to 40s in 22h. The gels were stained with
ethidium bromide and photographed. PFGE profiles were clustered with BioNumerics 4.0 (Applied
Maths) using the Dice coefficient and UPGMA.

Results and discussion

Isolation. A total of 18 broiler flocks were sampled in the broiler house. The study included 17
different poultry houses on 17 different farms. In the broiler house just before arrival of the 1-day-old
chicks, no Campylobacter was isolated from any of the samples. The Campylobacter status of a flock
was considered positive when at least one pool of caecal drops tested positive. The infection of the
broiler flocks increased during rearing from 3 flocks at day 14, to 4 flocks at day 28 and 7 flocks
(flocks 2, 3, 7, 9, 11, 12, 16) at 42 days, just before slaughtering. Almost exclusively C. jejuni was
detected during rearing, with the exception of one flock (flock 2) that was initially colonized with C.
jejuni (day 14) but replaced by C. coli at the end of rearing. On the farms with Campylobacter-positive
status, isolation was done most frequently in the environment from the puddles (3 out of 4 flocks
where sampling was done), the faecal material from other poultry houses (2 out of 2 flocks), the ditch
water (1 out of 1 flock) and footwear used outside the broiler house (1 out of 2 flocks).

Reproducibility of AFLP analysis. The reproducibility of the AFLP analysis was assessed by
performing the whole assay, starting with isolation of chromosomal DNA, three times with three C.
jejuni strains. The similarity between patterns obtained was at least 90%, as determined by the Pearson
product-moment correlation, and the results correlated perfectly with previous described
reproducibility values (On and Harrington 2000; Duim et al. 2001). A cut-off similarity value of 90%
was therefore used as the cut-off level for identical patterns.

Distribution of types. Of the 93 C. jejuni isolates studied, 20 different AFLP profiles were
obtained. For 4 clusters, similarity was between 80-90%, these clusters were given subscript letters.
Only one AFLP profile was obtained for the 4 C. coli isolates from flock 2.

In 6 out of the 7 flocks with Campylobacter-positive status, the nipple water inside the broiler
house was tested positive for Campylobacter. The nipple water was not tested positive for flock 16,
where colonization of the flock was not found earlier than at day 42. Table 1 gives an overview of the
AFLP profiles obtained for each flock during rearing. Flock 2 was first (at day 14) found colonized
with a strain from cluster P1/P2 that was replaced at day 42 by a C. coli strain that was clustered in S.
The latter strain was also isolated from the nipple water at day 42. Flock 3 was found colonized with a
strain from cluster F2 at all sampling periods. The same strain was also isolated from the nipple water
at 28 d and 42 d of rearing. For flock 7, the strain from cluster R9 colonizing the birds at 28 and 42
days of rearing was also isolated from the nipple water and the clean footwear at 42 days of rearing.
The typing results indicate that infection of the flock was probably caused by the ditch water, as a
strain from the same cluster was isolated from this site already at 14 days of rearing. The strains that
were isolated, also at 14 days of rearing, from the faecal material of the wild animals (cluster K) and
from the dung hill (cluster D) were grouped in other clusters by AFLP and are thus not the source of infection. Birds from another house on this farm were found colonized with a strain from another cluster (cluster L).

Table 1. Profiles by AFLP of *Campylobacter* isolates from flocks and the environment. *Campylobacter* was not recovered from any sample from flock 1, 4, 8, 14. Only sample types which were found *Campylobacter*-positive are mentioned in this table.

<table>
<thead>
<tr>
<th>Flock/no. houses&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Positive or negative flock</th>
<th>Sample type&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Age of the birds at sampling (days)</th>
<th>14</th>
<th>28</th>
<th>42</th>
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<sup>a</sup>- indicates that *Campylobacter* was not recovered from these samples.
<sup>b</sup>Flock number/number of poultry houses on the rearing farm.
<sup>c</sup>Samples of broiler flocks were obtained from caecal material. Clean footwear is footwear by the farmer used inside the broiler house. nd, not determined. Other: codc, container with dead chickens; df, dirty footwear; dh, dung hill; dr, drains of poultry house; dw, ditch water; fm, faecal material outside the rearing house; fmc, faecal material cows; fmwa, faecal material wild animals; fmoh, faecal material other houses; pu, puddles.
<sup>d</sup>*C. coli.*

**Flock 9** was colonized with two strains at day 28 of rearing, one from cluster H and one from cluster N<sub>1</sub>. These strains were also isolated from the dirty footwear at day 28 and from the nipple water at day 42. At day 42 of rearing, this flock was still found colonized with the strain from cluster N<sub>1</sub> (no more with a strain from cluster H although the clean footwear was still found contaminated with a
strain from this cluster), but also with a strain from cluster B. In the other two houses of this farm, one flock was found colonized with strains from cluster B and the other flock with strains from clusters G and N. The puddle was found contaminated with a strain from cluster N at 28 days of rearing, again indicating the importance of the puddle for transmission of Campylobacter. Strains from clusters Q and Q2 were isolated from caecal drops of flock 11 at day 14, but not later on during rearing. At day 28, the flock was found colonized with a strain from cluster I, while the nipple water and the clean footwear were found contaminated with a strain from cluster A. At day 42, the flock was still found colonized with a strain from cluster I, but also with a strain from cluster O. Nipple water, clean footwear and a container with dead chickens were also contaminated with a strain from cluster I. Already at 14 days of rearing, the puddle was found contaminated with a strain from cluster Q; again a strain from the same cluster as the one colonizing the birds. Birds from flock 12 were found colonized with a strain from cluster C at day 42. At the same moment, this strain was also found in the nipple water, clean footwear and also in the puddle. For flock 16, colonization of the broiler flock was obvious at day 42 of rearing with a strain from cluster M.

The surroundings of 7 flocks out of the 11 flocks with a Campylobacter-negative status were found contaminated with Campylobacter. The faecal material of wild animals was found contaminated in flock 5 and 17, the dung hill in flock 6 and 18, the faecal material of other houses in flock 10 and 13, the puddle and drains of the poultry house in flock 13, the faecal material of cows in flock 15 and the faecal material outside the rearing house and ditch water in flock 18.

Isolates that clustered together always originated from the same farm, with a few exceptions. Cluster E both contains isolates in the surroundings of flock 5 (faecal material of wild animals) and flock 13 (other poultry house, puddle and drains of poultry house). Cluster F contains closely related isolates colonizing flock 3 (F1) and another flock of farm 10 (F2). In the presence of flock 6, the dung hill was found contaminated with a strain from cluster K. When flock 7 was present, faecal material of wild animals was found contaminated with a strain from cluster K. At that moment, the dung hill was found contaminated with a strain from cluster D. Flocks 6 and 7 are actually 2 subsequent flocks in the same house.

Within a single farm only very limited diversity (1-4 AFLP types) was found. Several studies suggest a low biodiversity (one or two types) among the Campylobacter isolates from the same flock (e.g. Chuma et al (1993) and Shreeve et al (2000)), although Stern et al. (1997) and Thomas et al. (1997) found greater biodiversity. Single birds can be colonized by more than one PFGE type of Campylobacter (Hook et al. 2005). In our study, pools of 10 caecal drops were analyzed for Campylobacter. In flock 9, having a mixed infection of the flock with strains from cluster N1 and B, one pooled sample was found contaminated with both strains. Isolation of the same AFLP type from animals and environmental sources gave evidence for dispersal and circulation of Campylobacter in the environment. The ditch water (flock 7), puddle (flock 9, 11, 12), dirty footwear (flock 9), faecal material other house (flock 9) and container with dead chickens (flock 11) were shown to be contaminated with the same strain as the one colonizing the flock.

The clustering obtained by AFLP analysis was in very good agreement with the grouping of the isolates based on previously generated pulsed field gel electrophoresis fingerprints.

To conclude, AFLP analysis is an attractive tool which can be used for typing large numbers of Campylobacter strains and is extremely useful for epidemiological investigations. The most likely route of transmission of Campylobacter to the birds seems to be the poultry house surroundings. Although the surroundings of 7 flocks out of the 11 flocks were found contaminated with Campylobacter, the status of the flock remained negative. These farms might have good bio security rules applied or the Campylobacter strains were unable to colonize the flock.

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