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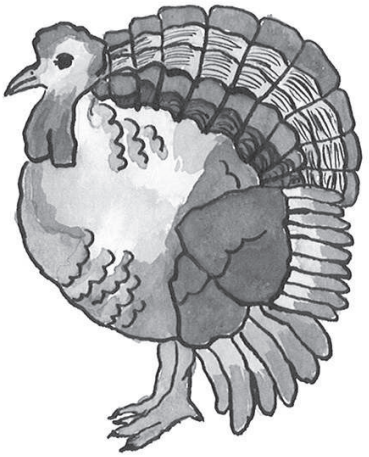

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Campylobacter jejuni and *C. coli* in Finnish poultry production

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To be presented with the permission of the Faculty of Veterinary Medicine, University of Helsinki, for public examination in Auditorio 2, Kampusranta 9 B, Seinäjoki on August 19th, 2011, at 12 o'clock noon.

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Abstract

Campylobacter, mainly *Campylobacter jejuni* and *C. coli*, are worldwide recognized as a major cause of bacterial food-borne gastroenteritis (World Health Organization 2010). Epidemiological studies have shown handling or eating of poultry to be significant risk factors for human infections. *Campylobacter* contamination can occur at all stages of a poultry meat production cycle.

In summer 1999, every broiler flock from all three major Finnish poultry slaughterhouses was studied during a five month period. Caecal samples were taken in the slaughterhouses from five birds per flock. A total of 1 132 broiler flocks were tested and 33 (2.9%) of those were *Campylobacter*-positive. Thirty-one isolates were identified as *C. jejuni* and two isolates were *C. coli.* The isolates were serotyped for heat-stable antigens (HS) and genotyped by pulsed-field gel electrophoresis (PFGE). The most common serotypes found were HS 6,7, 12 and 4-complex. Using a combination of *Sma*I and *Kpn*I patterns, 18 different PFGE types were identified.

Thirty-five Finnish *C. jejuni* strains with five *Sma*I/*Sac*II PFGE types selected among human and chicken isolates from 1997 and 1998 were used for comparison of their PFGE patterns, amplified fragment length polymorphism (AFLP) patterns, *Hae*III ribotypes, and HS serotypes. The discriminatory power of PFGE, AFLP and ribotyping with *Hae*III were shown to be at the same level for this selected set of strains, and these methods assigned the strains into the same groups. The PFGE and AFLP patterns within a genotype were highly similar, indicating genetic relatedness. An HS serotype was distributed among different genotypes, and different serotypes were identified within one genotype.

From one turkey parent flock, the hatchery, six different commercial turkey farms (together 12 flocks) and from 11 stages at the slaughterhouse a total of 456 samples were collected during one and the half year. For the detection of *Campylobacter* both conventional culture and a PCR method were used. No *Campylobacter* were detected in either of the samples from the turkey parent flock or from the hatchery samples using the culture method. Instead PCR detected DNA of *Campylobacter* in five faecal samples from the turkey parent flock and in one fluff and an eggshell sample. Six out of 12 commercial turkey flocks were found negative at the farm level but only two of those were negative at slaughter. *Campylobacter*-positive samples within the flock at slaughter were detected between 0% and 94%, with evisceration and chilling water being the most critical stages for contamination. All of a total of 121 *Campylobacter* isolates were shown to be *C. jejuni* using a multiplex PCR assay. PFGE analysis of all isolates with *Kpn*I restriction enzyme resulted in 11 PFGE types (I-XI) and *flaA*-SVR typing yielded nine *flaA*-SVR alleles. Three *Campylobacter*-positive turkey flocks were colonized by a limited number of *Campylobacter* genotypes both at the farm and slaughter level.

In conclusion, in our first study in 1999 a low prevalence of *Campylobacter* in Finnish broiler flocks was detected and it has remained at a low level during the study period until the present. In the turkey meat production, we found that flocks which were negative at the farm became contaminated with *Campylobacter* at the slaughter process. These results suggest that proper and efficient cleaning and disinfection of slaughter and processing premises are needed to avoid cross-contamination. Prevention of colonization at the farm by a high level of biosecurity control and hygiene may be one of the most efficient ways to reduce the amount of *Campylobacter*-positive poultry meat in Finland. In Finland, with a persistent low level of *Campylobacter*-positive flocks, it could be speculated that the use of logistic slaughtering, according to *Campylobacter* status at farm, might have be advantageous in reducing *Campylobacter* contamination of retail poultry products. However, the significance of the domestic poultry meat for human campylobacteriosis in Finland should be evaluated.

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This study started at the Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki during the period 1999-2001. Since then, the study was continued at the National Veterinary and Food Research Institute (EELA) later the Finnish Food Safety Authority Evira, Research Department, the Production Animal Health Unit and finally the Production Animal and Wildlife Unit. Since, 2006, the study was carried out in cooperation with Ruralia Institute, University of Helsinki. For the opportunity to carry out this project I thank Professor Hannu Korkeala, the head of the Department of Food Hygiene and Environmental Health, Professor Tuula Honkanen-Buzalski, previously the director general of EELA and the current head of the Research Department in Evira, Marja Fossi, DVM PhD, my former manager and Professor Antti Oksanen, my current manager, I also thank the head of Ruralia Institute Professor Sami Kurki. During all the years, the study was financially supported by the Finnish Graduate School of Applied Bioscience, the Finnish Veterinary Science Foundation, the Finnish Cultural Foundation, South Ostrobothnia Regional fund, the Oiva Kuusisto Foundation, Lapuan naisyhdistys r.y., and The Education Fund. I express my special thanks to these organisations, which made this work possible for me.

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Contents

List of original publications

This thesis is based on the following publications:

I Perko-Mäkelä, P., Hakkinen, M., Honkanen-Buzalski, T. and Hänninen M.-L. 2002. Prevalence of *Campylobacters* in chicken flocks during the summer of 1999 in Finland. Epidemiol Infect 129: 187-192.

II Hänninen, M.-L., Perko-Mäkelä, P., Rautelin, H., Duim, B. and Wagenaar, J. 2001. Genomic relatedness within five common Finnish *Campylobacter jejuni* pulsed-field gel electroforesis genotypes studies by amplied fragment length polymorphism analysis, ribotyping and serotyping. Appl Environ Mirobiol 67: 1581-1586.

III Perko-Mäkelä, P., Isohanni, P. Katzav, M., Lund, M., Hänninen, M.-L. and Lyhs, U. 2009. A longitudinal study of *Campylobacter* distribution in a turkey production chain. Acta Vet Scan 51:81.

IV Perko-Mäkelä, P., Alter, T., Isohanni, P., Zimmermann, S. and Lyhs, U. 2011. Distribution of *Campylobacter jejuni* isolates from turkey farms and different stages at slaughter using pulsed-field gel electrophoresis (PFGE) and *flaA*-short variable region (SVR) sequencing. Zoonoses Public Health. Article first published online: 13 JAN 2011, DOI: 10.1111/j.1863-2378.2010.01383.x

The publications are indicated in the text by their Roman numerals. The original articles have been reprinted with the permission of their copyright holders.

Abbreviations

AFLP	amplified fragment length polymorphism
bp	base pair
cfu	colony-forming unit
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid
EFSA	European Food Safety Authority
flaA	flagellin A gene
HACCP	hazard analysis and critical control points
HS	heat-stable
HL	heal-labile
mCCDA	modified charcoal cefoperazone deoxycholate agar
MLST	multilocus sequence typing
PCR	polymerase chain reaction
PFGE	pulsed-field gel electroporesis
SVR	short variable region
rRNA	ribosomal ribonucleic acid
ST	sequence type
UV	ultraviolet
UPGMA	unweighted pair group method using arithmetic averages
VBNC	viable but non-cultivable

1 Introduction

Poultry meat has become an everyday food for Finns over the last decades. Since 1995, the consumption of broiler meat has more than doubled and consumption of turkey meat is now almost four times higher. Nevertheless the amount of consumed meat is relatively low, 15.6kg broiler meat and 1.7kg of turkey meat per person per year. Most of the poultry meat consumed in Finland is sourced domestically. About 90% of poultry meat production is broiler meat and 10% is turkey meat. Other poultry has rather an insignificant role in Finland (http://www.siipi.net/).

Salmonella is a well-known food related zoonotic bacterium; especially poultry and eggs are high risk sources for *Salmonella* infection. In Finland, mandatory *Salmonella* control programme in poultry meat and egg production has been carried out since 1995. In 2009, 2 338 *Salmonella* cases with an incidence rate of 44/100 000 were reported. However, since 1999 the number of registered *Campylobacter* cases in Finland has been higher than that for *Salmonella*. In 2009, 4 048 campylobacteriosis cases were reported and the incidence was 76/100 000 (National Institute for Health and Welfare 2010) .

Several studies have shown the eating and handling of improperly cooked or raw poultry meat to be one of the most important sources for human campylobacteriosis (Kapperud et al. 2003, Michaud et al. 2004, EFSA Panel on Biological Hazards (BIOHAZ) 2010). Increasingly, other pathways for transmission than poultry have been pointed out to be important, for example, the environment, cattle and pets. However, poultry meat was shown to be an important source in Dioxin contamination in 1999 in Belgium (Vellinga and Van Loock 2002). Significant differences may occur between countries in the prevalence of *Campylobacter* in poultry at the farm and in retail poultry products (EFSA 2010a). To control and reduce consumer exposure to *Campylobacter* from contaminated poultry meat, different measures have been applied. At the farm level, biosecurity, defined as a set of preventive measures designed to reduce the risk of transmission of infectious diseases, is the often underlined factor. Interventions at slaughter, scheduled slaughtering or sorting of flocks according to *Campylobacter* status and different methods, such as steam treatment, to reduce the number of *Campylobacter* at the slaughter process have been evaluated (Northcutt et al. 2005, Sandberg et al. 2005, Smith et al. 2005, Arsenault et al. 2007, James et al. 2007, Katsma et al. 2007). In addition, good overall hygiene control, washing and chilling of the poultry carcasses and freezing of the meat are in use in processing plants to reduce the contamination level. In the EU, under Regulation (EC) No 853/2004, decontamination treatments are allowed to be considered as a supplement to good hygiene practices, but none of them are currently authorized in the EU (http://www.fsai.ie/uploadedFiles/Reg853_2004(1).pdf). In Finland, the mandatory *Campylobacter* monitoring programme for broiler slaughter batches started in 2004 (http://wwwb.mmm.fi/el/laki/j/10_EEO_2007.pdf). The programme implies no action for broiler meat originated from a *Campylobacter*-positive flock. To monitor *Campylobacter*

in turkey meat production, the slaughterhouse carries out its own control tests (personal communication, 2010).

Application of different genotyping methods of *Campylobacter* isolates from different stages of the poultry meat production chain provides information about the relationship of *Campylobacter* strains from different origins. Genotyping is an important tool to understand the epidemiology of human *Campylobacter* infections and the role of poultry as a source of infection. Different typing methods have been developed and used in epidemiological studies of *Campylobacter.* PFGE has been widely used and the protocols of Pulsenet (Ribot et al. 2001) and Campynet (http://campynet.vetinst.dk/) have been harmonizing the methods and make comparison more reliable. Other restriction-based methods such as AFLP and sequence-based methods such as *FlaA*-SVR and MLST have been useful typing schemes. Each method has its own limitations and may, however, show different relationships between strains (Meinersmann et al. 2005).

2 Review of the literature

2.1 *Campylobacter spp.*

As early as 1886, Theodor Escherich described nonculturable spiral shaped bacteria. The name 'campylobacter' is based on the morphology of the bacteria. The Greek word 'Campylo' means curved and 'bacter' means rod. *Campylobacter* (called *vibrios*) were successfully cultivated for the first time in 1913 by McFadyean and Stockman from aborted ewes (Butzler 2004, Skirrow 2006). After recognition that the organisms differ from *Vibrio* spp., the genus *Campylobacter* was established in 1963 (Sebald and Veron 1963, Moore et al. 2005). Taxonomy of the genus has been revised over the years (Butzler 2004, Vandamme et al. 1991, Vandamme and On 2001).The role of *Campylobacter* as an enteric pathogen remained undiscovered until the 1970s, mainly because of the difficulty of cultivating and isolating these bacteria from faecal samples. Using improved isolation methods in the cultivation of faecal samples of patients with enteric symptoms, as well as epidemiological studies, led to the conclusion that *Campylobacter (C.) jejuni and C. coli* are an important cause of human enteric illness (Skirrow 2006, Butzler et al. 1973, Skirrow 1977). To date, the genus *Campylobacter* comprises 17 validated species, most are human or animal pathogens or zoonotic pathogens (Debruyne et al. 2008).

Members of the genus *Campylobacter* are spiral curved, gram negative rods. The size of the cells is 0.2 to 0.8 μm wide and 0.5 to 5 μm long. Cells of most of the species are motile and have a single polar unsheathed flagellum at one or both ends. *Campylobacter* grow under microaerobic conditions, but some species grow anaerobically or aerobically. All *Campylobacter* grow at 37ºC, but for the thermophilic species *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* the optimum temperature is 42ºC. *Campylobacter* are fragile organisms, susceptible to a number of environmental conditions such as temperature, the presence of oxygen, pH, UV and humidity, but may survive in a viable but non-cultivable form (VBNC) in the environment (Talibart et al. 2000, Isohanni and Lyhs 2009). There is no one simple standard method for routine isolation of all *Campylobacter* species. The predominant species *C. jejuni* and *C. coli* grow in a microaerobic atmosphere on selective media. To study the presence of less common species, appropriate cultivation conditions need to be applied (Debruyne et al. 2008).

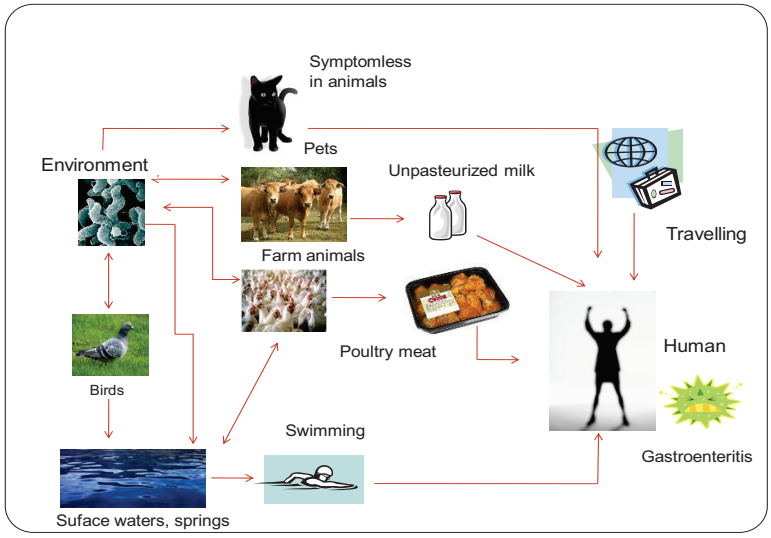
2.2 *Campylobacter* in humans

C. jejuni and C. coli are the most common causes of food-borne bacterial gastroenteritis in humans worldwide (Moore et al. 2005). In the European Food Safety Authority (EFSA) report on zoonoses in 2008, incidences of campylobacteriosis from <0.1 to 193.3/100 000

of the population in European countries was reported (EFSA 2010b). In Finland, the reported incidence in 2009 was 76/100 000 (National Institute for Health and Welfare 2010). The incubation time in campylobacteriosis is one to seven days and the infective dose of *C. jejuni* can be as low as 500 bacteria (Robinson 1981, Black et al. 1988). The main symptoms are cramp in the abdomen followed by diarrhoea. Also general symptoms such as fever, headache, dizziness and myalgia may occur. Late onset complications such as reactive arthritis, Reiter's syndrome, Guillain-Barré and Miller Fisher syndromes have been associated with *Campylobacter* enteritis (Blaser and Engberg 2008).

Campylobacter infections are mostly sporadic and this makes it challenging to define the sources of the infections. However the major sources have been identified. Food has been mentioned as the main transmission vector (Jacobs-Reitsma et al. 2008). The environment, travelling or direct contact with animals may also be pathways to acquire *Campylobacter* infection (Figure 1). EFSA stated that poultry is a major, if not the largest, single source of human infections. According to EFSA, the handling, preparation and consumption of broiler meat may account for 20% to 30% of human cases of campylobacteriosis, while 50% to 80% may be attributed to the chicken reservoir as a whole (EFSA Panel on Biological Hazards (BIOHAZ) 2010). However, the most recent reports from Finland suggest that poultry products and chicken as a reservoir in Finland have a less predominant role in human campylobacteriosis (Kärenlampi et al. 2003, Hakkinen et al. 2009, de Haan et al. 2010, Lyhs et al. 2010). Attribution of human illness to specific sources may also vary between different European regions (Pires et al. 2010).

Figure 1 Pathways to human *Campylobacter* infection (Figure: courtesy of Ulrike Lyhs)



2.3 *Campylobacter* in poultry production

2.3.1 Poultry production in Finland

In the late 1950s the first broiler eggs were smuggled into Finland by the football team of a paper mill (Toivio 2009). Organized poultry meat production started at the beginning of 1960s. Already then, production was based on contracts with the farms and slaughter companies (Toivio 2009, Perko 1997). All broiler production and about 95% of turkey production in Finland is nowadays based on contracts between farmers and slaughterhouses. Production is strictly scheduled, with scheduled dates of hatching and slaughter. Commercial poultry production technology is essentially similar all over Western Europe. Due to the weather conditions in Finland, rearing houses are insulated and a heating system is used. The average size of a commercial broiler farm is about 40 000 broilers and a turkey farm has about 9 600 birds (personal communication, 2010). Each farm has one or several rearing houses. The broiler- and turkey-production chains are described in detail in Figures 2 and 3. Broiler farms use in rearing the all in-all out strategy. Flocks of the same age are slaughtered within a few days and the houses are cleaned and disinfected while they are empty for a period of one to four weeks before a new flock comes in. Chicks will be sprayed with a commercial competitive exclusion product, a select mixture of bacteria derived from the caeca of an adult healthy broiler, to prevent *Salmonella*. No prophylactic vaccination against poultry diseases is in use at commercial broiler or turkey rearing farms in Finland (http://www.evira.fi/portal/fi/elaimet/elainten_terveys_ja_elaintaudit/rokoteneuvonta/elainlajikohtaiset_rokotteet/siipikarjarokotteet/). At turkey farms, females and males are reared in different groups, separated by various types of walls. After slaughter, the rearing house will be empty for a period of two to five weeks, cleaned and disinfected (personal communication, 2010). In Finland poultry is slaughtered at four big slaughterhouses (three for broilers and one for turkeys) and 13 small slaughterhouses specified for poultry (personal communication 2010).

Figure 2 Broiler meat production chain in Finland

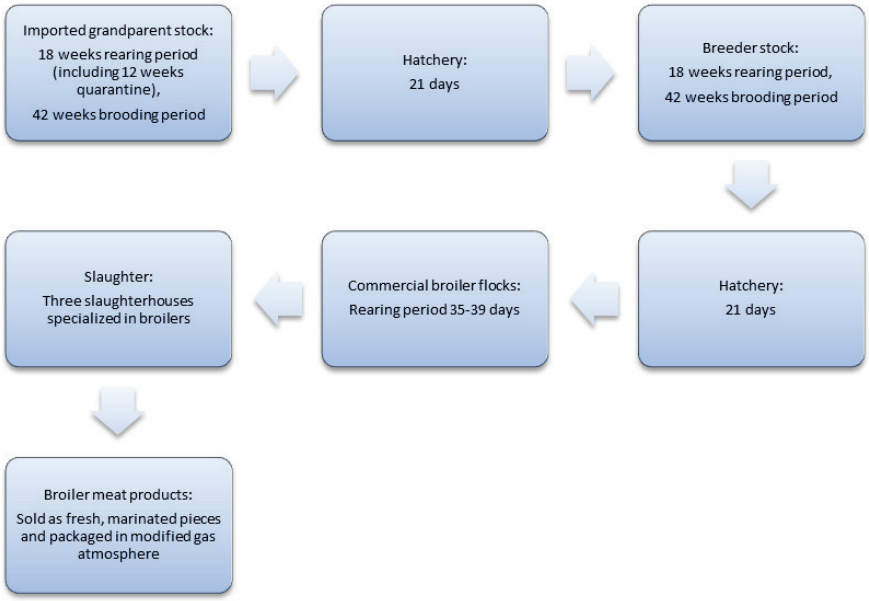
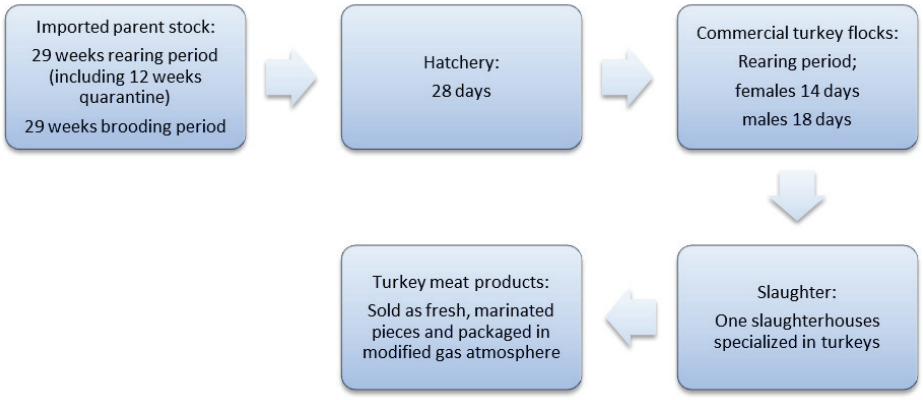


Figure 3 Turkey meat production chain in Finland



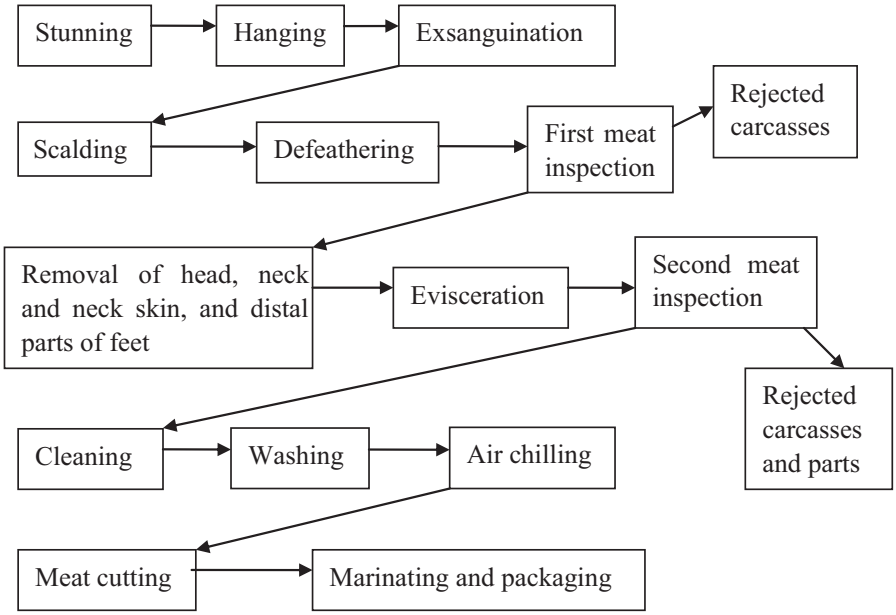
2.3.2 Slaughter

Poultry flocks can be split into a few slaughter batches and birds from one farm are slaughtered within subsequent batches. In Finland, split slaughter or thinning to make more space for the remaining birds is not used for broilers. Females and male turkeys are slaughtered separately because of the different slaughter age. Logistic slaughter is used only when the flock is known to be *Salmonella* positive based on the Finnish *Salmonella* control programme, in which case the flock is slaughtered at the end of the day in compliance with Finnish regulation 38/EEO/2006 (http://wwwb.mmm.fi/el/laki/j/Liha-asetus.pdf).

2.3.2.1 Broiler slaughter

Broilers are slaughtered at an age of 35 to 40 days. Broiler slaughterhouses are highly automated in Finland. The schematic flow chart of the slaughter process is shown in Figure 4. Two out of the three broiler slaughterhouses use carbon dioxide stunning and one uses electricity stunning. The water temperature used in scalding and defeathering is 54-56ºC. Evisceration can be highly automated, but at the second meat inspection site viscera and carcass must be linked together. Under Regulation (EC) No 853/2004, after inspection and evisceration, slaughtered poultry must be cleaned with water and chilled to 4ºC as soon as possible. In Finland, broiler slaughterhouses use air chilling to chill the carcasses (2ºC for three hours). After chilling, carcasses are transferred to the cutting room on the day of slaughter. Cutting and packaging of broiler meat is also highly automated. Most of the broiler meat is sold as fresh, processed and about 80% of the products are marinated and packaged in a modified atmosphere (Björkroth et al. 2005).

Figure 4 The schematic flow chart of the poultry slaughter process



2.3.2.2 Turkey slaughter

Turkey females are slaughtered at 13 to 15 weeks and males at 17 to 18 weeks of age. Turkey slaughter requires more manual work than broiler slaughter and the process is not highly automated. Electric stunning is used. The birds are hung by the legs before stunning. The water temperature used in scalding and defeathering is 54-56ºC. Evisceration and cleaning is performed manually. Turkey carcasses are chilled in a water tank at 2ºC for five minutes before hanging them for 24 hours in a refrigerated room at 2 ºC. The day after slaughter, meat cutting is done mainly manually. In 2007, all turkey slaughtering in Finland was centralized on one slaughterhouse with up-to-date and more automated slaughter technology.

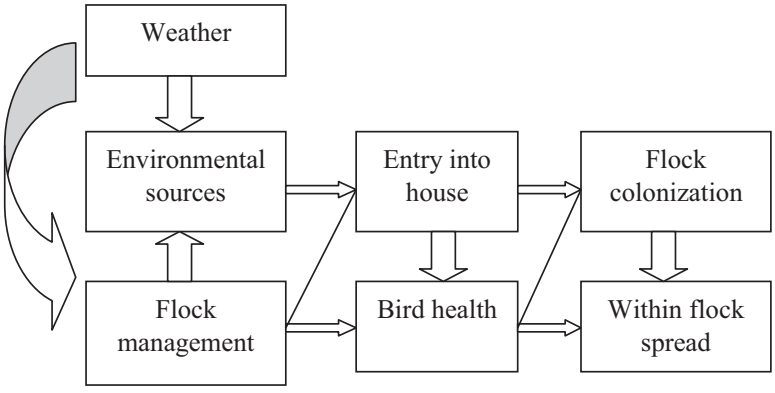
2.3.3 Campylobacter at farm

2.3.3.1 Colonization

Several studies have indicated that poultry flocks are free from *Campylobacter* at the beginning of the rearing period. Usually at two to three weeks of age, not earlier, *Campylobacter* could be cultivated from chicken faecal samples (Jacobs-Reitsma et al. 1995, Berndtson et al. 1996a, Evans and Sayers 2000). However, in experimental infections, two- to three-day-old broiler chicks were colonized by *C. jejuni* after the challenge (Ringoir et al. 2007). Several studies have shown that the maternal antibodies might have a protective role reflected by two- to three-week lag phase (Ringoir et al. 2007, Sahin et al. 2003). It has also been noted that flocks become increasingly colonized at around 10 days before slaughter. This is when the growth rate of the birds is greatest and the space for individual birds declines (Evans and Sayers 2000).

Spreading of *Campylobacter* is quick within the flock after the first colonization. In a study by Bullet et al. (2006) most birds were colonized within a week after *Campylobacter* were first detected in the flock. This is in agreement with the study of Van Gerwe et al. (2009), reporting that one colonized bird could, on average, infect 2.37 birds per day and the flock size 20 000 birds would be 95% colonized within one week (Figure 5). Birds carrying *Campylobacter* are asymptomatic colonizers without any clinical signs (Dhillon et al. 2006).

Figure 5 Causal path map showing likely pathways to colonization of broiler chickens by *Campylobacter* (according to Rushton et al. 2009).



Several studies have identified a seasonal variation of flocks colonized by *Campylobacter* (Kapperud et al. 1993, Hartnack et al. 2009, Jore et al. 2010). In Finland, as in other Northern European countries the seasonal peak and higher recovery rates have been detected during July, August and September (Jore et al. 2010) (Figure 6). The reason for seasonal variation is unknown, but may reflect levels of environmental contamination (Nylen et al. 2002). Rushton et al. (2009) reported that mean temperature and mean rainfall in the month of slaughter were the predictors of flock infection. Temperature was found to be highly correlated with the incidence of *Campylobacter*-positive broilers in the study of Jore et al. (2010). Weather factors might play a role either directly or indirectly also by increasing the susceptibility of heat-stressed birds for colonization. Additional reservoirs appearing and changes in practices due to weather conditions may explain the seasonal variation as well (Ellis-Iversen et al. 2009).

The prevalence of *Campylobacter* in broiler flocks varies in the different regions. Nordic countries like Norway, Finland, Sweden, and Denmark have reported a relatively low prevalence of 3.2%, 3.9%, 13.2% and 19.0%, respectively, in slaughtered flocks (EFSA 2010a). By contrast, other European countries have shown much higher occurrences of *Campylobacter* in broiler batches, for example, 48.9% in Germany, 76.1% in France, 78.9% in Poland and 88.0% in Spain (EFSA 2010a). Limited work has been carried out on investigating the prevalence of *Campylobacter* on turkey farms. In a Danish study, 48% to 80% of turkey flocks were *Campylobacter*-positive at the time of slaughter (Borck 2003).

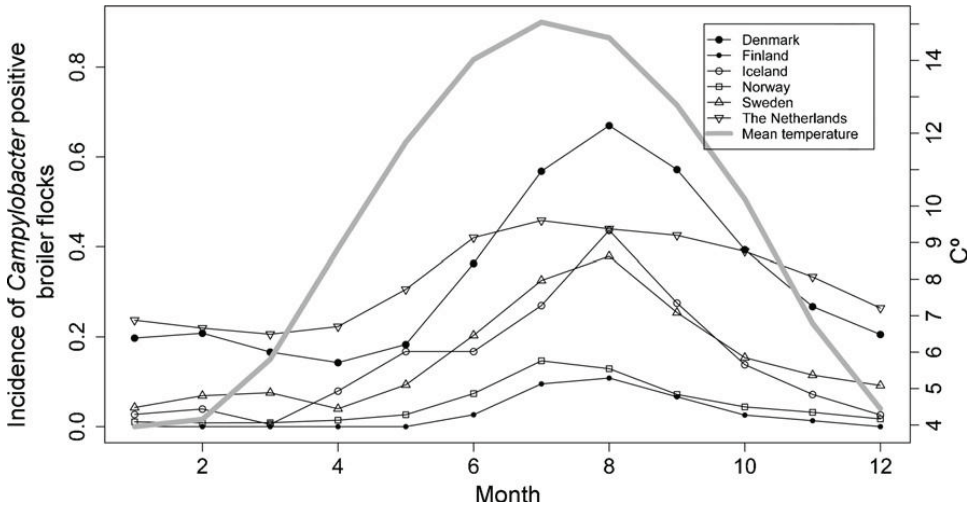


Figure 6 Mean monthly incidences of broiler flocks positive for *Campylobacter* spp. in Denmark, Finland, Iceland, Norway, Sweden, and the Netherlands during 2001–2007, compared with mean ambient temperature for the northern hemisphere (Jore et al. 2010). (The figure has been reprinted with the permission of copyright holder.)

2.3.3.2 Risk factors and sources for contamination

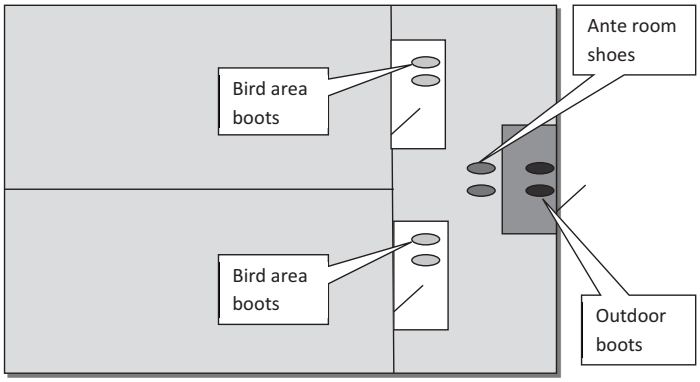
Many studies suggest that the outside environment of rearing houses is an ultimate source of colonization for poultry flocks and multiple factors are involved in the transmission of *Campylobacter* to poultry. The external environment, design and technical systems of rearing houses and animal management practices all play a role in the dynamics of the *Campylobacter* colonization of flocks (Rushton et al. 2009, Hansson et al. 2010).
Farm animals such as cattle, pigs and other poultry can be the reservoir of the *Campylobacter* and increase the risk for poultry houses nearby (van de Giessen et al. 1996, van de Giessen et al. 1998, Bouwknegt et al. 2004, Hald et al. 2004, Zweifel et al. 2008). Lynngstad et al. (2008) found that swine holdings located closer than 2 km were a risk factor for *Campylobacter* colonization. However, some studies have found that other animals on the farm were not associated with increased *Campylobacter* colonization risk or associated with a decreased risk of colonization (Kapperud et al. 1993, Guerin et al. 2007a). An Icelandic study reported that producers having other livestock in addition to broilers on a farm took precautions such as biosecurity and sanitation practices to prevent contamination of the broiler houses (Guerin et al. 2007a).

From environmental samples, *Campylobacter* is frequently isolated from puddles (Bull et al. 2006, Humphrey et al. 1993, Hiett et al. 2002b, Messens et al. 2009). *Campylobacter* survive in humid, moist conditions and mean rainfall in the month of slaughter has been suggested to be one predictor of colonization (Rushton et al. 2009). Concrete surrounding a poultry house may be able to reduce the areas where puddles can form and reduce the transfer of *Campylobacter* into the house (Bull et al. 2006).

Flies and other insects may act as a vector for *Campylobacter* transmission and the ventilation system might contribute to the possibility of insects entering poultry houses (Hald et al. 2004). Rushton et al. (2009) stated that natural ventilation is one predictor of colonization by increasing the number of flies entering a poultry house as forced ventilation might lead to higher mortality of flies.

Transmission of *Campylobacter* into a poultry house via a farm worker has been considered as one potential risk (Lyngstad et al. 2008, Johnsen et al. 2006a, Ridley et al. 2008a). The importance of proper hygiene practices and strict hygiene barriers has been established in many studies (Evans and Sayers 2000, Hansson et al. 2010). Johnsen et al. (2006a) discovered that transport personnel delivering day-old chicks passing through the hygiene barrier increased the risk of *Campylobacter* colonization. Figure 7 shows the hygiene barrier system used in poultry farms in Finland. The main aspect here is that footwear is changed after the anteroom before entering each separate hall.

Figure 7 Hygiene barrier system used in poultry farms in Finland (Figure: courtesy of Eija Kaukonen).



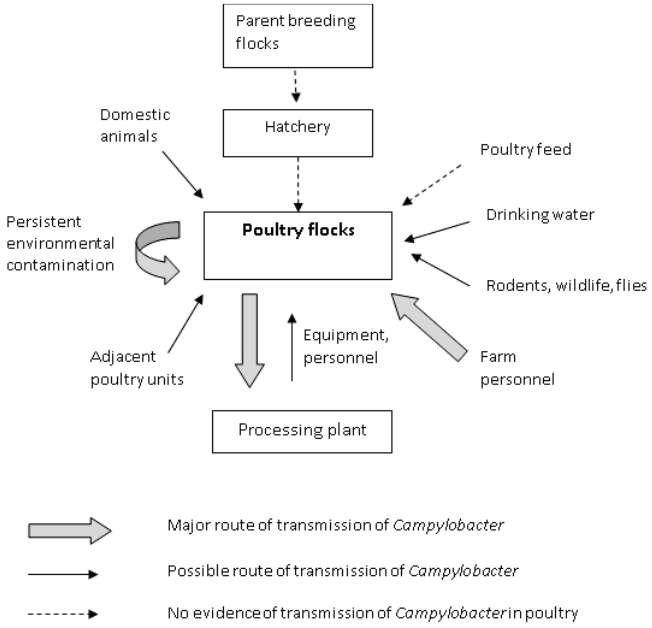
Drinking water source and the method of treatment have been found to be a risk factor for *Campylobacter* colonization in many studies. Lyngstad et al. (2008) reported that water from private sources was strongly associated with an increased risk of *Campylobacter* colonization and respectively Guerin et al. (2007a) stated that the use of municipal water reduces the risk. However, water treatments such as disinfectants might have a protective role in spreading *Campylobacter* within a flock rather than introduction into the flock (Ellis-Iversen et al. 2009).

Increasing farm size has been associated with *Campylobacter* risk on broiler farms. This has been established when the flock size was rather small (Guerin et al. 2007a). Berntdson et al. (1996b) found that the risk increased when the flock size was more than 25 000 birds. Thus, increased flock size may also be a surrogate for many other factors (Guerin et al. 2007a).

Horizontal transmission as described above (Figure 8) is the main route for colonization of *Campylobacter* to poultry flocks. Some studies, however, have pointed out the possibility of vertical transmission. In studies concerning vertical transmission, *C. jejuni* have been found on both outer and inner egg shell surfaces (Doyle 1984, Shanker et al. 1986) and in the reproductive tract of laying and broiler breeder hens (Jacobs-Reitsma 1997, Buhr et al. 2002). *Campylobacter* have also occurred in the reproductive tracts and semen of commercial turkeys (Cole et al. 2004). Hiet et al. (2002a) have shown the presence of *Campylobacter* DNA in fluff and eggshell samples. In contrast, Petersen et al. (2001) and Herman et al. (2003) reported no *Campylobacter*-positive samples collected in the hatchery e.g. incubator contents, swab samples from hatchery machinery and floors and

yolk sacs of diseased or dead chicks. Despite these observations, there is no clear evidence that vertical transmission or horizontal hatchery transmission does occur (Petersen et al. 2001, Smith et al. 2004, Callicott et al. 2006).

Figure 8 Routes of transmission of *Campylobacter* in broiler flocks



2.3.4 Campylobacter at slaughter process

It is widely acknowledged that contamination of the poultry carcasses and equipment with *Campylobacter* occurs during the slaughter process (Berndtson et al. 1996a, Stern et al. 2001, Reich et al. 2008). Implementation of HACCP programmes, separate processing of positive and negative poultry flocks, e.g. logistic or scheduled slaughter, is applied in order to prevent cross-contamination at slaughter in different countries (Katsma et al. 2007, Nauta et al. 2005). During the slaughter process, any event but more particularly the stages of scalding, defeathering and evisceration, can lead to *Campylobacter* contamination of the carcass (Stern and Robach 2003, Alter et al. 2005, Allen et al. 2007). Contacts with surfaces of the slaughter facilities and air are found as a potential source of the cross-contamination (Allen et al. 2007, Johnsen et al. 2006b, Posch et al. 2006, Peyrat

et al. 2008a). Allen et al. (2007) reported that *Campylobacter* were isolated from aerosols and droplets in the hanging, defeathering and evisceration areas even when *Campylobacter* were not isolated from the particular slaughtered flock. Scalding water is shown to contaminate the surface of carcasses even if scalding reduces the total number of bacteria on the skin (Alter et al. 2005, Berrang et al. 2000, Berrang et al. 2001, Bily et al. 2010). During broiler slaughter up to 78% of scalding water samples have been reported to be *Campylobacter*-positive with a mean bacterial count of 3.6 $\log_{10}$ cfu/ml. Rosenquist et al. (2006) showed that *Campylobacter* was present on the carcasses from contaminated broiler flocks throughout the slaughter process, but the counts increased during evisceration and decreased during air and water chilling. Other researchers have also reported increased contamination after evisceration (Ono and Yamamoto 1999, Klein et al. 2007b). After scalding and defeathering, 53.3% of the samples were *Campylobacter*-positive (mean bacterial count of 6.5 $\log_{10}$ cfu per carcass) and after evisceration 66.7% of the samples were positive (mean count of 6.0 $\log_{10}$ cfu per carcass) (Klein et al. 2007b). A correlation between the high concentration of *Campylobacter* in the intestinal contents and the high concentration on the neck skin of the carcasses has been reported by Siemer et al. (2004) and Rosenquist et al. (2006). Allen et al. (2007) highlighted that carcass contamination is related also to the within-flock prevalence. Contaminated carcasses from 100% colonized flocks had an average of 5.3 $\log_{10}$ cfu *Campylobacter* and carcasses from low prevalence flocks had an average of 2.3 $\log_{10}$ cfu *Campylobacter*. In broiler meat, contamination levels have even been over 4 $\log_{10}$ cfu per meat sample (EFSA 2010a, Klein et al. 2007b). Limited knowledge is available about the numbers of *Campylobacter* in turkey slaughter. Contamination levels of turkey carcasses have been reported with a rather high range from 2 to 7 $\log_{10}$ cfu/g from caecum, from 0.5 to 3.5 $\log_{10}$ cfu/g from neck skin and the levels of turkey meat samples ranged from 0.1 to 1.9 $\log_{10}$ cfu/g (Bily et al. 2010).

2.3.5 Finnish Campylobacter monitoring programme

Under Finnish regulation 10/EEO/2007 (http://wwwb.mmm.fi/el/laki/j/10_EEO_2007.pdf) slaughterhouses have to examine all slaughtered broiler flocks for *Campylobacter.* In the period from 1st June to 31st October, pooled caecal samples from ten birds are requested to be collected from all slaughter batches and in the winter time samples are taken less frequently. No action for broiler meat after positive result is demanded. If a farm has repeatedly positive results, the farmer has to evaluate their management and hygiene practice. The practices have to be inspected by municipal veterinarian. For turkeys, no obligatory programme exists in Finland, but the slaughterhouse monitors *Campylobacter* prevalence by own control.

2.4 Identification of *Campylobacter*

2.4.1 Phenotyping methods

2.4.1.1 Biochemical testing

Due to the relatively low activity in several conventional metabolic activity test and special growth requirements, species differentiation between *Campylobacter* species using classical phenotyping methods is rather difficult. To identify *C. jejuni* and *C. coli* several phenotypical tests have been described. Morphology by Gram staining, motility and catalase test should be performed in primary isolation. Further testing includes the hippurate hydrolysis test, growth at 25ºC, 37ºC and 42ºC, indoxyl acetate hydrolysis, and production of H_2S (Fitzgerald et al. 2008). The hippurate hydrolysis test has been used for differentiation between *C. jejuni* and *C. coli*. However, some hippurate negative *C. jejuni* isolates or false negative reactions make interpretation of the results of this test uncertain (Fields and Swerdlow 1999, Engvall et al. 2002, Nakari et al. 2008). Commercial tests for identifying *Campylobacter* species, for example, the bacterial identification test strip API Campy, are also available and have been a step forward in enhancing standardization, accuracy and reproducibility (Steinhauserova et al. 2000).

2.4.1.2 Serotyping

Serotyping has a long history of use in the typing of *Campylobacter*. The two serotyping systems differ on the basis of either using of heat-labile (HL) (Lior et al. 1982) or of soluble heat-stable (HS) antigens (Penner and Hennessy 1980, Penner et al. 1983). Schemes according to Penner and Hennessy (1980) are generally accepted and well-evaluated. The major disadvantages of both of these techniques are the high number of untypeable strains and the time-consuming and technically demanding requirements. Also antiserum reagents required for serotyping are not widely available (Wassenaar and Newell 2000). Serotyping alone does not exhibit a high discriminatory power, but could be improved in combination with a DNA-based method (Fussing et al. 2007) .

2.4.2 Species specific PCR

The polymerase chain reaction (PCR) method provides a rapid and highly sensitive method for the detection of species specific DNA sequences. PCR reaction amplifies copies of a fragment of DNA across several orders of magnitude. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA (Dieffenbach and Dveksler 2003).

PCR is relatively uncomplicated to use and a fast and robust method to identify *Campylobacter* at species level. An advantage is also the potential use in screening programmes (Linton et al. 1997, Lübeck et al. 2003).
A number of PCR assays have been developed and used to detect and identify *Campylobacter* (Linton et al. 1997, Vandamme et al. 1997, Klena et al. 2004, Miller et al. 2007). The presence of inhibitory compounds may affect the PCR reaction and give false-negative results. The use of an internal standard as a control of the PCR reaction increases the reliability of the technique (Denis et al. 2001). It is important to be aware that the PCR method may detect dead as well as viable bacteria (Waage et al. 1999). Real-time PCR assays are becoming of increasing importance since they assess the level of contamination with a given pathogen (Lübeck et al. 2003). Real-time PCR is based on the principles of conventional PCR but with continuous monitoring of product accumulation (Higuchi et al. 1992).

2.4.3 Genotyping methods

A number of different genotyping methods have been used for the typing of *Campylobacter* (Wassenaar and Newell 2000). *Campylobacter* is genetically very diverse and the genome is susceptible to genomic instability. This can confound molecular epidemiological investigations over an extended time period (Hänninen et al. 1998, Ridley et al. 2008b). Thus, combining two independent genotyping methods may have a greater discriminatory value than using only a single method (Wassenaar and Newell 2000).

2.4.3.1 Pulsed-field gel electrophoresis

The pulsed-field gel electrophoresis (PFGE) method involves the digestion of genomic DNA into pieces with restriction enzymes. A pulsing electric field applied across the gel drives the DNA pieces into the gel over a period of hours. The smallest pieces slip through the pores of the agarose gel more quickly. So the pieces are separated as distinct bands in the gel, based on the size. The resulting pattern of bands is the DNA “fingerprint". PFGE has proven to be useful and discriminatory for investigation of outbreaks of *C. jejuni.* (Fitzgerald et al. 2001). It has been used extensively for typing *Campylobacter* in studies associated with poultry (Posch et al. 2006, Borck and Pedersen 2005, Klein et al. 2007a, Lienau et al. 2007). The disadvantages of PFGE are high costs and time requirement; it is also a technically demanding method. Comparison of PFGE profiles from different laboratories and between studies has also been difficult. Distinct electrophoretic conditions may influence obtained profiles, different restriction enzymes are used to digest DNA and furthermore some *Campylobacter* isolates cannot be typed by PFGE (Wassenaar and Newell 2000). The widely-used restriction enzyme *Sma*I generates four to ten

fragments. *Kpn*I digest has more fragments than *Sma*I and is thus more discriminatory and it is often used as a secondary enzyme but has also been suggested as a primary choice for epidemiological studies (Michaud et al. 2001).

2.4.3.2 Sequencing of flaA short variable region

Analysis of the DNA sequence variation of the short variable region (SVR) of the *flaA* flagellin gene has proven to be a useful typing method for *Campylobacter* allowing relatively high sample throughput at reasonable cost (Meinersmann et al. 2005, Meinersmann et al. 1997). Sequence-based *flaA* typing avoids difficulties inherent in methods that rely on restriction fragment length polymorphisms of the flagellin genes (Wassenaar and Newell 2000). Since *flaA*-SVR is limited to analysis of variations in a single and highly variant gene, long-term time–location trends cannot be examined. However, this method can be very useful for discriminating more closely related *Campylobacter* isolates (Hiett et al. 2007). Among others, Ragimbeau (2008) and Wassenaar (2009) have found the *flaA*-SVR typing method useful in their epidemiological studies concerning *Campylobacter* from different sources.

2.4.3.3 Amplified fragment length polymorphism

The amplified fragment length polymorphism (AFLP) method is based on selective amplification of restriction fragments of chromosomal DNA. Target DNA is digested with two or more restriction enzymes. A PCR method is then used to amplify a subset of these fragments. One of the selective primers is labelled with a fluorescent compound. Amplified fragments are separated and detected by a suitable, usually sequencer-based system (Vos et al. 1995). The AFLP system can also be technically demanding and require expensive equipment to run. However, this technique is sensitive, reproducible and highly discriminatory and has been used for the identification and typing of *Campylobacter* in diverse animal and environmental studies including poultry (Siemer et al. 2004, Duim et al. 1999, Duim et al. 2001, Alter and Fehlhaber 2003).

2.4.3.4 Ribotyping

Ribotyping involves the cleaving of genomic DNA with a frequently cutting restriction enzyme, subsequent hybridization with a labelled ribosomal gene probe, and visualization of the resulting labelled patterns (Grimont and Grimont 1986). The method has a relatively low discriminatory power and the elaborate nature of the technique makes it a relatively unsuitable method for routine genotyping (Wassenaar and Newell 2000). Automation has made ribotyping more useable, but still the low level of diversity and relatively high cost

of automated ribotyping diminish its wider use for the study of *Campylobacter* (On et al. 2008).

2.4.3.5 Multilocus sequence typing

Multilocus sequence typing (MLST) is a sequence-based typing method based on partial sequence information at seven housekeeping loci (Maiden et al. 1998). For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). MLST has been proven useful for population characterization, lineage identification, and epidemiology of *C. jejuni* (Allen et al. 2007, Dingle et al. 2001, Kärenlampi et al. 2007).
The method is highly reproducible, scalable, and data are electronically portable between laboratories, enabling comparison of isolates via the internet MLST appears best in population genetic study but it is expensive. Due to the sequence conservation in housekeeping genes, MLST sometimes lacks the discriminatory power to differentiate bacterial strains, which limits its use in outbreak investigations (Urwin and Maiden 2003, Clark et al. 2005).

3 Aims of the study

The specific aims of the study dealing with *C. jejuni* and *C. coli* in Finnish poultry production were:

1. To study the occurrence of *Campylobacter* in broiler and turkey production in Finland (I, III).

2. To explore the persistence and diversity of *Campylobacter* at different stages of the turkey slaughter process (III, IV).

3. To compare conventional cultivation method with a PCR method for detection and to identify *Campylobacter* at different stages of the turkey production and different types of sample materials (III).

4. To compare the molecular typing methods as PFGE, AFLP, ribotyping, *flaA*-SVR sequencing and HS serotyping in order to find relatedness and diversity of *C. jejuni* isolates from Finnish poultry production (I, II, IV).

4 Materials and methods

4.1 Sampling of bacterial strains (I-IV)

In study I, contents of caecal samples were collected from three major broiler slaughterhouses by sampling five birds from each flock during the 5 month period, from May to September One *Campylobacter* isolate from each positive flock was taken for sero- and genotyping studies. Altogether 33 strains were collected.

In study II, thirty-five *C. jejuni* strains were selected from a large collection (Hänninen et al. 2000) of strains with known epidemiological backgrounds. The strains were collected from domestically acquired human infections and from chicken faecal and meat samples in the summers of 1997 and 1998.

In study III, on the first round of sampling in the turkey parent rearing farm, ten samples were taken from the chick transportation bed, including paper liners and faecal droppings. Thereafter in the subsequent samplings, ten swab samples were collected from fresh faecal droppings monthly over a period of seven months. After transfer of the birds to the brooding farm, ten swab samples were taken from fresh faecal droppings once a month, over a period of seven months. In the hatchery, eggshell and fluff were taken three times over a period of three weeks. One to two weeks prior to the slaughter of female and male turkey flocks, 20 swab samples were taken from fresh faecal droppings at six rearing farms (A-F). At the slaughterhouse, altogether 456 samples were collected during the slaughter process, including the processing environment (336), neck skin (120) and caecal samples (120). Swab samples were collected from the transportation crates after disinfection and from the rubber boots of the workers in the evisceration room. Gauze samples were taken from different surfaces of the evisceration and cutting room and from the floor of the chilling room. Process water samples of one litre were collected during the slaughter of each flock from the defeathering machine and the chilling tank, respectively. From the meat-cutting department, both environmental and meat samples (60) were taken. A total of 143 isolates obtained from turkey flocks at farms (22 isolates) and during slaughter (121 isolates) were selected and used for further identification by a PCR method. In study IV, a total of 121 *C. jejuni* isolates originating from farms (15 isolates) and the slaughterhouse (106 isolates) were typed by PFGE and *flaA*-SVR sequencing.

4.2 Detection of *Campylobacter*

4.2.1 *Culture method for detection of Campylobacter (I,III)*

All samples were tested by both direct plating on a selective medium (I, III) and an enrichment culture (III). Direct plating and isolation after enrichment was done on modified Charcoal Cefoperazone Deoxycholate agar plates (mCCDA) (Oxoid CM739) supplemented with SR 155 (Oxoid). Plates were incubated at 42 ± 1°C for 48 ± 4 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2), generated by CampyGen™ (Oxoid CN0035). For enrichment, Bolton selective enrichment broth (Oxoid CM0983) with selective supplement (Oxoid SR0183) and 5% lysed horse blood was used and incubated at 42 ± 1°C for 22 ± 2 h under microaerobic conditions generated by CampyGen™ (Oxoid). In study I, two presumptive *Campylobacter* colonies were subcultured and sent for further analysis to the National Veterinary and Food Research Institute and the Department of Food and Environmental Hygiene (I). Two to three presumptive colonies from each positive sample were isolated for detection and identification of *Campylobacter* to species level and subcultured on mCCDA agar (without supplement) (III, IV). One single *Campylobacter* isolate was further used for genotyping. For storage, all strains were frozen at -80°C in Brucella Broth (Scharlau Chemie 02-042, Barcelona, Spain) with 15% (v/v) glycerol solution.

4.2.2 *PCR detection of Campylobacter (III)*

For PCR, aliquots of 1 ml sample solute in saline or in Bolton broth, respectively, were collected from all farm and slaughterhouse samples both directly and after enrichment and centrifuged at 13,000 rpm for 8 min at room temperature. The supernatant was removed carefully and the pellet frozen at -80°C. (III). DNA isolation from the frozen pellet was carried out using a DNA isolation kit, MagneSil® KF Genomic System (Promega MD1460, Madison, WI, USA), with a Dynal MPC®-S magnetic stand (Dynal Biotech, Oslo, Norway) as described in Katzav et al. (2008). The detection of *Campylobacter* in the samples was based on amplification of the 16S rRNA gene using a set of oligonucleotide primers: C412F 5'-GGA TGA CAC TTT TCG GAG C-3' and 16S rRNA-campR2 5'-GGC TTC ATG CTC TCG AGT T-3' as described by Linton et al. (1996) and Lund et al. (2004), respectively. The internal amplification control (IAC) was prepared by isolating genomic DNA from *Yersinia ruckeri* (Gibello et al. 1999). This bacterium as a fish-adapted species is not found naturally in chickens. For detection of the internal control, the primers Yers F8 5'-CGA GGA GGA AGG GTT AAG TG- 3' and Yers R10 5'-AAG GCA CCA AGG CAT CTC TG-3' slightly modified from Gibello et al. (1999) were used. All the primers were synthesized by Oligomer Oy (Helsinki, Finland). The PCR conditions used in the present study are described by Lund et al. (2004) with a few modifications. Briefly, the PCR amplification was performed in 50 µl volumes

containing 5 µl of the DNA, 25 µl of a PCR master mix (Promega, Madison, WI, USA), 1 µl of a 25 mM $MgCl_2$ solution, 0.5 µl of a 10 mg ml^{-1} BSA solution (New England Biolabs, Ipswich, MA, USA), 20 pmol of each of the *Campylobacter* primers and 5 pmol of each of the internal control primers and 10 pg of genomic *Yersinia ruckeri* DNA primers. The PCR was performed in a Peltier Thermal Cycler (PTC-200; MJ Research Inc., Watertown, MA, USA). A DNA molecular weight marker 100 bp low ladder (P1473, Sigma-Aldrich, Saint Louis, MO, USA) was included in each gel (2% agarose gel). The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA). The PCR reaction for each sample was performed twice and considered positive if the PCR product formed a distinct band of the right size (857 bp). Samples with no internal control band were run again using a tenfold dilution of DNA.

4.3 Identification to species level

4.3.1 *Phenotypic methods (I, III)*

Biochemical confirmation was performed by a catalase test (3% H_2O_2), oxidase test (Kovacs reagent) and hippurate hydrolysis test (1% hippurate solution and ninhydrin reagent) according to the method of the National Committee of Food Analyses (1990, 2007) (I, III). To test their ability to grow in air, the colonies were streaked out onto blood plates (CASO agar, Casein- Peptone Soymeal-Peptone, Merck, Darmstadt, Germany with 5% bovine blood) and incubated aerobically at 37°C for up to three days. (III)

4.3.2 *Multiplex PCR (III, IV)*

In study III, for identification of the *Campylobacter* isolates to species level, a multiplex PCR assay with two sets of primers based on the method described by Vandamme et al. (1997) were used. The isolates were cultured on mCCDA agar without supplement and a colony was mixed with 20 µl of water and kept for 10 min at 100° C. The first primer set was *C. coli* specific: COL1 (5'-AG GCA AGG GAG CCT TTA ATC-3') and COL2 (5'-TAT CCC TAT CTA CAA ATT CGC-3'). The second set was *C. jejuni* specific: JUN3 (5'-CA TCT TCC CTA GTC AAG CCT-3') and JUN4 (5'-AAG ATA TGG CTC TAG CAA GAC 3'). All primers were synthesized by Oligomer Oy (Helsinki, Finland). PCR amplification was performed in 25 µl volumes containing 3 µl of template, 12.5 µl of a PCR master mix (Promega, Madison, WI, USA), 1.5 µl of water and 20 pmol of each primer. PCR was performed in a Peltier Thermal Cycler (PTC-200; MJ Research Inc., Watertown, MA, USA) and the conditions were according to Vandamme et al. (1997). A DNA molecular weight marker 100 bp low ladder (P1473, Sigma-Aldrich, Saint Louis,

MO, USA) was included in each gel. The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA) (III).
In study IV, for identification of the *Campylobacter* isolates to species level a multiplex PCR assay based on the method described by Wang et al. (2002) was used. Primers were 23SF (5'-TAT ACC GGT AAG GAG TGC TGG AG-3') and 23SR (5'- ATC AAT TAA CCT TCG AGC AC CG- 3') for *Campylobacter* (size 650 bp), CJF (5'-ACT TCT TTA TTG CTT GCT GC- 3') and CJR (5'-GCC ACA ACA AGT AAA GAA GC-3') for *C. jejuni* (size 323 bp), CCF (5'-GTA AAA CCA AAG CTT ATC GTG-3') and CCR (5'-TCC AGC AAT GTG TGC AAT G-3') for *C. coli* (size 126 bp) (Wang et al. 2002). All primers were synthesized by TIB MOLBIOL GmbH (Berlin, Germany). PCR amplification was performed in 25 µl volumes containing 2.5 µl of template DNA, 2.5 µl of 10 x NH_4- Buffer (Mg^{2+} free), 4.0 µl of $MgCl_2$ (50 mM), 1.5 µl of dNTP-Mix (10mM), 1.25 U of *Taq* DNA polymerase (all Bioline GmbH Luckenwalde, Germany), 0.5 µM of *C. jejuni* primers, 1 µM of C. coli primers and 0.2 µM of 23S rRNA primers. The volume was adjusted with sterile distilled water to give 25 µl. PCR was performed in a TProfessional Basic Thermal Cycler (Biometra, Göttingen, Germany) and the conditions were according to Wang et al. (2002). A DNA molecular weight marker (Hyperladder IV, Bioline**)** was included in each gel (2% agarose gel). The gel was documented by photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA).

4.4 Typing of *Campylobacter* isolates

4.4.1 *Serotyping of C. jejuni and C. coli isolates (I, II)*

For serotyping of all *C. jejuni* and *C. coli* isolates a commercially available serotyping kit (Campylobacter Antisera Seiken Set; Denka, Seiken, Japan) based on Penner's heat-stable serogroups was used according to the instructions of the kit producer. (I, II)

4.4.2 *Pulsed-field gel electrophoresis (I, II, IV)*

All isolates were typed by pulsed-field gel electrophoresis (PFGE) based on the method of Maslow et al. (1993) (I, II, IV). The isolates were grown on Brucella blood agar (1-2 days at 37°C) in a microaerobic atmosphere (I, II, IV). The bacterial cells were harvested and DNA plugs were prepared as described earlier (Hänninen et al. 1998, Maslow et al. 1993) (I).
In study II and IV the bacterial cells were harvested and treated with formaldehyde (II) and mercaptoethanol (IV) to inactivate endogenous nuclease. The DNA plug slices were digested with *Sma*I or *Kpn*I restriction enzymes (I), with *Sma*I and *Sac*II restriction enzymes (II), or with *Kpn*I restriction enzyme (IV) (New England Biolabs, Hertfordshire,

UK) as described by the manufacturer (I, II, IV). The DNA fragments were separated in with Gene Navigator (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in a 1% agarose gel (SeaKem Gold Agarose, Cambrex Bio Science) in 0.5×TBE buffer (45 mmol of Tris, 45 mmol of boric acid, 1 mmol of EDTA) at 200 V. Fragments were separated with a ramped pulse from 0.5 to 40 s for 19 h or 1 to 25 s for 20 h (I), 1 to 30 s for 20 h and of 1 to 20 s for 18 h (II), and 1 to 25s for 19h (IV). Lambda Ladder PFGE marker was used as a standard molecular weight marker in all gels (I, II, IV). If the isolates in study I had one or more differences in *Sma*I bands they were considered as different patterns and named as S1, S2 and so on. If they had five or more different bands in *Kpn*I they were considered as different patterns and named as genotype K1, K2 and so on. Together these two patterns were combined and named as genotype C1, C2 and so on. (I) A combined *Sma*I and *Sac*II pattern was designated as a PFGE type in study II. If strains had one to five differing fragments in their *Sma*I and *Sac*II patterns, they were designated as subtypes and marked with a letter (for example, genotypes VIa, VIb, Vic and so on) (II). In study I and II the pattern analysis were done visually. In study IV a computer program (BioNumerics, version 5.1, Applied Maths, Sint-Martens- Latem, Belgium) was used to identify the clusters of closely related and identical patterns. The gels were analyzed using UPGMA clustering using the Dice coefficient and 1% tolerance. PFGE clusters were defined at a similarity level of 90%. Clusters were assigned a Roman numeral (I to XI).

4.4.3 *Amplified fragment length polymorphism (II)*

The AFLP analysis was performed by using a protocol adapted from the AFLP microbial fingerprinting protocol of PE Applied Biosystems (Perkin-Elmer, Norwalk, Conn.). AFLP data were analyzed using GelCompar (Applied Maths, Kortrijk, Belgium) and a similarity matrix was created with the use of the Pearson product-moment correlation coefficient (*r*). The unweighted pair group method using average linkage was used to cluster the patterns (Vauterin and Vauterin 1992).

4.4.4 *Ribotyping (II)*

Purified chromosomal DNA in agar plugs prepared for PFGE was used for ribotyping. A 2-mm slide was cut from an agar plug, washed twice with the restriction buffer, and transferred into a tube with restriction buffer. DNA was digested with *Hae*III (Fitzgerald et al. 1996) according to the instructions of the manufacturer (Boehringer Mannheim, Mannheim, Germany). The digests were electrophoresed in 1.2% agarose gels (SeaKem ME Agarose; FMC BioProducts, Rockland, Maine) with TBE (45 mM Tris, 1 mM EDTA [pH adjusted to 8.0 with boric acid]) as the running buffer. DNA transfer and probing were performed as described in Hänninen et al. (1995).

4.4.5 *FlaA short variable region sequencing (IV)*

Typing was performed by amplifying the *flaA*-short variable region (SVR), followed by sequencing of the PCR product. The *flaA*-SVR was amplified using primers FLA4F (5´-GGA TTT CGT ATT AAC ACA AAT GGT GC-3´) and FLA625RU (5'- CAA GWC CTG TTC CWA CTG AAG-3´) as described previously (Nachamkin et al. 1993). PCR products were purified by using MiniElute PCR Purification Kit (Qiagen, Hilden, Germany). Sequence data were obtained using a 3730 DNA Analyzer (Applied Biosystems). The nucleotide region between primers FlaA242FU and FlaA625RU was used for allelic comparisons. Forward and reverse sequence results were confirmed by assembling them in Accelrys Gene v2.5 (Accelrys Inc., San Diego, USA). The nucleotide sequences were compared to the *C. jejuni flaA* database (http://pubmlst.org/campylobacter/flaA/) and allele numbers were assigned accordingly. Confirmed sequences were aligned using BioNumerics v5.1 (Applied Maths).

4.5 Statistical analysis

4.5.1 *Data analysis and calculations (III)*

For data analysis and calculations Microsoft® Excel 97 SR 2 was used. The level of agreement according to precision was expressed as the kappa statistic, defined as the proportion of potential agreement beyond chance exhibited by two tests. Diagnostic specificity was calculated as: d/(b + d) where d is the number of samples negative both by PCR and by culture and b is the number of samples positive by PCR, but negative by culture. The level of agreement between two tests was calculated as: (a + d)/n, where a is the number of samples positive both by PCR and by culture, d is the number of samples negative by both methods and n is the total number of samples under examination (Smith 1995, Martin et al. 1997).

4.5.2 *Calculation of the discrimination power of the genotyping methods (IV)*

The Simpson's index of diversity (Hunter and Gaston 1988) was used to calculate the discrimination power of PFGE and *flaA*-SVR method.

5 Results

5.1 *Campylobacter* in broiler production (I)

In study I, during the period from 1 May to 30 September 1999, the overall *Campylobacter*-positive broiler flock prevalence was 2.9% (33 of the total 1 132 broiler flocks studied). Out of 220 farms studied, 22 (10%) flocks were positive. Out of thirty-three isolates thirty-one were *C. jejuni* (94%) and two were *C. coli* (6%). Monthly variation in the number of *Campylobacter*-positive flocks is shown in Table 1.

Table 1 Monthly variation in the number of *Campylobacter*-positive flocks

Month	No. of flocks	No. of positive flocks	%
May	227	1	0.4
June	224	2	0.9
July	230	16	7.0
August	220	10	4.5
September	231	4	1.7
Total	1132	33	2.9

5.2 *Campylobacter* in turkey production (III)

In study III, none of the 150 samples from the turkey parent flock, collected during the rearing and brooding period, and of the 30 samples from the hatchery were *Campylobacter*-positive either by direct culture or culture following enrichment. However, using the PCR method, five samples from the parent flock in the brooding farm and one sample from the hatchery was *Campylobacter*-positive. The PCR products from these samples were sequenced and identified as *C. jejuni.* Three farms were found by cultivation and by PCR to be colonized with *Campylobacter* prior to slaughter. At the turkey slaughterhouse, *Campylobacter* were isolated from at least one sample in 10 out of the 12 flocks studied. However, from two of the flocks (B1 and D1) no *Campylobacter* were detected during the slaughter process. All *Campylobacter* isolates were identified as *C. jejuni*.

5.3 Persistence and diversity of *C. jejuni* at different stages of the turkey slaughter process (III,IV)

At the turkey processing plant, different types of samples were taken from 11 different sampling sites in study III. The highest percentage of positive samples was found among the environmental samples from the evisceration room (75%). Also faecal material collected from the transport crates (67%), the chilling water samples (67%) and the neck skin samples (62.5%) had high isolation rates by culture after enrichment (Table 2).

Table 2 Frequency of *C. jejuni* in samples at different stages of turkey meat production chain detected by culture and the PCR method

	Direct culture	Enrichment culture	PCR	PCR after enrichment
	No.of positive/ no.examined (%)	No.of positive/ no.examined (%)	No.of positive/ no.examined (%)	No.of positive/ no.examined (%)
Transportation crates	1/11* (9)	1/11* (9)	1/11* (9)	1/9* (11)
Fecal material from transportation crates	7/12 (58)	8/12 (67)	7/12 (58)	7/9 (78)
Water from defeathering machine	0/12 (0)	5/12 (42)	3/12 (25)	5/9 (56)
Caecal material	9/24 (37.5)	8/24 (33)	8/24 (67)	8/18 (44)
Neck skin	2/24 (8)	15/24 (62.5)	6/24 (25)	12/18 (67)
Environment (evisceration room)	6/12 (50)	9/12 (75)	7/12 (58)	9/9 (100)
Rubber boots (evisceration room)	3/12 (25)	6/12 (50)	3/12 (25)	5/9 (56)
Chilling water	3/12 (25)	8/12 (67)	3/12 (25)	7/9 (78)
Environment (chilling room)	0/12 (0)	6/12 (50)	0/12 (0)	5/9 (56)
Environment (meat cutting room)	0/12 (0)	5/12 (42)	0/12 (0)	5/9 (56)
Meat samples	0/60 (0)	17/60 (28)	4/60 (7)	13/45 (29)

* Eleven samples after washing and disinfection

Each *Campylobacter*-positive turkey farm had its own flock-related PFGE type when analyzed with *Kpn*I restriction enzyme in study IV. Farm A had PFGE type I, farm C PFGE type IX and farm E PFGE type V (Figure 10). These types were found on farms and at different slaughter stages. The male flock D2 was *Campylobacter*-negative on the farm. Isolates of *C. jejuni* positive samples of this flock obtained from eight different points of slaughter and from the meat cuttings formed a heterogeneous group of seven PFGE types (III-IX). However, these PFGE types were divided into only four different *flaA*-SVR alleles (36, 72, 161 and 508). PFGE types of the isolates from male flock E2 showed high similarity. The PFGE type V with *flaA* allele 161 was found at farm E and at all positive sampling sites during the process. Also PFGE type I, obtained from flock A2, persisted from the farm through the process. This isolate, however, yielded five different *flaA* alleles. In faecal samples occurred alleles 21 and 161, alleles 36 and 161 were found during the slaughter process and allele 15 from the cutting room and meat cuts (Table 4).

The isolates having PFGE type I, *flaA* allele 21, were also found from slaughterhouse samples (faecal material from the transport crates, neck skin samples and the environment of the chilling room) of flock B2. This flock, slaughtered three days after flock A1, was *Campylobacter*-negative on the farm. Also Flock F1 was *Campylobacter*-negative at farm level, but *C. jejuni* was isolated from the faecal material from the transport crates and the environment of the evisceration room during the slaughter process. These isolates shared PFGE type IX and *flaA* allele 36, which was mainly found in isolates of flock C2. Flock F1 was slaughtered one day after flock C2.

5.4 Comparison of conventional culture and PCR method for detection and identification of *Campylobacter* (III)

In study III, environmental samples from the chilling and cutting rooms were all negative in direct culture and PCR. However, following enrichment, 50% and 42% of the same samples from the chilling room, and 56% and 56% from the cutting room, were found to be positive for *Campylobacter* by culture and PCR, respectively. Water samples from the defeathering machine, neck skin samples, swab samples from the rubber boots of the workers in the evisceration room and meat cutting samples showed a higher percentage of *Campylobacter*-positive samples using PCR after enrichment (Table 2).

The diagnostic specificity for the comparison of PCR to direct culture was 0.88 with a level of agreement of 0.88 and for the comparison of both methods by selective enrichment was 0.88 with a level of agreement of 0.92.

5.5 Typing of *C. jejuni* and *C. coli* isolates from Finnish poultry production (I,II,IV)

5.5.1 *Serotyping and PFGE (SmaI and KpnI) (I)*

In study I, eight HS serotypes were identified out of 33 isolates. Six of the isolates were nonserotypable with the available set of sera. HS serotype 6,7 was the most common serotype found (7 out of 26) and HS serotypes 12, 4-complex and 27 were isolated more than once. Thirty *C. jejuni* and two *C. coli* isolates were genotyped with PFGE. *Sma*I enzyme identified 14 different patterns and *Kpn*I enzyme identified 15 different patterns. Together there were 18 different genotypes. The most common HS serotype, 6,7, was associated with *Sma*I genotype S2 (4/7) and three of the isolates were not digested with *Sma*I. All S2 isolates had highly similar patterns when digested with *Kpn*I. Furthermore, serotype 6,7 isolates which were not digested with *Sma*I had identical *Kpn*I patterns. Only one genotype was common for all three slaughterhouses. This genotype had HS serotype 27 or it was nonserotypable. HS serogroup 4-complex included three different genotypes (Table 3).

5.5.2 *PFGE (SmaI and SacII), AFLP, ribotyping and serotyping (II)*

A total of 35 *C. jejuni* strains that belonged to five different PFGE type groups were selected on the basis of their *Sma*I and *Sac*II patterns. AFLP analysis subdivided the strains into 10 AFLP types. Cluster analysis of AFLP patterns clearly separated distinct PFGE types and thus produced in most cases congruent results between the PFGE and AFLP analyses (Figure 9). Six different *Hae*III ribotypes, with two subtypes, were obtained from the strains. Data from PFGE, AFLP, and ribotypes were combined and designated as combined genotypes. A total of 13 combined genotypes were identified. Seven serotypes were identified among the strains studied and eight strains remained untypeable. Most common serotypes were HS serotype 1,44 and serotype 4 complex (Table 4). HS serotype 1,44 was identified among five different combined genotypes. HS serotype 4 complex was identified among the four combined genotypes. HS serotype 12 was associated with the one genotype, and combined genotypes G2 and G6 were serotype 57. The strains with related patterns of combined genotype of G7 and G8 had the same HS serotype 27.

Table 3 *Campylobacter*-positive farms and characterization of *Campylobacter* isolates by sero- and genotyping

Isolate	Month of isolation	Farm	No.of birds in the flock	No.of *Campylobacter* positive houses/ total no.of houses	Serotype (Penner)	PPGE genotype		
						Sma I pattern	*Kpn* I pattern	Combined
1831	May	A	15500	1/2	4,13,16,43,50	S3	K6	C6
1959	June	B	37500	1/1	6,7	S2	K3	C3
2059	June	C	15000	1/1	27	S2	K4	C4
2165	July	D	30000	1/1	12	S1	K1	C1
2166	July	E	15000	1/2	NS	S2	K5	C5
2186	July	B	3000	4/4	6,7	S2	K3	C3
2197	July	B	7000		6,7	S2	K3	C3
2199	July	B	15000		ND	S2	K3	C3
2213	July	B	10000		6,7	S2	K3	C3
2219	July	F	12500	1/1	4,13,16,43,50	S4	K6	C7
2227	July	G	30000	1/1	6,7	UD	K8	C9
2230	July	H	30000	1/1	12	S1	K1	C1
2232	July	I	11000	2/2	4,13,16,43,50	S5	K7	C8
2234	July	I	11000		ND	S2	K4	C4
2252	July	J	7000	1/1	41	S1	K2	C2
2347	July	K	15000	1/1	57	S11	K13	C15
2351	July	L	30000	2/2	12	S6	K9	C10
2360	July	M	30000	1/1	NS	S2	K5	C5
2361	July	N	30000	1/2	12	S6	K9	C10
2362	Aug.	H	30000	1/1	NS	S2	K4	C4
2447	Aug.	O	15000	2/2	*C. coli*	S14	K15	C18
2448	Aug.	O	15000		*C. coli*	S14	K15	C18
2449	Aug.	P	15000	1/1	6,7	UD	K8	C9
2450	Aug.	Q	30000	1/2	6,7	UD	K8	C9
2458	Aug.	B	44000	1/1	27	S2	K4	C4
2538	Aug.	R	30000	2/2	NS	S12	K13	C16
2539	Aug.	R	30000		ND	S7	K10	C11
2541	Aug.	S	30000	2/2	ND	S8	K11	C12
2542	Aug.	S	30000		ND	S1	K1	C1
2867	Sept.	J	7000	1/1	5	S9	K12	C13
2899	Sept.	T	27000	1/2	NS	-	-	-
2946	Sept.	U	8000	1/5	11	S10	K12	C14
2965	Sept.	V	15500	1/3	NS	S13	K14	C17

NS, nonserotypable; ND, not done; UD, undigested

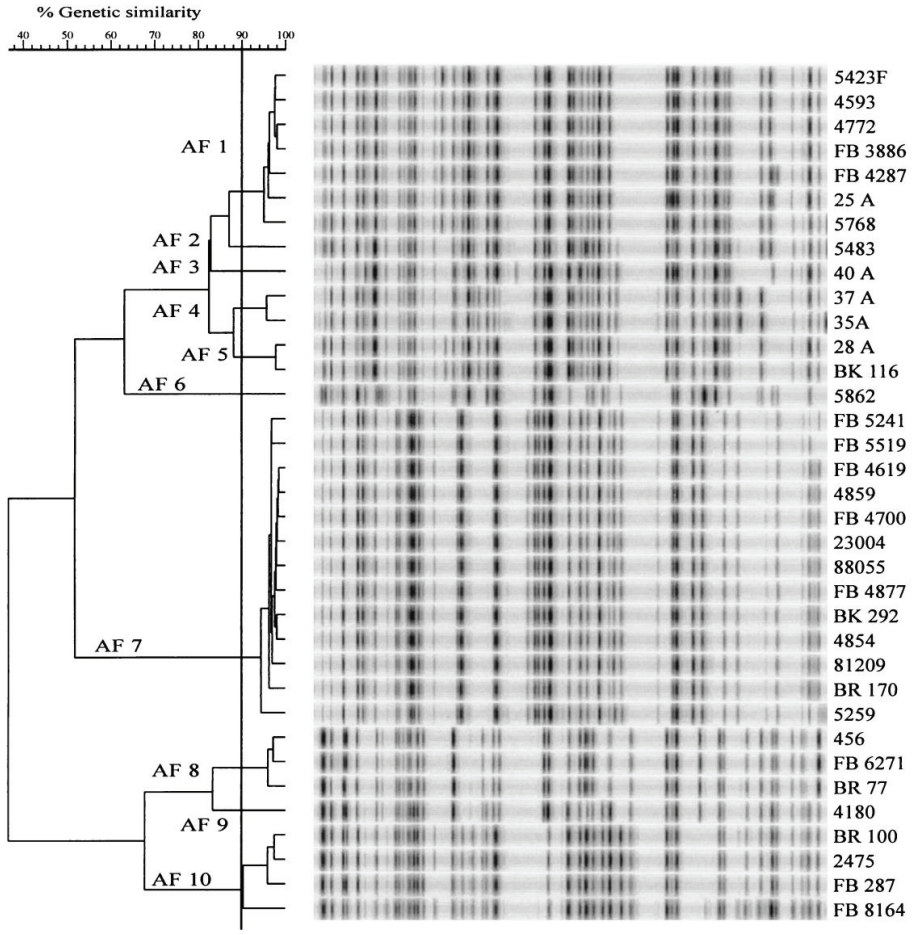
Table 4 *C. jejuni* strains, their sources, PFGE patterns, ribotypes, AFLP types and HS serotypes

Strain (n = 35)	Source data[a]	PFGE pattern (SmaI/SacII)	Ribotype (HaeIII)	AFLP type	Combined genotype	Serotype (HS)[b]
5423F	Patient, Pori, 98-07	I/K	A	AF1	G1	12
4593	Chicken, retail shop, Helsinki, producent A, 98-08	I/K	A	AF1	G1	12
4772	Chicken, retail shop, Helsinki, producent B, 98-08	I/K	A	AF1	G1	12
FB3886	Patient, Helsinki, 98-07	I/K	A	AF1	G1	1,44
FB4287	Patient, Helsinki, 98-07	I/K	A	AF1	G1	1,44
25A	Chicken fecal sample, 98-07	I/Kc	B	AF1	G2	57
5768	Chicken, retail shop, Helsinki, producent C, 98-09	I/K	A	AF1	G1	12
5483	Chicken, retail shop, Helsinki, producent A, 98-09	I/Ka	Aa	AF2	G3	15
40A	Chicken, fecal sample 98-11	I/K	A	AF3	G4	6,7
35A	Chicken, fecal sample 98-11	IV	C	AF4	G5	1,44
37A	Chicken, fecal sample 98-11	I/Kc	B	AF4	G6	57
28A	Chicken, fecal sample 98-08	I/Ka	Aa	AF5	G7	27
BK116	Chicken, retail shop, Helsinki, producent C, 97-08	I/K	A	AF5	G8	27
5862	Chicken, retail shop, Helsinki, 98-09	VII	E	AF6	G9	NS
FB5241	Patient, Helsinki, 98-08	VIa	D	AF7	G10	1,44
FB5519	Patient, Helsinki, 98-08	VIc	D	AF7	G10	1,44
FB4619	Patient, Helsinki, 98-07	VIa	D	AF7	G10	1,44
4859	Chicken, retail shop, Helsinki, 98-08	VIb	D	AF7	G10	NS
FB4700	Patient, Helsinki, 98-07	VIa	D	AF7	G10	1,44
25OO4	Patient, Pori, 98-07	VIa	D	AF7	G10	NS
88055	Patient, Pori, 98-07	VIa	D	AF7	G10	NS
FB4877	Patient, Helsinki, 98-07	VIc	D	AF7	G10	NS
BK292	Chicken, retail shop, Helsinki, 98-08	VIa	D	AF7	G10	4
4854	Patient, Helsinki, 98-07	VIc	D	AF7	G10	NS
81209	Patient, Pori, 98-07	VIc	D	AF7	G10	4
BR170	Chicken, retail shop, Helsinki, producent A, 98-08	VIb	D	AF7	G10	NS
5259	Chicken, retail shop, 98-08, Helsinki, producent B	VIc	D	AF7	G10	NS
FB6271	Patient, Helsinki, 97-07	T101a	F	AF8	G11	1,44
456	Patient, Helsinki, 97-07	T101a	F	AF8	G11	4
BR77	Chicken, retail shop, Helsinki, 97-07	T101a	F	AF8	G11	4
4180	Chicken, retail shop, Helsinki, 98-07	T101b	Fa	AF9	G12	4
BR100	Chicken, retail shop, Helsinki, 97-07	IV	C	AF10	G13	1,44
2475	Chicken, retail shop, Helsinki, 98-05	IV	C	AF10	G13	1,44
FB287	Patient, Helsinki, 98-06	IV	C	AF10	G13	1,44
FB8164	Patient, Helsinki, 97-08	IV	Ca	AF10	G13	1,44

[a] *C. jejuni* strains were obtained from chicken and human (patient) sources in the cities of Helsinki and Pori, as indicated, on the specified dates (year-month).

[b] HS, heat stable; NS, nonserotypeable.

Figure 9 AFLP patterns of 35 *C. jejuni* strains selected for the study



5.5.3 *PFGE (KpnI) and flaA-SVR typing (IV)*

In study IV, PFGE analysis of the *C. jejuni* with *Kpn*I restriction enzyme resulted in 11 PFGE types (I-XI) (D = 0.7295) and *flaA*-SVR typing yielded nine *flaA*-SVR alleles (D = 0.7098) (Table 5). Eleven distinct major clusters were defined at a similarity level of 95% from PFGE typing results. At the nucleotide level, the most prominent *flaA*-SVR alleles detected were *flaA* allele 36 (33.1%), *flaA* allele 161 (28.1%) and *flaA* allele 21 (24.8%). Statistical analysis showed that PFGE had a slightly better discriminatory power of 0.7295 compared to 0.7098 for *flaA*-SVR typing.

Table 5 PFGE types[4] and *flaA*-SVR alleles[5] identified among *C. jejuni* isolates from Finnish turkey rearing farms and at different stages of the slaughter line

				Slaughterhouse stages													
Farm-ID	No. of isolates	Flock	Date of slaughter	Faecal droppings at farm (f)	Transport crates (tc)	Faecal material from transport crates (ft)	Water from defeathering machine (wd)	Content of cecum (cc)	Neck skin (ns)	Environment (evisceration room) (ee)	Boots in evisceration room (be)	Chilling water (cw)	Environment (chilling room) (ech)	Environment (cutting room) (ecu)	Meat cuts (mc)
A	14	A1[1,2]	8/18/2006	I/21	I/161	I/21	-	I/21	I/161	I/21	I/161	-	-	-	I, II/21
	21	A2[1,3]	9/12/2006	I/21	-	I/21, 161	I/21, 252	I/161	I/36	I/161	I/161	I/161	I/36	I/15	I/15
B	-	B1	8/10/2006	-	-	-	-	-	-	-	-	-	-	-	-
	5	B2	8/22/2006	-	-	I/21	-	-	I/21	-	-	-	I/21	-	-
C	23	C1	8/25/2006	IX/21, 36	IX/36	IX/21	-	IX/21, 36	IX/21, 36	not typeable/21	IX/21	IX/21, 36	IX/36	-	IX/36
	19	C2	9/20/2006	IX/36		IX/36		IX/36	IX/36, 70	IX/36	IX, XI/22, 36	IX/36	IX/36	-	IX, XI/
D	-	D1	8/29/2006	-	-	-	-	-	-	-	-	-	-	-	-
	14	D2	10/3/2006	-	-	V, IX/36, 161	IX/36	-	IV/36	IX/36	IX/36	III, VI/36	VIII/72	-	VI, VII/36, 508
E	7	E1	9/1/2006	V/161	-	V/161	-	-	V/161	V/161	-	V/161	-	-	-
	15	E2	9/26/2006	V/161	-	V/161	V/161	V/161	V/161	V/161	V/161	V/161	V/161	-	-
F	2	F1	9/22/2006	-	-	IX/36	-	-	-	IX/36	-	-	-	-	-
	1	F2	10/23/2006	-	-	VI/36	-	-	-		-	-	-	-	-

[1] Letter indicate farm

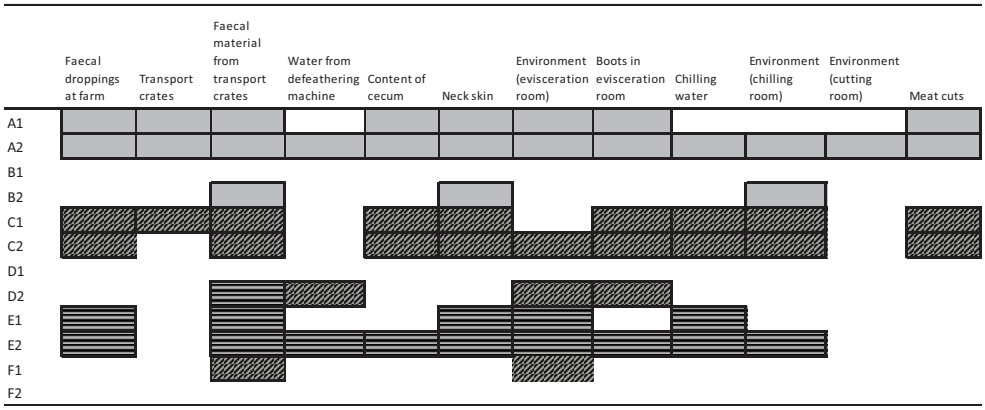
[2] Number one indicates female turkeys

[3] Number two indicates male turkeys

[4] Roman numerals indicate PFGE types

[5] Numbers indicate *flaA* alleles

Figure 10 Sequential spread of the dominant *C. jejuni* PFGE types [1] isolated from Finnish turkey rearing farms and at different stages of the slaughter line



[1] The shading pattern in each box is related to a different PFGE type. The same pattern means the same PFGE type.

PFGE type I , PFGE type IX , PFGE type V

6 Discussion

6.1 *Campylobacter* in poultry production

During study I, in 1999, all slaughtered flocks of the three major Finnish poultry companies were studied for the first time for *Campylobacter* with both cultivation method and sample size being harmonized. The results showed that approximately 3% of the flocks were positive indicating a very low *Campylobacter* contamination level in chickens. From 2004 on, samples have been taken according to the Finnish *Campylobacter* monitoring programme. The prevalence of *Campylobacter* in broiler slaughter batches from 2004 to 2010 has varied monthly from zero to 13.9% and annually from 5.6% to 6.6% (http://www.zoonoosikeskus.fi/attachments/zoonoosit/kampylobakteeri/kampylobakteeri_2.pdf). Also other Nordic countries like Norway, Sweden, and Denmark have reported a relatively low prevalence of *Campylobacter* in broiler 3.2%, 13.2% and 19.0%, respectively (EFSA 2010a). Similar studies are not published from turkey production. However, in Denmark, 80% of turkey flocks were *Campylobacter*-positive at the time of slaughter (Borck 2003).

Campylobacter contamination may occur at all stages of a poultry production. In study III, *Campylobacter* DNA was detected by PCR from five faecal samples collected during the turkey parent flocks brooding period. It is likely that the brooding flock had been in contact with *Campylobacter,* but the infection had not spread within the flock. Self-limitation of colonization and detection of antibodies against *C. jejuni* without colonization has previously been described (Newell and Fearnley 2003). *Campylobacter* DNA was also detected by PCR in one fluff and eggshell sample that supports the findings of Hiett et al. (2002a). The bacterium was not isolated either from the present brooding flocks or from the hatchery, and it was not possible to determine whether it was viable or dead. Thus, no further conclusions can be made on vertical transmission based on this study.

According to several studies, (Evans and Sayers 2000, Rushton et al. 2009, Hansson et al. 2010, van de Giessen et al. 1998, Berndtson et al. 1996b, Hartnett et al. 2001) *Campylobacter* is introduced sporadically into the flock from an external site of the environment. Strict hygiene and biosecurity are suggested to be the most successful measures against environmental contamination (Berndtson et al. 1996b, Hartnett et al. 2001, Guerin et al. 2007b). The presence of a hygiene barrier has been pointed out to be the most important single biosecurity measure (Hald et al. 2000). The risk for *Campylobacter* contamination is high when strict biosecurity barriers are loosened and a poultry flock may come into contact with the environment via people and equipment on the farm. The possibility of compromising biosecurity during partial depopulation or "thinning" has yielded conflicting data. Several authors have demonstrated that the catching team can introduce the bacterium into the house and, therefore, partial

depopulation has been considered a risk factor for *Campylobacter* colonization (Hald et al. 2001, Jacobs-Reitsma et al. 2001). In contrast, it has also been demonstrated that it does not necessarily influence *Campylobacter* colonization in the flock (Russa et al. 2005). On Finnish turkey farms, the flocks are usually divided and females and males are reared in separate groups, but in the same house. Females are slaughtered two to four weeks before the males. After the turkey females have been slaughtered, the males can use the area where the females have been. This area could be seen as a risk for contamination since the personnel catching the turkeys can break the hygiene barriers during collection of the female birds. In study III, three flocks were *Campylobacter*-negative before slaughter of the females and remained negative when the males were tested two to three weeks later. Hansson et al. (2007) found no differences in the presence of *Campylobacter* in the environment between producers who frequently or rarely deliver *Campylobacter*-positive slaughter batches. Thus, the results of study III could be explained by good hygiene control of the catching equipment and personnel at the negative farms.

In Finland, the poultry industry is well organized and because of a strict *Salmonella* control programme, farmers are educated to understand the importance of biosecurity barriers and hygiene control in the prevention of environmental contamination. For example, hygiene gates are in wide use. The construction of insulated poultry houses prevents environmental contamination. Snow-covered earth in winter might decrease the possible outside sources of contamination. Competitive exclusion, to prevent *Salmonella*, has been in wide use for over 30 years (personal communication, 2010). This also might have an impact on decreasing the colonization of *Campylobacter* in chicken (Shane 2000).

6.2 Detection and diversity of *C. jejuni* at different stages of the turkey slaughter process

In study III, the number of *Campylobacter*-positive samples within a flock at slaughter varied between 0% and 94%. High variation *Campylobacter* findings in the turkey flocks at the slaughterhouse has also been demonstrated previously (Borck and Pedersen 2005, Atanassova et al. 2007).

The evisceration stage, with a *Campylobacter* detection rate of 100% by PCR after enrichment, was found as a critical stage during the slaughter process where the spread of bacteria can lead to carcass contamination. Also 56% of samples taken from rubber boots of the workers at the evisceration room were positive. These findings are in agreement with Alter et al. (2005) reporting a 72% *Campylobacter* isolation rate from turkey carcasses after evisceration. The high contamination level at the evisceration stage is easily explained by the rupture of intestines during the processing.

Neck skin samples are mentioned as good targets to indicate *Campylobacter* contamination at the slaughterhouse (Berndtson et al. 1996a). In study III, neck skin

samples were more often positive (67% by the PCR method after enrichment) than caecal samples (44% by the PCR method after enrichment). Hansson et al. (2005) found more positive samples from broiler neck skin (50%) than from cloacal (41%) samples. They concluded that if cloacal samples were negative, the neck skin samples might have been contaminated from the slaughterhouse environment.

In study III, the detection rate of *Campylobacter* in the chilling water was 78%, by PCR after enrichment. In the slaughterhouse studied here, the turkey carcasses were chilled by placing them first in a water tank for five minutes before hanging them for 24 hours in a room at 2°C. More positive samples from the chilling water than from the chilling room environment were observed, suggesting the chilling water as being a source of carcass contamination. Extended air-chilling might lead to drying of the carcass surface and the environment of the chilling room resulting in a reduction of *Campylobacter* (Allen et al. 2007, Klein et al. 2007b, Sanchez et al. 2002). Alter et al. (2005) confirmed a significant decrease of *Campylobacter*-positive poultry carcasses after the final chilling period. Comparative studies on the effect of air chilling (2ºC) or ice-water immersion (2ºC) on the *Campylobacter* load on carcasses reported similar or moderately higher reduction rates by immersion chilling compared to air chilling (Rosenquist et al. 2006, Berrang et al. 2008).

It has been shown that contamination at the slaughterhouse cannot be avoided when a *Campylobacter*-positive poultry flock is processed (Herman et al. 2003). Allen et al. (2007) isolated *Campylobacter* at a slaughterhouse from aerosols, particles and droplets in the hanging, plucking and evisceration areas also during the processing of a *Campylobacter*-negative flock. Since enrichment was needed to recover the bacteria, it seems that some processing steps like the scalding and chilling process had an adverse effect on the bacteria. A similar decreasing effect was also established in studies of *Campylobacter* prevalence on chicken carcasses during processing (reviewed by Guerin et al. 2010). Bily et al. (2010) found that slaughtering and cutting operations led to low amounts of *Campylobacter* on the final skinless turkey breast meat. In our study (IV), some clones (I21 and IX36) were found through the process and in the meat cuts and in the environment of cutting room. Thus, stress factors such as high temperature of the scalding and defeathering water (54-56ºC), drying of the carcass skin during air chilling (24 hours at 2ºC), could not eliminate *Campylobacter* completely. These findings indicate the resistance of certain *Campylobacter* clones to environmental and technological stresses (Alter et al. 2005, Callicott et al. 2008, Hunter et al. 2009).

Before slaughter, three turkey farms (A, C, E) were *Campylobacter*-positive and three (B, D, F) were *Campylobacter*-negative. Positive flocks were colonized by a limited number of *C. jejuni* types (PFGE types I, V and IX; flaA alleles 21, 36 and 161) from the farm along the entire processing line to the end-products (meat cuts) (Figure 10, Table 5). This confirms the traceability of flock-specific strains and is in agreement with earlier reports (Lienau et al. 2007). Dominance of certain clonal types has also been reported by other authors (Borck and Pedersen 2005, Lienau et al. 2007, Newell et al. 2001).

Individual chickens may harbour a multiplicity of different strains (De Cesare et al. 2008) and the poultry flock is often colonized with several subtypes (Jacobs-Reitsma et al. 1995, Hiett et al. 2002b). However, only one *C. jejuni* subtype is present in the majority of *Campylobacter*-positive broiler flocks in Finland (Hakkinen and Kaukonen 2009). In study III, only one strain from each turkey farm was genotyped. Since only one flock related subtype was seen also during the process of positive farms (A, C and E) (Figure 10), it seems that only one subtype was present in those flocks. This might be explained by only one environmental exposure of *Campylobacter* or the same source for colonization.

At the slaughterhouse studied, all turkeys originated from the same flock and only one flock per day was slaughtered. Thus, cross-contamination from another, potentially positive, flock slaughtered earlier the same day was not possible. Cleaning and disinfection procedures were performed daily. However, there is evidence that contamination at a slaughterhouse can withstand cleaning and disinfection. *Campylobacter*-negative flocks, B2 and F1, became contaminated during processing by the same subtypes of *C. jejuni* introduced into the slaughterhouse by positive flocks A1 and C2, even if slaughtered on following days. Contamination from a flock slaughtered the day before is also reported by Lindmark et al. (2006). Peyrat et al. (2008a, 2008b) recovered *C. jejuni* from the equipment surfaces after cleaning and disinfection in three out of four slaughterhouses visited. It is possible that *Campylobacter*, as well as other bacteria, persist on surfaces in poultry processing facilities forming a biofilm (Jeffrey et al. 2001, Cools et al. 2005, Sanders et al. 2007). Thus, the release of the bacterium from such biofilms may also contaminate products which touch the surface of the processing equipment.

Isolates from flock D2 formed a heterogeneous group of seven PFGE types (II-VIII) or four *flaA* alleles (36, 72, 161, 508). Farm D was *Campylobacter*-negative at farm level. The Female flock D1 was negative also at the processing plant. The Male flock D2 was negative at the farm and also caecal samples at slaughter were negative. All other post transport samples of this flock (except the environment of the cutting room) were, however, positive indicating high cross contamination during the processing. Bily et al. (2010) stated that if the main contamination of dominant *Campylobacter* types disappears due to environmental stress factors, the selection of pre-existing genotypes could be detected. In study III, two *Campylobacter*-negative flocks (B1 and D1) also remained negative during the slaughter process. Samples taken in the morning before slaughter would have given us more information about the possible persistent contamination at processing line.

6.3 Comparison of conventional culture and PCR method for detection and identification of *Campylobacter*

As we found a high level of agreement between different detection methods, this showed that there were no significant differences between the conventional culture and the PCR method in the samples analyzed in study III. This is in agreement with Schnider et al. (2010), who had a similar number of positive samples with the real-time PCR method and enrichment-based culture method in the detection of *Campylobacter* in broiler neck skin samples. However, the need for enrichment in our study for the detection of *Campylobacter* at certain processing steps, also PCR detection, might indicate low numbers of *Campylobacter* at the farm level and in the slaughterhouse. Thus, a combination of enrichment and PCR assay seems to be the optimal method for detection of *Campylobacter* in this situation.

The difficulties in the identification of *C. jejuni* and *C. coli* with the hippurate hydrolysis test have been reported in several studies (Steinhauserova et al. 2001, Waino et al. 2003). Nakari et al. (2008) stated that the standardized hippurate test could reliably identify hippurate-positive strains. However, hippurate negative *C. jejuni* strains cannot be reliable identified with phenotypic methods. The same uncertainly was seen in our study. For study IV, 121 *Campylobacter* strains were studied and 89 were hippurate positive and 35 hippurate negative. With the multiplex PCR method of Wang et al. (2002), all strains were identified as *C. jejuni*.

6.4 Relatedness of *C. jejuni* isolates from Finnish poultry production using different molecular typing methods

C. jejuni is a naturally transformable bacterium and genomic rearrangements and recombinants are frequently occurring events creating a novel subpopulation of strains (Wassenaar et al. 1998, Hänninen et al. 1999, Schouls et al. 2003).
To increase the potential to adapt to new environments, colonize the gut in different hosts and survive outside the gut in transmission phase between hosts *C. jejuni* may undergo genetic variation (Taylor 1992). These variations are probably important in the transmission route from broiler to man, where *Campylobacter* must survive several hostile environments (Hansson et al. 2008). The diversity in PGFE and AFLP banding patterns is most likely caused by genomic rearrangements. These genetic changes may have occurred in the bacterial population in the intestine of individual birds. Hänel et al. (2009) showed that novel PFGE types and *flaA*-types were formed during the passage through the chicken gut. In addition, there is evidence that instability and related changes in the macrorestriction profiles may occur due to the influence of in vitro stress factors e.g. during isolation and extensive subculturing of *Campylobacter* (Wassenaar et al. 1998, Höök et al. 2005). These mechanisms may contribute to the observed small variation in the number and size of fragments, as was noted in study II in all selected genotypes with

otherwise-similar PFGE or AFLP patterns and in study IV in PFGE patterns. This minor genomic variability, however, may lead to overestimation of genetic diversity of *C. jejuni.*

The results of comparative analysis of PFGE and AFLP patterns of *C. jejuni* in study II showed that both methods produced congruent results in most cases, thus having similar levels of sensitivity. In one group, AFLP subdivided PFGE type I/K strains into three subclusters (AF1, AF3, and AF5). In the group PFGE VI, however, PFGE analysis was more discriminatory than AFLP, because PFGE subdivided the strains into three subtypes and AFLP analysis showed a high relatedness of the patterns. An explanation for the high discriminatory power of AFLP is the large number of fragments used in the analysis. Ribotype analysis was shown to have a level of discriminatory power similar to that of the PFGE and AFLP methods used. Other ribotyping studies have revealed that ribotyping was less discriminatory than PFGE (Gibson et al. 1995, de Boer et al. 2000) or AFLP (de Boer et al. 2000). In these studies a highly diverse collection of *C. jejuni* strains was used, whereas in the present study (II), we had a limited number of strains and they represented a restricted set of PFGE genotypes, which may explain the difference in discrimination by ribotyping.

In study IV, we applied PFGE using *Kpn*I restriction enzyme in combination with *flaA*-short variable region (SVR) sequencing. The *flaA*-SVR typing differentiated the isolates into nine different sequence types and PFGE differentiated into 11 clusters. We found that PFGE had a slightly better discriminatory power of 0.7295 compared to 0.7098 for *flaA*-SVR typing. These results are consistent with other studies investigating the discriminatory powers of PFGE compared to *flaA*-SVR typing (Miller et al. 2010). The majority of *flaA*-SVR alleles displayed a distinct association with a specific PFGE type. Nonetheless, a linear relationship for all strains among both typing methods could not be established. The *flaA*-SVR method alone cannot track recombinant effects and is by itself poorly suited for the investigation of the molecular epidemiology of *Campylobacter* strains (Levesque et al. 2008).

Certain *Campylobacter* strains with shared genotypes and phenotypes may become locally predominant and form temporary clonal groupings, probably due to specific characteristics that are advantageous for their colonization and pathogenicity. PFGE groups selected to study II were commonly found and persistent during a period of three years. Those genotypes differed from each other by all of the genotyping methods used. This indicates that chosen PFGE type groups represent genetic lineage among highly diverse genotypes of *C. jejuni* isolated during study period. Other studies in Finland suggest that certain *C. jejuni* serotypes and genotypes are persistent among Finnish human, chicken and cattle isolates (Rautelin and Hänninen 1999, Vierikko et al. 2004, Nakari et al. 2005, Schönberg-Norio et al. 2006, Hakkinen et al. 2007). Over time, stable and common types have also been reported in a Swedish study by Hansson et al. (2008) and in Denmark by Fussing et al. (2007). The predominant HS serotypes identified among human isolates in Finland have been 2, 4-complex and 1,44. However, only 4-complex was found in chicken isolates

in study I, where HS serotypes 6,7, 12, 4-complex and 27 were the most common. In later studies, HS serotypes 6,7 and 12 have shown to be associated with MLST type ST-45 and found to be related with human infections in Finland (Kärenlampi et al. 2003, Kärenlampi et al. 2007).

7 Conclusions

- In our first study in 1999 a low prevalence of *Campylobacter* in Finnish broiler flocks was detected and it has remained at a low level during the study period until the present. A combination of various preventive methods and external factors may explain the low prevalence of *Campylobacter*-positive broiler flocks in Finland. Strict hygiene control and biosecurity barriers are in use to prevent the contamination of *Salmonella* and *Campylobacter.* Due to the cold climate, an airtight shell of buildings is needed. This might have an influence on the prevention of *Campylobacter* transmission. Since complete elimination of thermophilic *Campylobacter* from the poultry production chain does not seem feasible, a reduction of contamination at the farm level by a high level of biosecurity control and hygiene may be one of the most efficient ways to reduce the amount of contaminated poultry meat in Finland.

- During the slaughter process of turkeys, especially evisceration and water chilling were found to be risk factors for the *C. jejuni* contamination of the meat products. To reduce the cross-contamination of *Campylobacter*-negative flocks during the slaughter, hygiene measures, efficient cleaning and disinfection of the processing premises are needed. However, cross-contamination of turkey carcasses coming from different flocks but slaughtered at same slaughterhouse seems to be unavoidable with present slaughter logistics. Thus, regarding poultry, a single flock infected with *Campylobacter* may constitute a contamination risk for *Campylobacter*-negative flocks in the slaughter process. Even though risk assessments generally regard logistic slaughter as non-effective in poultry meat production, it is an additional control option for Finland showing a very low prevalence of *Campylobacter* in poultry flocks.

- No significant difference between the conventional cultivation and PCR method in detection *Campylobacter* was seen. The need for enrichment for detection of *C. jejuni* at certain processing stages at the slaughterhouse, also when performing PCR, might indicate low numbers of *Campylobacter* at the farm and the slaughterhouse level.

- Either PFGE or AFLP analyses were shown to have a high level of discriminatory power. However, a combination of different genotyping methods is advisable to specify genetic relatedness of strains. PFGE analysis using *Kpn*I restriction enzyme together with *flaA*-SVR method was shown to be feasible.

- In future, quantitative studies of *Campylobacter* at farm and slaughterhouse level as well as studies on the spreading of *Campylobacter* colonization in and within the flock could provide useful information in a low-level prevalence country like Finland for intervention actions at the farms.

- To clarify the role of Finnish poultry and poultry meat as the reservoir and the source for human campylobacteriosis, equal and comparable detection and genotyping methods should be used. Furthermore, a close cooperation between the poultry industry and research institutes should continue and intensify. The food industry and authorities have duty to maintain food safety, but also consumers have their own responsibility for handling foodstuff properly.

8 References

Allen, V.M., Bull, S.A., Corry, J.E., Domingue, G., Jorgensen, F., Frost, J.A., Whyte, R., Gonzalez, A., Elviss, N. and Humphrey, T.J. (2007) *Campylobacter* spp. contamination of chicken carcasses during processing in relation to flock colonisation. *Int J Food Microbiol* **113**, 54-61.

Alter, T. and Fehlhaber, K. (2003) Use of AFLP analysis for the molecular biological fine typing of *Campylobacter jejuni* strains from turkey flocks]. *Berl Munch Tierarztl Wochenschr* **116**, 454-459.

Alter, T., Gaull, F., Froeb, A. and Fehlhaber, K. (2005) Distribution of *Campylobacter jejuni* strains at different stages of a turkey slaughter line. *Food Microbiol* **22**, 345-351.

Arsenault, J., Letellier, A., Quessy, S. and Boulianne, M. (2007) Prevalence and risk factors for *Salmonella* and *Campylobacter* spp. carcass contamination in broiler chickens slaughtered in Quebec, Canada. *J Food Prot* **70**, 1820-1828.

Atanassova, V., Reich, F., Beckmann, L. and Klein, G. (2007) Prevalence of *Campylobacter* spp. in turkey meat from a slaughterhouse and in turkey meat retail products. *FEMS Immunol Med Microbiol* **49**, 141-145.

Berndtson, E., Danielsson-Tham, M.L. and Engvall, A. (1996a) *Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process. *Int J Food Microbiol* **32**, 35-47.

Berndtson, E., Emanuelson, U., Engvall, A. and Danielsson-Tham, M.-. (1996b) A 1-year epidemiological study of *campylobacters* in 18 Swedish chicken farms. *Prev Vet Med* **26**, 167-185.

Berrang, M.E., Buhr, R.J. and Cason, J.A. (2000) *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. *Poult Sci* **79**, 286-290.

Berrang, M.E., Buhr, R.J., Cason, J.A. and Dickens, J.A. (2001) Broiler carcass contamination with *Campylobacter* from feces during defeathering. *J Food Prot* **64**, 2063-2066.

Berrang, M.E., Meinersmann, R.J., Smith, D.P. and Zhuang, H. (2008) The effect of chilling in cold air or ice water on the microbiological quality of broiler carcasses and the population of *Campylobacter*. *Poult Sci* **87**, 992-998.

Bily, L., Petton, J., Lalande, F., Rouxel, S., Denis, M., Chemaly, M., Salvat, G. and Fravalo, P. (2010) Quantitative and qualitative evaluation of *Campylobacter* spp.

contamination of turkey cecal contents and carcasses during and following the slaughtering process. *J Food Prot* **73**, 1212-1218.

Björkroth, J., Ristiniemi, M., Vandamme, P. and Korkeala, H. (2005) *Enterococcus* species dominating in fresh modified-atmosphere-packaged, marinated broiler legs are overgrown by *Carnobacterium* and *Lactobacillus* species during storage at 6 degrees C. *Int J Food Microbiol* **97**, 267-276.

Black, R.E., Levine, M.M., Clements, M.L., Hughes, T.P. and Blaser, M.J. (1988) Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* **157**, 472-479.

Blaser, M.J. and Engberg, J. (2008) Clinical aspects of *Campylobacter jejuni* and *Campylobacter coli* infections. In *Campylobacter* ed. Nachamkin, I., Szymanski, C.M. and Blaser, M.J. pp. 99-121. Washington,DC: ASM Press.

Borck, B. (2003) *Campylobacter* spp. in the Danish turkey production.

Borck, B. and Pedersen, K. (2005) Pulsed-field gel electrophoresis types of *Campylobacter* spp. in Danish turkeys before and after slaughter. *Int J Food Microbiol* **101**, 63-72.

Bouwknegt, M., van de Giessen, A.W., Dam-Deisz, W.D., Havelaar, A.H., Nagelkerke, N.J. and Henken, A.M. (2004) Risk factors for the presence of *Campylobacter* spp. in Dutch broiler flocks. *Prev Vet Med* **62**, 35-49.

Buhr, R.J., Cox, N.A., Stern, N.J., Musgrove, M.T., Wilson, J.L. and Hiett, K.L. (2002) Recovery of *Campylobacter* from segments of the reproductive tract of broiler breeder hens. *Avian Dis* **46**, 919-924.

Bull, S.A., Allen, V.M., Domingue, G., Jorgensen, F., Frost, J.A., Ure, R., Whyte, R., Tinker, D., Corry, J.E., Gillard-King, J. and Humphrey, T.J. (2006) Sources of *Campylobacter* spp. colonizing housed broiler flocks during rearing. *Appl Environ Microbiol* **72**, 645-652.

Butzler, J.P. (2004) *Campylobacter*, from obscurity to celebrity. *Clin Microbiol Infect* **10**, 868-876.

Butzler, J.P., Dekeyser, P., Detrain, M. and Dehaen, F. (1973) Related vibrio in stools. *J Pediatr* **82**, 493-495.

Callicott, K.A., Friethriksdottir, V., Reiersen, J., Lowman, R., Bisaillon, J.R., Gunnarsson, E., Berndtson, E., Hiett, K.L., Needleman, D.S. and Stern, N.J. (2006) Lack of evidence for vertical transmission of *Campylobacter* spp. in chickens. *Appl Environ Microbiol* **72**, 5794-5798.

Callicott, K.A., Hargardottir, H., Georgsson, F., Reiersen, J., Frigriksdottir, V., Gunnarsson, E., Michel, P., Bisaillon, J.R., Kristinsson, K.G., Briem, H., Hiett, K.L.,

Needleman, D.S. and Stern, N.J. (2008) Broiler *Campylobacter* contamination and human campylobacteriosis in Iceland. *Appl Environ Microbiol* **74**, 6483-6494.

Clark, C.G., Bryden, L., Cuff, W.R., Johnson, P.L., Jamieson, F., Ciebin, B. and Wang, G. (2005) Use of the oxford multilocus sequence typing protocol and sequencing of the flagellin short variable region to characterize isolates from a large outbreak of waterborne *Campylobacter* spp. strains in Walkerton, Ontario, Canada. *J Clin Microbiol* **43**, 2080-2091.

Cole, K., Donoghue, A.M., Blore, P.J. and Donoghue, D.J. (2004) Isolation and prevalence of *Campylobacter* in the reproductive tracts and semen of commercial turkeys. *Avian Dis* **48**, 625-630.

Cools, I., Uyttendaele, M., Cerpentier, J., D'Haese, E., Nelis, H.J. and Debevere, J. (2005) Persistence of *Campylobacter jejuni* on surfaces in a processing environment and on cutting boards. *Lett Appl Microbiol* **40**, 418-423.

de Boer, P., Duim, B., Rigter, A., van Der Plas, J., Jacobs-Reitsma, W.F. and Wagenaar, J.A. (2000) Computer-assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. *J Clin Microbiol* **38**, 1940-1946.

De Cesare, A., Parisi, A., Bondioli, V., Normanno, G. and Manfreda, G. (2008) Genotypic and phenotypic diversity within three *Campylobacter* populations isolated from broiler ceca and carcasses. *Poult Sci* **87**, 2152-2159.

de Haan, C.P., Kivisto, R., Hakkinen, M., Rautelin, H. and Hänninen, M.L. (2010) Decreasing trend of overlapping multilocus sequence types between human and chicken *Campylobacter jejuni* isolates over a decade in Finland. *Appl Environ Microbiol* **76**, 5228-5236.

Debruyne, L., Gevers, D. and Vandamme, P. (2008) Taxonomy of the family *Campylobacteracae*. In *Campylobacter* ed. Nachamkin, I., Szymanski, C.M. and Blaser, M.J. pp. 3-25. Washington,DC: ASM Press.

Denis, M., Refregier-Petton, J., Laisney, M.J., Ermel, G. and Salvat, G. (2001) *Campylobacter* contamination in French chicken production from farm to consumers. Use of a PCR assay for detection and identification of *Campylobacter jejuni* and *Camp. coli*. *J Appl Microbiol* **91**, 255-267.

Dhillon, A.S., Shivaprasad, H.L., Schaberg, D., Wier, F., Weber, S. and Bandli, D. (2006) *Campylobacter jejuni* infection in broiler chickens. *Avian Dis* **50**, 55-58.

Dieffenbach, C.W. and Dveksler, G.S. (2003) *PCR Primer, a laboratory manual.* New York: Cold Sring Harbor laboratory Press.

Dingle, K.E., Colles, F.M., Wareing, D.R., Ure, R., Fox, A.J., Bolton, F.E., Bootsma, H.J., Willems, R.J., Urwin, R. and Maiden, M.C. (2001) Multilocus sequence typing system for *Campylobacter jejuni*. *J Clin Microbiol* **39**, 14-23.

Doyle, M.P. (1984) Association of *Campylobacter jejuni* with laying hens and eggs. *Appl Environ Microbiol* **47**, 533-536.

Duim, B., Vandamme, P.A., Rigter, A., Laevens, S., Dijkstra, J.R. and Wagenaar, J.A. (2001) Differentiation of *Campylobacter* species by AFLP fingerprinting. *Microbiology* **147**, 2729-2737.

Duim, B., Wassenaar, T.M., Rigter, A. and Wagenaar, J. (1999) High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. *Appl Environ Microbiol* **65**, 2369-2375.

EFSA (2010a) Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008 - Part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA Journal* **8**.

EFSA (2010b) The Community Summary Report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008 . *EFSA Journal* **8**, 1-410.

EFSA Panel on Biological Hazards (BIOHAZ) (2010) Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. *EFSA Journal* **8**, 1-89.

Ellis-Iversen, J., Jorgensen, F., Bull, S., Powell, L., Cook, A.J. and Humphrey, T.J. (2009) Risk factors for *Campylobacter* colonisation during rearing of broiler flocks in Great Britain. *Prev Vet Med* **89**, 178-184.

Engvall, E.O., Brändström, B., Gunnarsson, A., Mörner, T., Wahlström, H. and Fermér, C. (2002) Validation of a polymerase chain reaction/restriction enzyme analysis method for species identification of thermophilic *campylobacters* isolated from domestic and wild animals. *J Appl Microbiol* **92**, 47-54.

Evans, S.J. and Sayers, A.R. (2000) A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. *Prev Vet Med* **46**, 209-223.

Fields, P.I. and Swerdlow, D.L. (1999) *Campylobacter jejuni*. *Clin Lab Med* **19**, 489-504, v.

Fitzgerald, C., Owen, R.J. and Stanley, J. (1996) Comprehensive ribotyping scheme for heat-stable serotypes of *Campylobacter jejuni*. *J Clin Microbiol* **34**, 265-269.

Fitzgerald, C., Stanley, K., Andrew, S. and Jones, K. (2001) Use of pulsed-field gel electrophoresis and flagellin gene typing in identifying clonal groups of *Campylobacter*

jejuni and *Campylobacter coli* in farm and clinical environments. *Appl Environ Microbiol* **67**, 1429-1436.

Fitzgerald, C., Whichard, J. and Nachamkin, I. (2008) Diagnosis and antimicrobial susceptibility of *Campylobacter* species. In *Campylobacter* ed. Nachamkin, I., Szymanski, C.M. and Blaser, M.J. pp. 227-243. Washington,DC: ASM Press.

Fussing, V., Moller Nielsen, E., Neimann, J. and Engberg, J. (2007) Systematic serotyping and riboprinting of *Campylobacter* spp. improves surveillance: experiences from two Danish counties. *Clin Microbiol Infect* **13**, 635-642.

Gibello, A., Blanco, M.M., Moreno, M.A., Cutuli, M.T., Domenech, A., Dominguez, L. and Fernandez-Garayzabal, J.F. (1999) Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. *Appl Environ Microbiol* **65**, 346-350.

Gibson, J.R., Fitzgerald, C. and Owen, R.J. (1995) Comparison of PFGE, ribotyping and phage-typing in the epidemiological analysis of *Campylobacter jejuni* serotype HS2 infections. *Epidemiol Infect* **115**, 215-225.

Grimont, F. and Grimont, P.A. (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann Inst Pasteur Microbiol* **137B**, 165-175.

Guerin, M.T., Martin, W., Reiersen, J., Berke, O., McEwen, S.A., Bisaillon, J.R. and Lowman, R. (2007a) A farm-level study of risk factors associated with the colonization of broiler flocks with *Campylobacter* spp. in Iceland, 2001-2004. *Acta Veterinaria Scandinavica* **49**, 18.

Guerin, M.T., Martin, W., Reiersen, J., Berke, O., McEwen, S.A., Bisaillon, J.R. and Lowman, R. (2007b) House-level risk factors associated with the colonization of broiler flocks with *Campylobacter* spp. in Iceland, 2001 - 2004. *BMC Vet Res* **3**, 30.

Guerin, M.T., Sir, C., Sargeant, J.M., Waddell, L., O'Connor, A.M., Wills, R.W., Bailey, R.H. and Byrd, J.A. (2010) The change in prevalence of *Campylobacter* on chicken carcasses during processing: a systematic review. *Poult Sci* **89**, 1070-1084.

Hakkinen, M. and Kaukonen, E. (2009) *Campylobacters* in Finlans broiler flocks: contamination level, divercity, and role of breeders in transmission. .

Hakkinen, M., Nakari, U.M. and Siitonen, A. (2009) Chickens and cattle as sources of sporadic domestically acquired *Campylobacter jejuni* infections in Finland. *Appl Environ Microbiol* **75**, 5244-5249.

Hald, B., Rattenborg, E. and Madsen, M. (2001) Role of batch depletion of broiler houses on the occurrence of *Campylobacter* spp. in chicken flocks. *Lett Appl Microbiol* **32**, 253-256.

Hald, B., Skovgard, H., Bang, D.D., Pedersen, K., Dybdahl, J., Jespersen, J.B. and Madsen, M. (2004) Flies and *Campylobacter* infection of broiler flocks. *Emerg Infect Dis* **10**, 1490-1492.

Hald, B., Wedderkopp, A. and Madsen, M. (2000) Thermophilic *Campylobacter* spp. in Danish broiler production: a cross-sectional survey and a retrospective analysis of risk factors for occurrence in broiler flocks. *Avian Pathol* **29**, 123-131.

Hanel, I., Borrmann, E., Muller, J., Muller, W., Pauly, B., Liebler-Tenorio, E.M. and Schulze, F. (2009) Genomic and phenotypic changes of *Campylobacter jejuni* strains after passage of the chicken gut. *Vet Microbiol* **136**, 121-129.

Hänninen, M.L., Hakkinen, M. and Rautelin, H. (1999) Stability of related human and chicken *Campylobacter jejuni* genotypes after passage through chick intestine studied by pulsed-field gel electrophoresis. *Appl Environ Microbiol* **65**, 2272-2275.

Hänninen, M.L., Pajarre, S., Klossner, M.L. and Rautelin, H. (1998) Typing of human *Campylobacter jejuni* isolates in Finland by pulsed-field gel electrophoresis. *J Clin Microbiol* **36**, 1787-1789.

Hänninen, M.L., Perko-Mäkelä, P., Pitkälä, A. and Rautelin, H. (2000) A three-year study of *Campylobacter jejuni* genotypes in humans with domestically acquired infections and in chicken samples from the Helsinki area. *J Clin Microbiol* **38**, 1998-2000.

Hänninen, M.L., Salmi, S., Mattila, L., Taipalinen, R. and Siitonen, A. (1995) Association of *Aeromonas* spp. with travellers' diarrhoea in Finland. *J Med Microbiol* **42**, 26-31.

Hansson, I., Ederoth, M., Andersson, L., Vågsholm, I. and Olsson Engvall, E. (2005) Transmission of *Campylobacter* spp. to chickens during transport to slaughter. *J Appl Microbiol* **99**, 1149-1157.

Hansson, I., Engvall, E.O., Vågsholm, I. and Nyman, A. (2010) Risk factors associated with the presence of *Campylobacter*-positive broiler flocks in Sweden. *Prev Vet Med* **96**, 114-121.

Hansson, I., Persson, M., Svensson, L., Engvall, E.O. and Johansson, K.E. (2008) Identification of nine sequence types of the 16S rRNA genes of *Campylobacter jejuni subsp. jejuni* isolated from broilers. *Acta Veterinaria Scandinavica* **50**, 10.

Hansson, I., Vågsholm, I., Svensson, L. and Olsson Engvall, E. (2007) Correlations between *Campylobacter* spp. prevalence in the environment and broiler flocks. *J Appl Microbiol* **103**, 640-649.

Hartnack, S., Doherr, M.G., Alter, T., Toutounian-Mashad, K. and Greiner, M. (2009) *Campylobacter* monitoring in German broiler flocks: an explorative time series analysis. *Zoonoses Public Health* **56**, 117-128.

Hartnett, E., Kelly, L., Newell, D., Wooldridge, M. and Gettinby, G. (2001) A quantitative risk assessment for the occurrence of *Campylobacter* in chickens at the point of slaughter. *Epidemiol Infect* **127**, 195-206.

Herman, L., Heyndrickx, M., Grijspeerdt, K., Vandekerchove, D., Rollier, I. and De Zutter, L. (2003) Routes for *Campylobacter* contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. *Epidemiol Infect* **131**, 1169-1180.

Hiett, K.L., Cox, N.A. and Stern, N.J. (2002a) Direct polymerase chain reaction detection of *Campylobacter* spp. in poultry hatchery samples. *Avian Dis* **46**, 219-223.

Hiett, K.L., Stern, N.J., Fedorka-Cray, P., Cox, N.A., Musgrove, M.T. and Ladely, S. (2002b) Molecular subtype analyses of *Campylobacter* spp. from Arkansas and California poultry operations. *Appl Environ Microbiol* **68**, 6220-6236.

Hiett, K.L., Stern, N.J., Fedorka-Cray, P., Cox, N.A. and Seal, B.S. (2007) Molecular phylogeny of the *flaA* short variable region among *Campylobacter jejuni* isolates collected during an annual evaluation of poultry flocks in the Southeastern United States. *Foodborne Pathog Dis* **4**, 339-347.

Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R. (1992) Simultaneous amplification and detection of specific DNA sequences. *Biotechnology (N Y)* **10**, 413-417.

Höök, H., Fattah, M.A., Ericsson, H., Vågsholm, I. and Danielsson-Tham, M.L. (2005) Genotype dynamics of *Campylobacter jejuni* in a broiler flock. *Vet Microbiol* **106**, 109-117.

Humphrey, T.J., Henley, A. and Lanning, D.G. (1993) The colonization of broiler chickens with *Campylobacter jejuni*: some epidemiological investigations. *Epidemiol Infect* **110**, 601-607.

Hunter, P.R. and Gaston, M.A. (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* **26**, 2465-2466.

Hunter, S.M., Berrang, M.E., Meinersmann, R.J. and Harrison, M.A. (2009) Genetic diversity of *Campylobacter* on broiler carcasses collected preevisceration and postchill in 17 U.S. poultry processing plants. *J Food Prot* **72**, 49-54.

Isohanni, P.M. and Lyhs, U. (2009) Use of ultraviolet irradiation to reduce *Campylobacter jejuni* on broiler meat. *Poult Sci* **88**, 661-668.

Jacobs-Reitsma, W., Lyhs, U. and Wagenaar, J. (2008) *Campylobacter* in the food supply. In *Campylobacter* ed. Nachamkin, I., Szymanski, C.M. and Blaser, M.J. pp. 627-644. Washington,DC: ASM Press.

Jacobs-Reitsma, W., Wilpshaar, E., Gussinklo, B., Wagenaar, J. and Stegeman, A. (2001) Epidemiological investications into the colonisation of Dutch broiler flocks with *Campylobacter*. *Int J Med Microbiol* **291**, 42-43.

Jacobs-Reitsma, W.F. (1997) Aspects of epidemiology of *Campylobacter* in poultry. *Vet Q* **19**, 113-117.

Jacobs-Reitsma, W.F., van de Giessen, A.W., Bolder, N.M. and Mulder, R.W. (1995) Epidemiology of Campylobacter spp. at two Dutch broiler farms. *Epidemiol Infect* **114**, 413-421.

James, C., James, S.J., Hannay, N., Purnell, G., Barbedo-Pinto, C., Yaman, H., Araujo, M., Gonzalez, M.L., Calvo, J., Howell, M. and Corry, J.E. (2007) Decontamination of poultry carcasses using steam or hot water in combination with rapid cooling, chilling or freezing of carcass surfaces. *Int J Food Microbiol* **114**, 195-203.

Jeffrey, J.S., Tonooka, K.H. and Lozanot, J. (2001) Prevalence of *Campylobacter* spp. from skin, crop, and intestine of commercial broiler chicken carcasses at processing. *Poult Sci* **80**, 1390-1392.

Johnsen, G., Kruse, H. and Hofshagen, M. (2006a) Genetic diversity and description of transmission routes for *Campylobacter* on broiler farms by amplified-fragment length polymorphism. *J Appl Microbiol* **101**, 1130-1139.

Johnsen, G., Kruse, H. and Hofshagen, M. (2006b) Genotyping of *Campylobacter jejuni* from broiler carcasses and slaughterhouse environment by amplified fragment length polymorphism. *Poult Sci* **85**, 2278-2284.

Jore, S., Viljugrein, H., Brun, E., Heier, B.T., Borck, B., Ethelberg, S., Hakkinen, M., Kuusi, M., Reiersen, J., Hansson, I., Engvall, E.O., Lofdahl, M., Wagenaar, J.A., van Pelt, W. and Hofshagen, M. (2010) Trends in *Campylobacter* incidence in broilers and humans in six European countries, 1997-2007. *Prev Vet Med* **93**, 33-41.

Kapperud, G., Espeland, G., Wahl, E., Walde, A., Herikstad, H., Gustavsen, S., Tveit, I., Natas, O., Bevanger, L. and Digranes, A. (2003) Factors associated with increased and decreased risk of *Campylobacter* infection: a prospective case-control study in Norway. *Am J Epidemiol* **158**, 234-242.

Kapperud, G., Skjerve, E., Vik, L., Hauge, K., Lysaker, A., Aalmen, I., Ostroff, S.M. and Potter, M. (1993) Epidemiological investigation of risk factors for *Campylobacter* colonization in Norwegian broiler flocks. *Epidemiol Infect* **111**, 245-255.

Kärenlampi, R., Rautelin, H., Hakkinen, M. and Hänninen, M.L. (2003) Temporal and geographical distribution and overlap of Penner heat-stable serotypes and pulsed-field gel electrophoresis genotypes of *Campylobacter jejuni* isolates collected from humans and chickens in Finland during a seasonal peak. *J Clin Microbiol* **41**, 4870-4872.

Kärenlampi, R., Rautelin, H., Schönberg-Norio, D., Paulin, L. and Hänninen, M.L. (2007) Longitudinal study of Finnish *Campylobacter jejuni* and *C. coli* isolates from humans, using multilocus sequence typing, including comparison with epidemiological data and isolates from poultry and cattle. *Appl Environ Microbiol* **73**, 148-155.

Katsma, W.E., De Koeijer, A.A., Jacobs-Reitsma, W.F., Mangen, M.J. and Wagenaar, J.A. (2007) Assessing interventions to reduce the risk of *Campylobacter* prevalence in broilers. *Risk Anal* **27**, 863-876.

Katzav, M., Isohanni, P., Lund, M., Hakkinen, M. and Lyhs, U. (2008) PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products. *Food Microbiol* **25**, 908-914.

Klein, G., Beckmann, L., Vollmer, H.M. and Bartelt, E. (2007a) Predominant strains of thermophilic *Campylobacter* spp. in a German poultry slaughterhouse. *Int J Food Microbiol* **117**, 324-328.

Klein, G., Reich, F., Beckmann, L. and Atanassova, V. (2007b) Quantification of thermophilic *Campylobacter* spp. in broilers during meat processing. *Antonie Van Leeuwenhoek* **92**, 267-273.

Klena, J.D., Parker, C.T., Knibb, K., Ibbitt, J.C., Devane, P.M., Horn, S.T., Miller, W.G. and Konkel, M.E. (2004) Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene lpxA. *J Clin Microbiol* **42**, 5549-5557.

Levesque, S., Frost, E., Arbeit, R.D. and Michaud, S. (2008) Multilocus sequence typing of *Campylobacter jejuni* isolates from humans, chickens, raw milk, and environmental water in Quebec, Canada. *J Clin Microbiol* **46**, 3404-3411.

Lienau, J.A., Ellerbroek, L. and Klein, G. (2007) Tracing flock-related *Campylobacter* clones from broiler farms through slaughter to retail products by pulsed-field gel electrophoresis. *J Food Prot* **70**, 536-542.

Lindmark, H., Diedrich, I.C., Andersson, L., Lindqvist, R. and Engvall, E.O. (2006) Distribution of *Campylobacter* genotypes on broilers during slaughter. *J Food Prot* **69**, 2902-2907.

Linton, D., Lawson, A.J., Owen, R.J. and Stanley, J. (1997) PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *J Clin Microbiol* **35**, 2568-2572.

Linton, D., Owen, R.J. and Stanley, J. (1996) Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res Microbiol* **147**, 707-718.

Lior, H., Woodward, D.L., Edgar, J.A., Laroche, L.J. and Gill, P. (1982) Serotyping of Campylobacter jejuni by slide agglutination based on heat-labile antigenic factors. *J Clin Microbiol* **15**, 761-768.

Lübeck, P.S., Cook, N., Wagner, M., Fach, P. and Hoorfar, J. (2003) Toward an international standard for PCR-based detection of food-borne thermotolerant *Campylobacters*: validation in a multicenter collaborative trial. *Appl Environ Microbiol* **69**, 5670-5672.

Lund, M., Nordentoft, S., Pedersen, K. and Madsen, M. (2004) Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. *J Clin Microbiol* **42**, 5125-5132.

Lyhs, U., Katzav, M., Isohanni, P., Heiska, H. and Maijala, R. (2010) The temporal, PFGE and resistance pattern associations suggest that poultry products are only a minor source of human infections in western Finland. *Food Microbiol* **27**, 311-315.

Lyngstad, T.M., Jonsson, M.E., Hofshagen, M. and Heier, B.T. (2008) Risk factors associated with the presence of *Campylobacter* species in Norwegian broiler flocks. *Poult Sci* **87**, 1987-1994.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M. and Spratt, B.G. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**, 3140-3145.

Martin, S.W., Meek, A.H. and Willberg, P. (1997) *Veterinary epidemiology, principles and methods.* Iowa: Iowa State University Press.

Maslow, J.N., Slutsky, A.M. and Arbeit, R.D. (1993) Application of pulsed-field gel electrophoresis to molecular epidemiology. In *Diagnostic molecular microbiology. Principles and applications.* ed. Pershing, D.H., Smith, T.F. and White, T.J. pp. 563-572. Washington D.C.: American Society for Microbiology.

Meinersmann, R.J., Helsel, L.O., Fields, P.I. and Hiett, K.L. (1997) Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. *J Clin Microbiol* **35**, 2810-2814.

Meinersmann, R.J., Phillips, R.W., Hiett, K.L. and Fedorka-Cray, P. (2005) Differentiation of *Campylobacter* populations as demonstrated by flagellin short variable region sequences. *Appl Environ Microbiol* **71**, 6368-6374.

Messens, W., Herman, L., De Zutter, L. and Heyndrickx, M. (2009) Multiple typing for the epidemiological study of contamination of broilers with thermotolerant *Campylobacter*. *Vet Microbiol* **138**, 120-131.

Michaud, S., Menard, S. and Arbeit, R.D. (2004) *Campylobacteriosis*, Eastern Townships, Quebec. *Emerg Infect Dis* **10**, 1844-1847.

Michaud, S., Menard, S., Gaudreau, C. and Arbeit, R.D. (2001) Comparison of *Sma*I-defined genotypes of *Campylobacter jejuni* examined by *Kpn*I: a population-based study. *J Med Microbiol* **50**, 1075-1081.

Miller, R.S., Miller, W.G., Behringer, M., Hariharan, H., Matthew, V. and Oyarzabal, O.A. (2010) DNA identification and characterization of *Campylobacter jejuni* and *Campylobacter coli* isolated from caecal samples of chickens in Grenada. *J Appl Microbiol* **108**, 1041-1049.

Miller, W.G., Parker, C.T., Heath, S. and Lastovica, A.J. (2007) Identification of genomic differences between *Campylobacter jejuni subsp. jejuni* and *C. jejuni subsp. doylei* at the nap locus leads to the development of a *C. jejuni* subspeciation multiplex PCR method. *BMC Microbiol* **7**, 11.

Moore, J.E., Corcoran, D., Dooley, J.S., Fanning, S., Lucey, B., Matsuda, M., McDowell, D.A., Megraud, F., Millar, B.C., O'Mahony, R., O'Riordan, L., O'Rourke, M., Rao, J.R., Rooney, P.J., Sails, A. and Whyte, P. (2005) *Campylobacter*. *Vet Res* **36**, 351-382.

Nachamkin, I., Bohachick, K. and Patton, C.M. (1993) Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *J Clin Microbiol* **31**, 1531-1536.

Nakari, U.M., Puhakka, A. and Siitonen, A. (2008) Correct identification and discrimination between *Campylobacter jejuni* and *C. coli* by a standardized hippurate test and species-specific polymerase chain reaction. *Eur J Clin Microbiol Infect Dis* **27**, 513-518.

National Institute for Health and Welfare (2010) Infectious diseases in Finland 1995-2009. http://www.thl.fi/thl-client/pdfs/a4c2f994-438c-4f3e-9d72-7d0bf17f551c.

Nauta, M., van der Fels-Klerx, I. and Havelaar, A. (2005) A poultry-processing model for quantitative microbiological risk assessment. *Risk Anal* **25**, 85-98.

Newell, D.G. and Fearnley, C. (2003) Sources of *Campylobacter* colonization in broiler chickens. *Appl Environ Microbiol* **69**, 4343-4351.

Newell, D.G., Shreeve, J.E., Toszeghy, M., Domingue, G., Bull, S., Humphrey, T. and Mead, G. (2001) Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. *Appl Environ Microbiol* **67**, 2636-2640.

Nordic committee of Food Analyses (2007) Thermotolerant *Campylobacter*. Detection, semi-quantitative and quantative determination in foods and drinking water. *NCFA Method no.119, 3rd ed.*

Nordic Committee on food Analysis (1990) *Campylobacter jejuni/coli* detection in foods. *NCFA Method no.119, 2nd ed.*

Northcutt, J.K., Smith, D.P., Musgrove, M.T., Ingram, K.D. and Hinton, A., Jr. (2005) Microbiological impact of spray washing broiler carcasses using different chlorine concentrations and water temperatures. *Poult Sci* **84**, 1648-1652.

Nylen, G., Dunstan, F., Palmer, S.R., Andersson, Y., Bager, F., Cowden, J., Feierl, G., Galloway, Y., Kapperud, G., Megraud, F., Molbak, K., Petersen, L.R. and Ruutu, P. (2002) The seasonal distribution of *Campylobacter* infection in nine European countries and New Zealand. *Epidemiol Infect* **128**, 383-390.

On, S.L., McCarthy, N.D., Miller, W.G. and Gilpin, B.J. (2008) Molecular Epidemiology of *Campylobacter* Species. In *Campylobacter* ed. Nachamkin, I., Szymanski, C.M. and Blaser, M.J. pp. 191-211. Washington,DC: ASM Press.

Ono, K. and Yamamoto, K. (1999) Contamination of meat with *Campylobacter jejuni* in Saitama, Japan. *Int J Food Microbiol* **47**, 211-219.

Penner, J.L. and Hennessy, J.N. (1980) Passive hemagglutination technique for serotyping Campylobacter fetus subsp. jejuni on the basis of soluble heat-stable antigens. *J Clin Microbiol* **12**, 732-737.

Penner, J.L., Hennessy, J.N. and Congi, R.V. (1983) Serotyping of *Campylobacter jejuni* and *Campylobacter coli* on the basis of thermostable antigens. *Eur J Clin Microbiol* **2**, 378-383.

Perko, J. (1997) Kultamunaa etsimässä, Munakunta 1921-1996. Kokemäki: Munakunta.

Petersen, L., Nielsen, E.M. and On, S.L. (2001) Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks. *Vet Microbiol* **82**, 141-154.

Peyrat, M.B., Soumet, C., Maris, P. and Sanders, P. (2008a) Phenotypes and genotypes of *Campylobacter* strains isolated after cleaning and disinfection in poultry slaughterhouses. *Vet Microbiol* **128**, 313-326.

Peyrat, M.B., Soumet, C., Maris, P. and Sanders, P. (2008b) Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int J Food Microbiol* **124**, 188-194.

Pires, S.M., Vigre, H., Makela, P. and Hald, T. (2010) Using outbreak data for source attribution of human salmonellosis and campylobacteriosis in Europe. *Foodborne Pathog Dis* **7**, 1351-1361.

Posch, J., Feierl, G., Wuest, G., Sixl, W., Schmidt, S., Haas, D., Reinthaler, F.F. and Marth, E. (2006) Transmission of *Campylobacter* spp. in a poultry slaughterhouse and genetic characterisation of the isolates by pulsed-field gel electrophoresis. *Br Poult Sci* **47**, 286-293.

Ragimbeau, C., Schneider, F., Losch, S., Even, J. and Mossong, J. (2008) Multilocus sequence typing, pulsed-field gel electrophoresis, and *fla* short variable region typing of clonal complexes of *Campylobacter jejuni* strains of human, bovine, and poultry origins in Luxembourg. *Appl Environ Microbiol* **74**, 7715-7722.

Reich, F., Atanassova, V., Haunhorst, E. and Klein, G. (2008) The effects of *Campylobacter* numbers in caeca on the contamination of broiler carcasses with *Campylobacter*. *Int J Food Microbiol* **127**, 116-120.

Ribot, E.M., Fitzgerald, C., Kubota, K., Swaminathan, B. and Barrett, T.J. (2001) Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. *J Clin Microbiol* **39**, 1889-1894.

Ridley, A.M., Allen, V.M., Sharma, M., Harris, J.A. and Newell, D.G. (2008a) Real-time PCR approach for detection of environmental sources of *Campylobacter* strains colonizing broiler flocks. *Appl Environ Microbiol* **74**, 2492-2504.

Ridley, A.M., Toszeghy, M.J., Cawthraw, S.A., Wassenaar, T.M. and Newell, D.G. (2008b) Genetic instability is associated with changes in the colonization potential of *Campylobacter jejuni* in the avian intestine. *J Appl Microbiol* **105**, 95-104.

Ringoir, D.D., Szylo, D. and Korolik, V. (2007) Comparison of 2-day-old and 14-day-old chicken colonization models for *Campylobacter jejuni*. *FEMS Immunol Med Microbiol* **49**, 155-158.

Robinson, D.A. (1981) Infective dose of *Campylobacter jejuni* in milk. *Br Med J (Clin Res Ed)* **282**, 1584.

Rosenquist, H., Sommer, H.M., Nielsen, N.L. and Christensen, B.B. (2006) The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. *Int J Food Microbiol* **108**, 226-232.

Rushton, S.P., Humphrey, T.J., Shirley, M.D., Bull, S. and Jorgensen, F. (2009) *Campylobacter* in housed broiler chickens: a longitudinal study of risk factors. *Epidemiol Infect* **137**, 1099-1110.

Russa, A.D., Bouma, A., Vernooij, J.C., Jacobs-Reitsma, W. and Stegeman, J.A. (2005) No association between partial depopulation and *Campylobacter* spp. colonization of Dutch broiler flocks. *Lett Appl Microbiol* **41**, 280-285.

Sahin, O., Luo, N., Huang, S. and Zhang, Q. (2003) Effect of *Campylobacter*-specific maternal antibodies on *Campylobacter jejuni* colonization in young chickens. *Appl Environ Microbiol* **69**, 5372-5379.

Sanchez, M.X., Fluckey, W.M., Brashears, M.M. and McKee, S.R. (2002) Microbial profile and antibiotic susceptibility of *Campylobacter* spp. and *Salmonella* spp. in broilers processed in air-chilled and immersion-chilled environments. *J Food Prot* **65**, 948-956.

Sandberg, M., Hofshagen, M., Ostensvik, O., Skjerve, E. and Innocent, G. (2005) Survival of *Campylobacter* on frozen broiler carcasses as a function of time. *J Food Prot* **68**, 1600-1605.

Sanders, S.Q., Boothe, D.H., Frank, J.F. and Arnold, J.W. (2007) Culture and detection of *Campylobacter jejuni* within mixed microbial populations of biofilms on stainless steel. *J Food Prot* **70**, 1379-1385.

Schnider, A., Overesch, G., Korczak, B.M. and Kuhnert, P. (2010) Comparison of real-time PCR assays for detection, quantification, and differentiation of *Campylobacter jejuni* and *Campylobacter coli* in broiler neck skin samples. *J Food Prot* **73**, 1057-1063.

Schouls, L.M., Reulen, S., Duim, B., Wagenaar, J.A., Willems, R.J., Dingle, K.E., Colles, F.M. and Van Embden, J.D. (2003) Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: strain diversity, host range, and recombination. *J Clin Microbiol* **41**, 15-26.

Sebald, M. and Veron, M. (1963) Base Dna Content and Classification of Vibrios. *Ann Inst Pasteur (Paris)* **105**, 897-910.

Shane, S.M. (2000) *Campylobacter* infection of commercial poultry. *Rev Sci Tech* **19**, 376-395.

Shanker, S., Lee, A. and Sorrell, T.C. (1986) *Campylobacter jejuni* in broilers: the role of vertical transmission. *J Hyg (Lond)* **96**, 153-159.

Siemer, B.L., Harrington, C.S., Nielsen, E.M., Borck, B., Nielsen, N.L., Engberg, J. and On, S.L. (2004) Genetic relatedness among *Campylobacter jejuni* serotyped isolates of diverse origin as determined by numerical analysis of amplified fragment length polymorphism (AFLP) profiles. *J Appl Microbiol* **96**, 795-802.

Skirrow, M.B. (2006) John McFadyean and the centenary of the first isolation of *Campylobacter* species. *Clin Infect Dis* **43**, 1213-1217.

Skirrow, M.B. (1977) *Campylobacter* enteritis: a "new" disease. *Br Med J* **2**, 9-11.

Smith, D.P., Cason, J.A. and Berrang, M.E. (2005) Effect of fecal contamination and cross-contamination on numbers of coliform, *Escherichia coli*, *Campylobacter*, and *Salmonella* on immersion-chilled broiler carcasses. *J Food Prot* **68**, 1340-1345.

Smith, K., Reimers, N., Barnes, H.J., Lee, B.C., Siletzky, R. and Kathariou, S. (2004) *Campylobacter* colonization of sibling turkey flocks reared under different management conditions. *J Food Prot* **67**, 1463-1468.

Smith, R.D. (1995) *Veterinary Clinical Epidemiology.* Florida: CRC Press Inc.

Steinhauserova, I., Ceskova, J., Fojtikova, K. and Obrovska, I. (2001) Identification of thermophilic *Campylobacter* spp. by phenotypic and molecular methods. *J Appl Microbiol* **90**, 470-475.

Steinhauserova, I., Fojtikova, K. and Klimes, J. (2000) The incidence and PCR detection of *Campylobacter upsaliensis* in dogs and cats. *Lett Appl Microbiol* **31**, 209-212.

Stern, N.J., Fedorka-Cray, P., Bailey, J.S., Cox, N.A., Craven, S.E., Hiett, K.L., Musgrove, M.T., Ladely, S., Cosby, D. and Mead, G.C. (2001) Distribution of *Campylobacter* spp. in selected U.S. poultry production and processing operations. *J Food Prot* **64**, 1705-1710.

Stern, N.J. and Robach, M.C. (2003) Enumeration of *Campylobacter* spp. in broiler feces and in corresponding processed carcasses. *J Food Prot* **66**, 1557-1563.

Talibart, R., Denis, M., Castillo, A., Cappelier, J.M. and Ermel, G. (2000) Survival and recovery of viable but noncultivable forms of *Campylobacter* in aqueous microcosm. *Int J Food Microbiol* **55**, 263-267.

Taylor, D.E. (1992) Genetics of Campylobacter and Helicobacter. *Annu Rev Microbiol* **46**, 35-64.

Toivio, H. (2009) Suomen broilerituotannon historia 1959-2009. Sastamala: Suomen Broileryhdistys.

Urwin, R. and Maiden, M.C. (2003) Multi-locus sequence typing: a tool for global epidemiology. *Trends Microbiol* **11**, 479-487.

van de Giessen, A.W., Bloemberg, B.P., Ritmeester, W.S. and Tilburg, J.J. (1996) Epidemiological study on risk factors and risk reducing measures for *Campylobacter* infections in Dutch broiler flocks. *Epidemiol Infect* **117**, 245-250.

van de Giessen, A.W., Tilburg, J.J., Ritmeester, W.S. and van der Plas, J. (1998) Reduction of *Campylobacter* infections in broiler flocks by application of hygiene measures. *Epidemiol Infect* **121**, 57-66.

van Gerwe, T., Miflin, J.K., Templeton, J.M., Bouma, A., Wagenaar, J.A., Jacobs-Reitsma, W.F., Stegeman, A. and Klinkenberg, D. (2009) Quantifying transmission of *Campylobacter jejuni* in commercial broiler flocks. *Appl Environ Microbiol* **75**, 625-628.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. and De Ley, J. (1991) Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41**, 88-103.

Vandamme, P. and On, S.L. (2001) Recommendations of the subcommittee on the taxonomy of *Campylobacter* and related bacteria. *Int J Syst Evol Microbiol* **51**, 719-721.

Vandamme, P., Van Doorn, L.J., al Rashid, S.T., Quint, W.G., van der Plas, J., Chan, V.L. and On, S.L. (1997) *Campylobacter hyoilei* Alderton et al. 1995 and *Campylobacter coli* Veron and Chatelain 1973 are subjective synonyms. *Int J Syst Bacteriol* **47**, 1055-1060.

Vauterin, L. and Vauterin, P. (1992) Computer-aided objective comparison of electrophoretic patterns for grouping and identification of microorganisms. *Europ Microbiol* **1**, 37-41.

Vellinga, A. and Van Loock, F. (2002) The dioxin crisis as experiment to determine poultry-related *Campylobacter* enteritis. *Emerg Infect Dis* **8**, 19-22.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J. and Kuiper, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407-4414.

Waage, A.S., Vardund, T., Lund, V. and Kapperud, G. (1999) Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. *Appl Environ Microbiol* **65**, 1636-1643.

Waino, M., Bang, D.D., Lund, M., Nordentoft, S., Andersen, J.S., Pedersen, K. and Madsen, M. (2003) Identification of *Campylobacteria* isolated from Danish broilers by phenotypic tests and species-specific PCR assays. *J Appl Microbiol* **95**, 649-655.

Wang, G., Clark, C.G., Taylor, T.M., Pucknell, C., Barton, C., Price, L., Woodward, D.L. and Rodgers, F.G. (2002) Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus subsp. fetus*. *J Clin Microbiol* **40**, 4744-4747.

Wassenaar, T.M., Fernandez-Astorga, A., Alonso, R., Marteinsson, V.T., Magnusson, S.H., Kristoffersen, A.B. and Hofshagen, M. (2009) Comparison of *Campylobacter fla*-SVR genotypes isolated from humans and poultry in three European regions. *Lett Appl Microbiol* **49**, 388-395.

Wassenaar, T.M., Geilhausen, B. and Newell, D.G. (1998) Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. *Appl Environ Microbiol* **64**, 1816-1821.

Wassenaar, T.M. and Newell, D.G. (2000) Genotyping of *Campylobacter* spp. *Appl Environ Microbiol* **66**, 1-9.

World Health Organization (2010), http://www.who.int/mediacentre/factsheets.

Zweifel, C., Scheu, K.D., Keel, M., Renggli, F. and Stephan, R. (2008) Occurrence and genotypes of *Campylobacter* in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms. *Int J Food Microbiol* **125**, 182-187.

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Prevalence of campylobacters in chicken flocks during the summer of 1999 in Finland

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SUMMARY

In order to determine the prevalence of campylobacter positive broiler flocks in Finland, every flock from all three major slaughterhouses was studied during the period from 1 May to 30 September 1999. Caecal samples were taken in the slaughterhouses from five birds per flock. A total of 1132 broiler flocks were tested and 33 (2·9%) of those were campylobacter positive. Thirty-one isolates were *C. jejuni* and two isolates were *C. coli*. Isolates were serotyped for heat-stable antigens (Penner) and genotyped with pulsed-field gel electrophoresis (PFGE). The most common serotypes were serotypes 6, 7, 12 and 4-complex. Together with *Sma*I and *Kpn*I patterns there were 18 different PFGE genotypes. Simultaneous monitoring of chicken flocks and typing of the isolates produced data which can be used to study the epidemiology of campylobacters in chicken as well as their role in human infections.

INTRODUCTION

In Finland, as in many Western European countries the number of reported human campylobacter cases has increased during recent years. Latterly the number of campylobacter cases has exceeded that of the reported number of salmonella cases [1, 2]. In 1999, 3303 campylobacter and 2801 salmonella infections were reported in Finland [2]. In epidemiological studies handling or eating poultry have been shown to be significant risk factors for human infections [3, 4]. The contamination rate of poultry at the retail level varies between different countries from 14–98% [5]. In Finland, the contamination rate at retail markets in the Helsinki area during the seasonal peak in July to August in 1996–9 was 10–30% [6].

Decreasing the prevalence of campylobacter colonized broiler flocks is considered to be one of the most effective ways to reduce the number of campylobacter positive poultry products [7, 8]. This ensures the microbiological safety of fresh chicken for human consumption. Although slaughtering technique and processing hygiene have improved, the contamination of carcasses from intestinal contents is not likely to be completely prevented [9, 10].

Prevalence studies on campylobacter positive poultry flocks in Europe have been made and results vary from 18% in Norway to 82% in The Netherlands [11]. In many studies a seasonal variation of the prevalence of campylobacter colonized flocks has been seen. Higher recovery rates have been detected during the summer months, June, July and August, compared to winter [7, 8, 12].

There are only limited data on the prevalence of campylobacters in chicken flocks in Finland. In 1988 Aho and Hirn [13] published a study in which they reported that 24% (117/490) of caecal samples at slaughter were campylobacter positive. The three major slaughterhouses do their own-check studies but there has not been any permanent monitoring programme controlled by authority in which all slaughter-

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houses have participated. In 1999, from 1 May to 30 September, every flock from all three major slaughterhouses which account for 98% of Finnish chicken meat production were studied in order to determine the prevalence of campylobacter positive broiler flocks. In order to get more data on diversity of campylobacter isolates and to compare similarity of chicken and human isolates for epidemiological purposes, serotyping with heat stable antigens and genotyping with pulsed-field gel electrophoresis was performed. This study was a co-operation between the slaughterhouses, the National Veterinary and Food Research Institute and the Department of Food and Environmental Hygiene.

MATERIALS AND METHODS

Study design

The study population consisted of 1132 broiler flocks which included all the slaughtered flocks of the three major Finnish poultry companies from 1 May to 30 September in 1999. These three companies produce approximately 98% of the broiler meat produced in Finland. Broiler chickens are slaughtered at the age of 35–42 days and the entire flock is slaughtered on the same day or on 2 subsequent days.

Sampling

Caecal samples were taken from slaughterhouses by sampling of five birds from each flock. The size of the flock varied from 3500–45000 birds, the most usual flock size being 15000 or 30000 birds. The number of studied caecal samples, five, was estimated to detect campylobacter positive flocks at a confidence level of 97·5% from population size up to 45000 birds with an estimated prevalence of 60% within the infected flock. If at least one of the five samples was positive the flock was classified as positive.

Caecal samples were taken by broiler-company personnel at the point of meat inspection of viscera. Individual samples were cultured in the laboratories of the participating slaughterhouses.

Bacteriological methods

Caecal samples were analysed for campylobacter using a modified version of the procedure described by Bolton et al. [14] for isolation of campylobacter from faeces. Caecal contents were cultured by direct plating on modified CCD agar (Oxoid Ltd., Basingstoke, Hamphire, UK). The plates were incubated in a microaerobic atmosphere at 42 °C for 48 h. Two typical colonies were subcultured and sent for further analysis to the National Veterinary and Food Research Institute and the Department of Food and Environmental Hygiene.

Isolates were identified to the species level by the use of Gram-staining, phase contrast microscopy for motility, oxidase, catalase, hippurate hydrolysis and susceptibility to nalidixic acid (30 μg/ml) according to a modified procedure of the Nordic Committee on Food Analysis [15]. One isolate from each positive flock was taken for sero- and genotyping studies.

Serotyping

Campylobacter jejuni isolates were serotyped with a commercial reagent for the serotyping of heat stable antigens (Penner) of campylobacters by the passive haemagglutination method (Denka Seiken Co., Ltd. Tokyo, Japan).

Genotyping with pulsed-field gel electrophoresis

For genotyping with PFGE all isolates were cultured on brucella blood agar (Oxoid) plates incubated at 37 °C in a microaerobic atmosphere for 24–40 h. The bacterial cells were harvested and DNA plugs were prepared as described earlier [16, 17]. The DNA plug slices were digested with *Sma*I or *Kpn*I restriction enzymes (New England Biolabs, Hertfordshire, UK) as described by the manufacturer. *Sma*I and *Kpn*I fragments were separated with a ramped pulse of 0·5–40 sec for 19 h or 1–25 sec for 20 h, respectively. Otherwise, electrophoresis conditions were as described earlier [16].

If the isolates had one or more differences in *Sma*I bands they were considered as different patterns and named as S1, S2 and so on. If they had five or more different bands in *Kpn*I they were considered as differing patterns and named as K1, K2 and so on. Together these two patterns were combined and named as genotype C1, C2 and so on. The schema has been used in our earlier studies [16].

RESULTS

The overall campylobacter-positive flock prevalence was 2·9% (33 of the total 1132 flocks studied) during the period from 1 May to 30 September 1999. The

Table 1. *Campylobacter positive farms and characterization of campylobacter isolates by sero- and genotyping*

Isolate	Month of isolation	Farm	No. of birds in the flock	No.of campylobacter positive houses/ total no. of houses	Serotype (Penner)	PFGE genotype		
						*Sma*I pattern	*Kpn*I pattern	Combined
1831	May	A	15500	1/2	4, 13, 16, 43, 50	S3	K6	C6
1959	June	B	37500	1/1	6, 7	S2	K3	C3
2059	June	C	15000	1/1	27	S2	K4	C4
2165	July	D	30000	1/1	12	S1	K1	C1
2166	July	E	15000	1/2	NS	S2	K5	C5
2186	July	B	3000	4/4	6, 7	S2	K3	C3
2197	July	B	7000		6, 7	S2	K3	C3
2199	July	B	15000		ND	S2	K3	C3
2213	July	B	10000		6, 7	S2	K3	C3
2219	July	F	12500	1/1	4, 13, 16, 43, 50	S4	K6	C7
2227	July	G	30000	1/1	6, 7	UD	K8	C9
2230	July	H	30000	1/1	12	S1	K1	C1
2232	July	I	11000	2/2	4, 13, 16, 43, 50	S5	K7	C8
2234	July	I	11000		ND	S2	K4	C4
2252	July	J	7000	1/1	41	S1	K2	C2
2347	July	K	15000	1/1	57	S11	K13	C15
2351	July	L	30000	1/2	12	S6	K9	C10
2360	July	M	30000	1/1	NS	S2	K5	C5
2361	July	N	30000	1/2	12	S6	K9	C10
2362	Aug.	H	30000	1/1	NS	S2	K4	C4
2447	Aug.	O	15000	2/2	*C. coli*	S14	K15	C18
2448	Aug.	O	15000		*C. coli*	S14	K15	C18
2449	Aug.	P	15000	1/1	6, 7	UD	K8	C9
2450	Aug.	Q	30000	1/2	6, 7	UD	K8	C9
2458	Aug.	B	44000	1/1	27	S2	K4	C4
2538	Aug.	R	30000	2/2	NS	S12	K13	C16
2539	Aug.	R	30000		ND	S7	K10	C11
2541	Aug.	S	30000	2/2	ND	S8	K11	C12
2542	Aug.	S	30000		ND	S1	K1	C1
2867	Sept.	J	7000	1/1	5	S9	K12	C13
2899	Sept.	T	27000	1/2	NS	—	—	—
2946	Sept.	U	8000	1/5	11	S10	K12	C14
2965	Sept.	V	15500	1/3	NS	S13	K14	C17

NS, nonserotypable; ND, not done; UD, undigested.

sizes of campylobacter positive flocks varied: < 10000 (5), 10000–15500 (11), 15600–20000 (0), 20000–30000 (12), and > 30000 (2) (Table 1). During this time approximately 19700000 broilers were slaughtered and 606000 (3%) of these were campylobacter positive, if the whole flock was assumed to be positive when one sample was positive. The monthly variation in the number of campylobacter positive flocks is shown in Table 2. Thirty-one of the isolates were *C. jejuni* (94%) and two were *C. coli* (6%). Positive flocks were from 22 farms. The total number of studied farms was 220. Three of the farms had positive flocks subsequently (B, H and J, Table 1). Thirteen of the positive farms had two or more broiler houses. In eight of these farms only one of the houses was positive for campylobacter. Five farms that had several houses had every house positive for campylobacter (Table 1).

Serotypes

Eight serotypes were identified among 26 isolates which were serotyped (Table 2). Six of the isolates were nonserotypable with the available set of serospecific sera. Serotype 6, 7 was the most common serotype found (7/26) and serotypes 12, 4-complex and 27 were isolated more than once. Serotypes 6, 7 and 27 were found in June, July and August. Serotype

Table 2. *Monthly variation in the number of campylobacter positive flocks*

Month	No. of flocks	No. of positive flocks	%
May	227	1	0·4
June	224	2	0·9
July	230	16	7·0
Aug.	220	10	4·5
Sept.	231	4	1·7
Total	1132	33	2·9

12 was seen in July and August. The 4-complex serotype was seen in May and July. In September unique serotypes 5 and 11 were found. Serotypes of subsequent campylobacter positive flocks at farm B were 6, 7 in June and 27 in August, at farm J, 41 in July and 5 in September, and at farm H, 12 in July and nonserotypable in August (Table 2). Serotypes 6, 7, 27 and 4-complex were identified in the samples of two slaughterhouses and serotype 12 occurred only in the samples of one slaughterhouse.

Genotypes

Thirty *C. jejuni* and two *C. coli* isolates were genotyped with PFGE. *Sma*I enzyme identified 14 different patterns and *Kpn*I identified 15 different patterns. Together there were 18 different genotypes. The most common serotype 6, 7 was associated with *Sma*I genotype S2 (4/7) and three of the isolates were not digested with *Sma*I. All S2 isolates had highly similar patterns when digested with *Kpn*I (K3) and they were named as combined genotype C3. Furthermore serotype 6, 7 isolates which were not digested with *Sma*I (UD) had identical *Kpn*I patterns (K8) and they were named as combined genotype C9. All genotype C3 isolates originated from one farm from slaughterhouse 2. Genotype C9 isolates originated from three different farms but from the same slaughterhouse, 3.

Serotype 12 included two different combined genotypes C1 and C10. They were all from slaughterhouse 3 and from four different farms. One farm which had genotype C1 in July also had a positive flock in August, but the isolate was nonserotypable and its genotype was C4. The genotype C4 was also found in June, July and August. Genotype C4 was the only one which was found in the areas of all three slaughterhouses. This genotype had serotype 27 or it was nonserotypable. Serogroup 4-complex included three different genotypes C6, C7 and C8. These types were found in two different slaughterhouses.

DISCUSSION

Systematic monitoring on the prevalence of campylobacter serotypes and genotypes in Finnish chicken farms from the entire country was performed. There are only a few countries in Europe who monitor the prevalence of campylobacter positive flocks from the entire country. In Denmark [18] and Sweden [19] prevalences have been followed for several years. Our study period was during five summer months because it is known from several other studies that there is a clear seasonal variation in the prevalence of campylobacter positive flocks [5, 7, 8] and in the human cases [4, 6] especially within the Nordic countries, Norway, Sweden, Denmark [7, 8, 10].

The results showed that approximately 3% of the flocks were positive indicating a very low campylobacter contamination level in chickens. Previous studies have shown that Sweden and Norway also have a low campylobacter prevalence [7, 8]. According to several studies [8, 9, 20, 21] campylobacter infection is introduced sporadically into the flock from an external site in the environment. Strict hygiene and biosecurity are suggested to be one of the most successful measures against environmental contamination [8, 9, 20] and the presence of a hygiene barrier has been suggested to be the most important single biosecurity measure [10]. Moreover Gibbens et al. [22] found out in their trial, that hygiene and biosecurity measures helped to control campylobacter infection in a poultry flock.

In Finland, the poultry industry is well organized and because of a strict salmonella control programme, farmers are educated to understand the importance of biosecurity barriers and hygiene control in the prevention of environmental contamination. For example boot dips are widely used as a biosecurity barrier. The construction of chicken houses prevents environmental contamination. In addition due to cold winters, houses are well insulated thus preventing the vector animals from entering, and the inside environment may be standardized. Snow-covered earth in winter decreases the possible outside sources of contamination. Competitive exclusion, to prevent salmonella, has been in wide use for over 20 years. This also might have an impact on decreasing

colonization of campylobacters in chicken [12]. In conclusion, a combination of various preventive factors may explain the low prevalence of campylobacter positive flocks in Finland.

Only three of the farms had two subsequent campylobacter positive flocks. In Finland the poultry rearing is a batch all-in, all-out system. The empty period between flocks is approximately 2 weeks. During this time houses are cleaned, disinfected and litter is changed. Dividing the flock into cohort batches for slaughter and an empty period shorter than 14 days have been found as risk factors for campylobacter contamination [10].

Even though we only followed the prevalence of positive flocks from May to September the typical seasonal variation was seen. In May and June only a few positive flocks were identified. The prevalence increased in July and August and decreased in September. Furthermore, in humans, most domestic campylobacter infections occur in June to August [16].

The number of isolates identified was rather low, but they probably represented most of the chicken isolates circulating in the chicken food chain during this period. Heterogeneity of sero/genotypes seen in other studies [8, 23] was also found in our study. Among 30 *C. jejuni* isolates 18 combined genotypes (*Sma*I and *Kpn*I) were identified. Common serotypes were subdivided into differing genotypes and certain genotypes were associated with serotypes 6, 7, 12 and 27 as also found in our earlier study [24].

Common identified serotypes were serotypes 6, 7, 12, 4-complex and serotype 27. These serotypes have also been found in our earlier studies on Finnish human *C. jejuni* isolates from 1995 and 1996 [24]. These serotypes were also seen among Finnish human and chicken isolates with known PFGE genotypes from 1997 and 1998 [25]. Our sero/genotyping studies over a 5 year period suggest that certain sero/genotypes are persistent among Finnish human and chicken isolates [6, 24, 25] and these sero/genotypes were identified in the present study as well. Further sero/genotyping studies of Finnish isolates will provide more data on the importance of these types. Simultaneous sero/genotyping of human and chicken isolates will provide data on persistent *C. jejuni* strains and their role in the contamination of broiler flocks and in human infections.

To confirm the low prevalence of *C. jejuni* within chicken farms in Finland, further monitoring studies would be needed. According to this study, sero/genotyping indicated that certain types found among chicken isolates are persistent. Combination of sero- and genotyping can be a useful tool to follow the persistence of certain strains in the Finnish environment and circulation of certain sero/genotypes among chicken farms.

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REFERENCES

1. Rohner P, Pittet D, Pepey B, Nije-Kinge T, Auckenthaler R. Etiological agents of infectious diarrhea: implications for requests for microbial culture. J Clin Microbiol 1997; **35**: 1427–32.
2. National Public Health Institute, Finland. National infectious disease registry. Available at: http://www.ktl.fi/ttr/. Accessed 8 March 2002.
3. Neimann J, Engberg J, Mölbak K, Wegener HC. Food borne risk factors associated with sporadic campylobacteriosis in Denmark. Dansk Veterinärtidskrift 1998; **81**: 702–5.
4. Studahl A, Andersson Y. Risk factors for indigenous campylobacter infection: a Swedish case-control study. Epidemiol Infect 2000; **125**: 269–75.
5. Jacobs-Reitsma W. Campylobacter in the food supply. In: Nachamkin I, Blaser MJ, eds. Campylobacter. Washington D.C.: American Society for Microbiology, 2000: 497–509.
6. Hänninen M-L, Perko-Mäkelä P, Pitkälä A, Rautelin H. A three-year study of *Campylobacter jejuni* genotypes in humans with domestically acquired infections and in chicken samples from Helsinki area. J Clin Microbiol 2000; **38**: 1998–2000.
7. Kapperud G, Skjerve E, Vik L, et al. Epidemiological investigation of risk factors for campylobacter colonization in Norwegian broiler flocks. Epidemiol Infect 1993; **111**: 245–55.
8. Berndtson E. Campylobacter in broiler chickens the mode of spread in chicken flocks with special reference to food hygiene (dissertation). Uppsala, Swedish University of Agricultural Science, 1996.
9. Giessen AW van de, Tilburg JJHC, Ritmeester WS, Plas J van der. Reduction of campylobacter infections in broiler flocks by application of hygiene measures. Epidemiol Infect 1998; **121**: 57–66.
10. Hald B, Wedderkopp A, Madsen M. Thermofilic *Campylobacter* spp. in Danish broiler production: a cross-sectional survey and a retrospective analysis of risk factors for occurrence in broiler flocks. Avian Pathol 2000; **29**: 123–31.

11. Newell DG, Wagenaar JA. Poultry infections and their control at the farm level. In: Nachamkin I, Blaser MJ, eds. Campylobacter. Washington D.C.: American Society for Microbiology, 2000: 497–509.
12. Shane SH. Campylobacter infection of commercial poultry. Rev Sci Tech 2000; **19**: 376–95.
13. Aho M, Hirn J. Prevalence of campylobacteria in the Finnish broiler chicken chain from the producer to the consumer. Acta Vet Scand 1988; **29**: 451–62.
14. Bolton FJ, Hutchinson DN, Coates D. Blood-free selective medium for isolation of *Campylobacter jejuni* from feces. J Clin Microbiol 1984; **19**: 169–71.
15. The Nordic Committee on Food Analysis. *Campylobacter jejuni/coli* detection in foods. NMKL Method no. 119, 2nd edn, 1990.
16. Hänninen M-L, Pajarre S, Klossner M-L, Rautelin H. Typing of human *Campylobacter jejuni* isolates in Finland by pulsed-field gel electrophoresis. J Clin Microbiol 1998; **36**: 1787–9.
17. Maslow JN, Slutsky AM, Arbeit RD. Application of pulsed-field gel electrophoresis to molecular epidemiology. In: Persing DH, Smith TF, Tenover FC, White TJ, eds. Diagnostic molecular microbiology: principles and applications. Washington D.C.: American Society for Microbiology, 1993: 563–72.
18. Danish Zoonoosis Centre. Available at: http://130.226.165.6/annualreport1999/003.html. Accessed 8 March 2002.
19. Swedish Institute for Infectious Diseases. Annual report 1999 Available at: http://www.smittskyddsinstitutet.se/download/pdf/rapp99.pdf. Accessed 12 March 2002.
20. Hartnett E, Kelly L, Newell D, Wooldridge M, Gettinby G. A quantitative risk assessment for the occurrence of campylobacter in chickens at the point of slaughter. Epidemiol Infect 2001; **127**: 195–206.
21. Evans SJ, Sayers AR. A longitudinal study of campylobacter infection of broiler flocks in Great Britain. Prev Vet Med 2000; **46**: 209–23.
22. Gibbens JC, Pascoe SJS, Evans SJ, Davies RH, Sayers AR. A trial of biosecurity as a means to control campylobacter infection of broiler chickens. Prev Vet Med 2001; **48**: 85–99.
23. Nielsen EM, Nielson NL. Serotypes and typability of *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry products. J Food Microbiol 1999; **46**: 199–205.
24. Rautelin H, Hänninen M-L. Comparison of a commercial test for serotyping heat-stable antigens of *Campylobacter jejuni* with genotyping by pulsed-field gel electrophoresis. J Med Microbiol 1999; **48**: 617–21.

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Genomic Relatedness within Five Common Finnish *Campylobacter jejuni* Pulsed-Field Gel Electrophoresis Genotypes Studied by Amplified Fragment Length Polymorphism Analysis, Ribotyping, and Serotyping

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Thirty-five Finnish *Campylobacter jejuni* strains with five *Sma*I/*Sac*II pulsed-field gel electrophoresis (PFGE) genotypes selected among human and chicken isolates from 1997 and 1998 were used for comparison of their PFGE patterns, amplified fragment length polymorphism (AFLP) patterns, *Hae*III ribotypes, and heat-stable (HS) serotypes. The discriminatory power of PFGE, AFLP, and ribotyping with *Hae*III were shown to be at the same level for this selected set of strains, and these methods assigned the strains into the same groups. The PFGE and AFLP patterns within a genotype were highly similar, indicating genetic relatedness. The same HS serotypes were distributed among different genotypes, and different serotypes were identified within one genotype. HS serotype 12 was only associated with the combined genotype G1 (PFGE-AFLP-ribotype). These studies using polyphasic genotyping methods suggested that common Finnish *C. jejuni* genotypes form genetic lineages which colonize both humans and chickens.

Campylobacter jejuni is the leading cause of human bacterial gastroenteritis in developed countries (22, 29). Serious consequence of campylobacteriosis can be the development of the Guillain-Barré and Miller-Fisher syndromes (33). Most human infections are apparently sporadic, and the distribution of cases shows a seasonal variation. In the Northern hemisphere the human cases occur mostly from June to September (19, 29). *C. jejuni* is commonly found in the intestinal contents of many domestic and wild animals (27), and there may also be a seasonal variation in the infection rate of poultry (3, 18) and the fecal excretion of *C. jejuni* in cattle and calves. (28). Although in a few cases, the transmission routes from animal hosts and environmental sources to humans have not been determined, epidemiological studies and data from outbreaks indicate that contaminated drinking water, unpasteurized milk, and eating or handling contaminated poultry products are important risk factors associated with human infections (19, 29).

Subtyping of *C. jejuni* strains supports epidemiological studies for tracing sources and transmission routes of infections. Serotyping, phage typing, and molecular typing of *Campylobacter* isolates from human and animal sources have revealed that *C. jejuni* is a highly heterogeneous organism (7, 11, 23). For example, approximately 70 heat-stable and more than 100 heat-labile serotypes have been identified for *C. jejuni* and *C. coli* (22). Application of several typing techniques for comparison of strains obtained from humans and animals have revealed that there is an overlap of serotypes and phage types indicating either common infection sources or transmission of the organism from animal reservoirs to humans through food chains, drinking water, or direct animal contact (11, 21).

Genotyping techniques have shown distinct levels of discriminatory power when applied for studies on *C. jejuni*. One of the most discriminating techniques has been shown to be pulsed-field gel electrophoresis (PFGE), which uses rare-cutting restriction enzymes and shows sequence variation in restriction sites located over the whole genome (4, 20). However, with *Sma*I, an enzyme commonly used for PFGE of *C. jejuni*, only a limited number of fragments is generated, which limits the discriminatory power of this technique (9, 11). To increase the discrimatory power, *Kpn*I (10) or *Sac*II analysis (11) can be used in combination with *Sma*I. Ribotyping, based on restriction fragment length polymorphism (RFLP) analysis of ribosomal loci, is a less discriminatory method than PFGE for *C. jejuni* (4, 9) since *C. jejuni* only has three copies of ribosomal genes, which decreases the number of fragments obtained for a pattern (6). Amplified fragment length polymorphism (AFLP) is a rather new technique used for *Campylobacter* typing which, by combination of DNA restriction with one or more restriction enzymes and the use of a selective PCR, amplifies a subset of chromosomal fragments. AFLP has been recently applied to studies on *C. jejuni* strains from different sources and was shown to be a highly discriminatory technique for analysis of both *C. jejuni* and *C. coli* strains (5).

In the present study three genotyping methods—PFGE, AFLP, and ribotyping and serotyping—were applied to a set of selected *C. jejuni* strains. The selected strains represented five combined *Sma*I/*Sac*II PFGE genotype groups that were com-

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TABLE 1. *C. jejuni* strains, their sources, PFGE patterns, ribotypes, AFLP types, and HS serotypes

Strain (n = 35)	Source data[a]	PFGE pattern (*Sma*I/*Sac*II)	Ribotype (*Hae*III)	AFLP type	Combined genotype	Serotype (HS)[b]
5423F	Patient, Pori, 98-07	I/K	A	AF1	G1	12
4593	Chicken, retail shop, Helsinki, producent A, 98-08	I/K	A	AF1	G1	12
4772	Chicken, retail shop, Helsinki, producent B, 98-08	I/K	A	AF1	G1	12
FB3886	Patient, Helsinki, 98-07	I/K	A	AF1	G1	1,44
FB4287	Patient, Helsinki, 98-07	I/K	A	AF1	G1	1,44
25A	Chicken fecal sample, 98-07	I/Kc	B	AF1	G2	57
5768	Chicken, retail shop, Helsinki, producent C, 98-09	I/K	A	AF1	G1	12
5483	Chicken, retail shop, Helsinki, producent A, 98-09	I/Ka	Aa	AF2	G3	15
40A	Chicken, fecal sample, 98-11	I/K	A	AF3	G4	6,7
35A	Chicken, fecal sample, 98-11	IV	C	AF4	G5	1,44
37A	Chicken, fecal sample, 98-11	I/Kc	B	AF4	G6	57
28A	Chicken, fecal sample, 98-08	I/Ka	Aa	AF5	G7	27
BK116	Chicken, retail shop, Helsinki, producent C, 97-08	I/K	A	AF5	G8	27
5862	Chicken, retail shop, Helsinki, 98-09	VII	E	AF6	G9	NS
FB5241	Patient, Helsinki, 98-08	VIa	D	AF7	G10	1,44
FB5519	Patient, Helsinki, 98-08	VIc	D	AF7	G10	1,44
FB4619	Patient, Helsinki, 98-07	VIa	D	AF7	G10	1,44
4859	Chicken, retail shop, Helsinki, 98-08	VIb	D	AF7	G10	NS
FB4700	Patient, Helsinki, 98-07	VIa	D	AF7	G10	1,44
23OO4	Patient, Pori, 98-07	VIa	D	AF7	G10	NS
88055	Patient, Pori, 98-07	VIa	D	AF7	G10	NS
FB4877	Patient, Helsinki, 98-07	VIc	D	AF7	G10	NS
BK292	Chicken, retail shop, Helsinki, 98-08	VIa	D	AF7	G10	4
4854	Patient, Helsinki, 98-07	VIc	D	AF7	G10	NS
81209	Patient, Pori, 98-07	VIc	D	AF7	G10	4
BR170	Chicken, retail shop, Helsinki, producent A, 98-08	VIb	D	AF7	G10	NS
5259	Chicken, retail shop, 98-08, Helsinki, producent B	VIc	D	AF7	G10	NS
FB6271	Patient, Helsinki, 97-07	T101a	F	AF8	G11	1,44
456	Patient, Helsinki, 97-07	T101a	F	AF8	G11	4
BR77	Chicken, retail shop, Helsinki, 97-07	T101a	F	AF8	G11	4
4180	Chicken, retail shop, Helsinki, 98-07	T101b	Fa	AF9	G12	4
BR100	Chicken, retail shop, Helsinki, 97-07	IV	C	AF10	G13	1,44
2475	Chicken, retail shop, Helsinki, 98-05	IV	C	AF10	G13	1,44
FB287	Patient, Helsinki, 98-06	IV	C	AF10	G13	1,44
FB8164	Patient, Helsinki, 97-08	IV	Ca	AF10	G13	1,44

[a] *C. jejuni* strains were obtained from chicken and human (patient) sources in the cities of Helsinki and Pori, as indicated, on the specified dates (year-month).
[b] HS, heat stable; NS, nonserotypeable.

monly found in Finnish patients and chicken isolates in 1997 and 1998 (14). The interstrain relatedness within selected PFGE genotype groups was further studied with the use of other molecular typing methods and heat-stable serotyping.

MATERIALS AND METHODS

Bacterial strains. Thirty-five *C. jejuni* strains were selected from a large collection of strains with known epidemiological backgrounds and whose *Sma*I/*Sac*II PFGE genotypes had been determined (14). The strains were collected from human infections that were domestically acquired and from chicken fecal and meat samples in the summers of 1997 and 1998. The origins of the strains are presented in Table 1.

Typing *C. jejuni* isolates by PFGE. For PFGE analysis, the isolates were grown on brucella blood agar (Oxoid, Ltd., Basingstoke, Hampshire, England) for 2 days at 37°C in a microaerobic atmosphere. The bacterial cells were harvested and treated with formaldehyde to inactivate endogeneous nucleases (8). Otherwise, DNA was prepared as described by Maslow et al. (20). The DNA fragments were separated with GeneNavigator (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in 1% agarose gel in 0.5× TBE (45 mmol Tris, 45 mmol boric acid, 1 mmol EDTA) at 200 V. *Sma*I and *Sac*II fragments were separated with ramped pulses of 1 to 30 s for 20 h and of 1 to 20 s for 18 h, respectively. A combined *Sma*I/*Sac*II pattern was designated as a PFGE genotype. If strains had one to five differing fragments in their *Sma*I and *Sac*II patterns, they were designated as subtypes and marked with a letter (for example, genotypes VIa, VIb, VIc, etc.).

AFLP analysis. The AFLP analysis was performed by using a protocol adapted from the AFLP microbial fingerprinting protocol of PE Applied Biosystems (Perkin-Elmer, Norwalk, Conn.). A more detailed description of the used procedure has been published earlier (5). AFLP data were analyzed using GelCompar (Applied Maths, Kortrijk, Belgium), and a similarity matrix was created with the use of the Pearson product-moment correlation coefficient (r). The unweighted pair group method using average linkage was used to cluster the patterns (30).

Ribotyping. Purified chromosomal DNA in agar plugs prepared for PFGE was used for ribotyping. In brief, a 2-mm slide was cut from an agar plug, washed two times with the restriction buffer, and transferred into a tube with restriction buffer. DNA was digested with *Hae*III (6) according to the instructions of the manufacturer (Boehringer Mannheim, Mannheim, Germany). The digests were electrophoresed in 1.2% agarose gels (SeaKem ME Agarose; FMC BioProducts, Rockland, Maine) with TBE (45 mM Tris, 1 mM EDTA [pH adjusted to 8.0 with boric acid]) as running buffer. DNA transfer and probing were performed as described earlier (13).

Serotyping. A commercially available serotyping kit (Campylobacter Antisera Seiken Set; Denka, Seiken, Japan) based on Penner's heat-stable serogroups was used as described earlier (26).

RESULTS

PFGE patterns. A total of 35 strains that belonged to five different PFGE genotype groups were selected on the basis of their *Sma*I and *Sac*II patterns. The distribution of the strains within PFGE types is shown in Table 1.

PFGE genotype I/K included eight strains, isolated from patients and chickens in the summer of 1998 (Table 1), which showed identical PFGE patterns (Fig. 1 and 2, lanes 1 and 4;

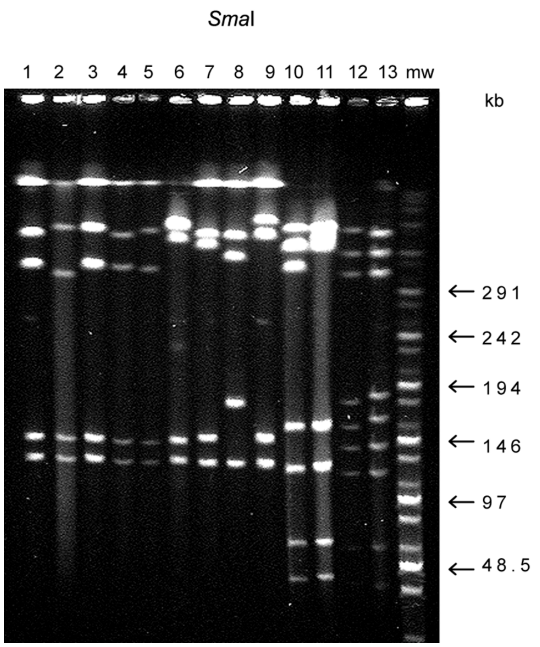

FIG 1. *Sma*I PFGE patterns of *C. jejuni* strains. Lanes 1 to 5, *Sma*I pattern I, strains 4772 (lane 1), 25A (lane 2), 5483 (lane 3), 40A (lane 4), and 28A (lane 5); lanes 6 to 9, pattern VII, strains 5862 (lane 6), FB5241 (lane 7), 4859 (lane 8), and FB5519 (lane 9); lanes 10 and 11, pattern T101, strain FB6271 (lane 10) and 4180 (lane 11); lanes 12 and 13, pattern IV, strains FB287 (lane 12) and strain 35A (lane 13); mw, molecular size marker.

partial digestion seen in Fig. 2, lane 1). In addition, two strains with the highly related PFGE patterns I/Ka and I/Kb differed from pattern I/K by four fragments in only their *Sac*II profiles (Fig. 1 and 2, lanes 3 and 5, respectively). Two more strains were of the related PFGE type I/Kc and had a *Sac*II pattern which differed from the pattern K by five fragments (Fig. 1 and 2, lane 2).

Thirteen strains represented the genotype VI with three closely related groups designated VIa, VIb, and VIc (Table 1). Their *Sma*I and *Sac*II patterns differed from each other by two to five fragments (Fig. 1 and 2, lanes 7, 8, and 9). Strain 5862 was assigned to type VII. It showed a closely related *Sma*I pattern (Fig. 1, lane 6) with the group VI strains, but the *Sac*II pattern differed by more than 10 fragments from the other patterns of this group (Fig. 2, lane 6).

PFGE genotype IV included five strains (Table 1; Fig. 1 and 2, lanes 12 and 13), and PFGE genotype T101 had two subtypes, a and b (Fig. 1 and 2, lanes 10 and 11; Table 1), that differed by one fragment in their *Sma*I profiles (double band on T101b) and by one fragment in their *Sac*II profiles.

AFLP. AFLP analysis subdivided the 35 *C. jejuni* strains into 10 AFLP types (AF1 to AF10). AFLP fingerprints were identified as distinct types when the banding patterns shared less than 90% homology, as has been shown by Duim et al. (5). Cluster analysis of AFLP patterns clearly separated distinct PFGE types and thus produced in most cases congruent results between the PFGE and AFLP analyses. The only exception was strain 35A (PFGE IV), which clustered into the AF4 type (Table 1; Fig. 1 and 2, lane 13, and Fig. 3).

AFLP patterns of six strains with the the PFGE genotype I/K were clustered at a >90% similarity level (AF1), but patterns of two strains of this PFGE group were clustered only with an 82% similarity level with other strains of the I/K group (AF3 and AF5; Fig. 3). Strains 25A and 37A, with PFGE types I/Kc (Fig. 1, lane 2), were clustered in the AFLP analysis into two clusters, AF1 and AF4, respectively (Fig. 3). In the AFLP pattern analysis, all PFGE genotype VI strains were clustered into the same group AF7 with highly similar profiles (Fig. 3). The pattern of strain 5862 (AF6) clustered between AF7 and AF1 to AF5, being only distantly related to the AF7 strains, thus further confirming that this strain does not belong to the same lineage as the other strains in this group. Three T101a genotype strains from humans and chickens had similar AFLP patterns (AF8), and the AFLP pattern of PFGE genotype T101b was related with a similarity level of 82% with the genotype T101a (Fig. 3, AF9).

Ribotyping. *Hae*III ribotypes of the strains are shown in Fig. 4 and Table 1. Ribotypes of eight strains of PFGE/AFLP genotypes I/K/AF1, I/K/AF3, and I/K/AF5 were identical (ribotype A; Fig. 4, lanes 1 and 2), whereas two strains (5483 and 28A) had a slightly different ribotype (ribotype Aa, Fig. 4, lane 4). Also, the PFGE types (I/Ka and IKb) of these two strains were slightly different from the pattern I/K (Fig. 1 and 2, lanes

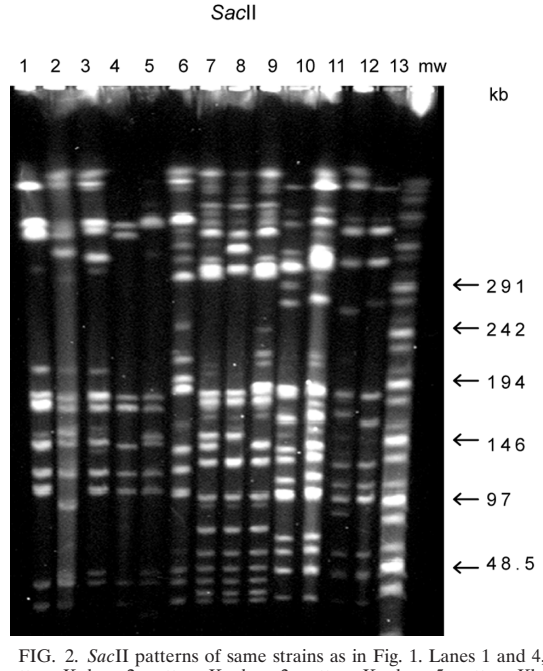

FIG. 2. *Sac*II patterns of same strains as in Fig. 1. Lanes 1 and 4, pattern K; lane 2, pattern Kc; lane 3, pattern Ka; lane 5, pattern Kb; lane 6, pattern VII; lane 7, pattern VIa; lane 8, pattern VIb; lane 9, pattern VIc; lane 10, pattern T101a; lane 11, pattern T101b; lanes 12 and 13, pattern IV. mw, molecular size marker.

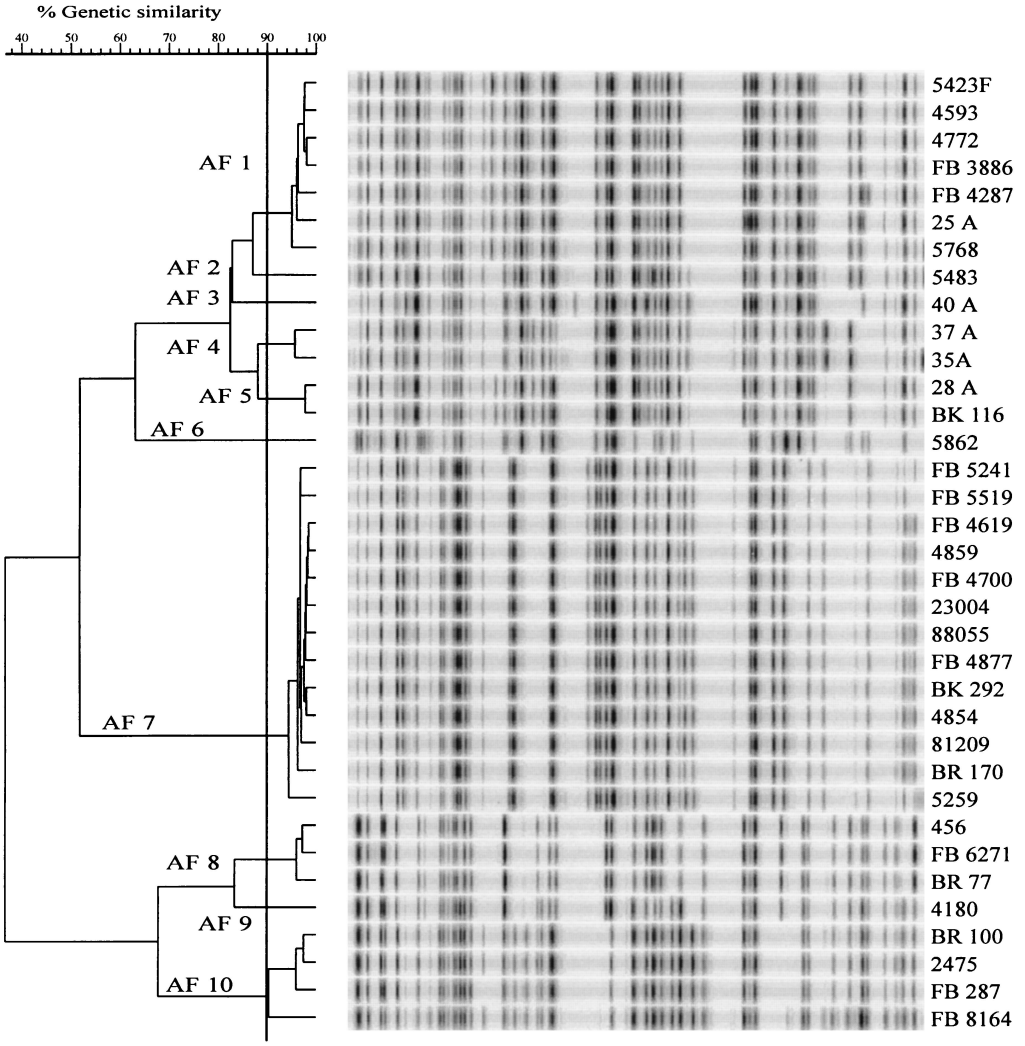

FIG. 3. AFLP patterns of 35 *C. jejuni* strains selected for the study.

1, 3, and 5). Two strains with PFGE genotypes I/Kc and AFLP genotypes AF1 and AF4 were of ribotype B (Fig. 4, lane 3). All strains of PFGE type VI and AFLP type AF7 had the identical ribotype D (Fig. 4, lanes 7, 8, 10, and 11; Table 1). The ribotype of the strain 5862 (PFGE/AFLP genotype VII/AF6) was E (Fig. 4, lane 9; Table 1). All three strains of PFGE/AFLP genotype IV/AF10 had highly similar ribotypes C and Ca (Fig. 4, lanes 5 and 6). Three strains of PFGE/AFLP genotypes T101a/AF8 and T101b/AF9 had highly similar ribotypes F and Fa, respectively (Fig. 4, lanes 12, 13 and 14; Table 1).

Combined genotypes. Data from PFGE, AFLP, and ribotypes were combined and designated as combined genotypes, G1, G2, etc. (Table 1). A total of 13 combined genotypes were identified.

Serotypes. Seven serotypes were identified among the strains studied, and eight strains remained untypeable (Table 1, NS). Heat-stable serotype 1,44 was identified among five different combined genotypes: G1, G5, G10, G11, and G13. Serotype 4 was identified among the combined genotypes G10, G11, and G12. Serotype 12 was associated with the G1 genotype, and two PFGE genotype I/Kc strains were of serotype 57. The strains with related patterns of combined genotype of G7 and G8 had the same serotype 27.

DISCUSSION

The results of comparative analysis of PFGE and AFLP patterns of *C. jejuni* showed that both methods produced con-

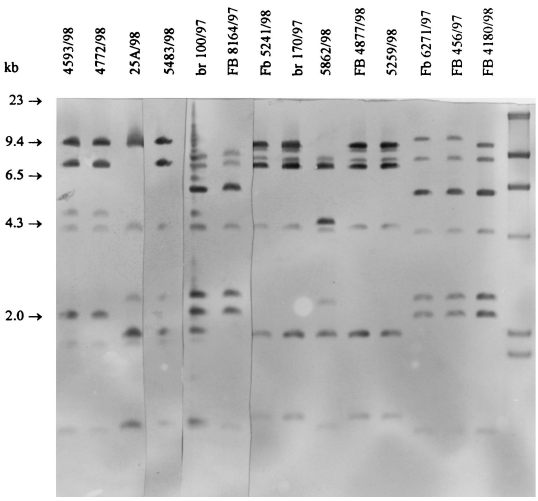

FIG. 4. *Hae*III ribopattern types of *C. jejuni* strains selected for studies. Lane 1, strain 4593, type A; lane 2, strain 4772, type A; lane 3, strain 25A, type B; lane 4, strain 5483, type Aa; lane 5, strain BR100, type C; lane 6, strain FB8164, type Ca; lane 7, strain FB5241, type D; lane 8, strain BR170, type D; lane 9, strain 5862, type E; lane 10, strain FB4877, type D; lane 11, strain 5259, type D; lane 12, FB6271, type F; lane 13, strain FB456, type F; lane 14, strain FB4180, type Fa; lane 15, molecular size marker (2.0, 2.3, 4.3, 6.5, 9.4, and 23 kb).

gruent results in most cases, thus having similar levels of sensitivity. In one group, AFLP subdivided PFGE type I/K strains into three subclusters (AF1, AF3, and AF5). In the group PFGE VI, however, PFGE analysis was more discriminatory than AFLP because PFGE subdivided the strains into three subtypes and AFLP analysis showed a high relatedness of the patterns. An explanation for the high discriminatory power of AFLP is the large number of fragments used in the analysis. Ribotype analysis was shown to have a level of discriminatory power similar to that of the other genetic methods used. Other ribotyping studies have revealed that ribotyping was less discriminatory than PFGE (4, 9) or AFLP (4). In these studies a highly diverse collection of *C. jejuni* strains was used, whereas in the present study the strains represented a restricted set of PFGE genotypes, which may explain the difference in discrimination by ribotyping.

The *C. jejuni* strains were systematically collected after human infections that were domestically acquired in two geographic areas and from chicken samples between 1995 to 1998 in Finland (14). We determined the genotype diversity among these *C. jejuni* strains, which PFGE genotypes were commonly found each year, and how persistent the genotypes were during the study period. On the basis of these data, representatives of five common PFGE genotypes found in 1997 and 1998 were chosen for AFLP analysis, ribotyping, and serotyping. The present extensive genetic analysis revealed that the five chosen genotypes differed from each other by all of the genotyping methods used, and in most cases the majority of strains within one PFGE genotype shared fragments in the AFLP and *Hae*III ribotype patterns. This indicated that PFGE genotype groups I/K, IV, VI, and T101 represent genetic lineages among highly diverse genotypes of *C. jejuni* isolated during a period of 1 year and that these genotypes seemed to persist from 1 year to another. The strain 5862 of PFGE genotype VII was related to PFGE genotype VI but was shown by polyphasic genotype analysis to be only distantly related to genotype VI. Polyphasic genetic analysis of predominant genotypes is recommended because this approach gives information on the relatedness of assigned genotypes and on the homogeneity within a genotype and helps to choose the most applicable genotyping method(s) for future monitoring studies.

Heat-stable serotyping revealed that identical serotypes were distributed among different genotypes and on the opposite several serotypes were identified within one genotype, as has been noted earlier (23, 26). Extensive serotyping data on Finnish strains is not available, but heat-stable serotypes 1, 4, and 6 complexes have been predominant in England (7, 23), Denmark (21), and the United States (25). In the present study serotypes 1,44 and 4 were distributed among most of the selected common Finnish genotypes. Penner serotype 12 consisted only of combined genotype G1, which suggests that this serotype belongs to a stable genotype, similar to that seen for the heat-labile serotypes 4 and 7 (17) and the heat-stable serotype 55 (12). When a more extensive international database for *C. jejuni* genotypes and serotypes becomes available, the comparison of typing data from different countries will be possible and information on common genotypes and serotypes occurring in different countries will be provided.

Population genetic analysis using multilocus enzyme electrophoresis has suggested a heterogenic stucture for *C. jejuni* (2). Certain strains with shared genotypes and phenotypes, however, may become locally predominant and form temporary clonal groupings, probably due to specific characteristics that are advantageous for their colonization in animals or for their environmental transmission and pathogenicity for humans. *C. jejuni* has been shown to be naturally transformable (31). For the flagellin locus recombination by intra- and interstrain transfer of DNA has been described (15). Recent analysis of the whole genome sequence of the *C. jejuni* strain NCTC 11168 has revealed that the strain has 23 hypervariable homopolymeric tracts within the chromosomal DNA. These sequences can be sensitive to slipped-strand mispairing during genome replication of *C. jejuni* (24). Slipped-strand mispairing, as well as recombination or large-scale genomic rearrangements (plasticity), may be useful in the adaptation of the organism for colonization and survival in the gut of a variety of hosts. Slightly changed fragment patterns in the PFGE and AFLP genotypes with otherwise highly related patterns may result from single nucleotide changes in the restriction site or from large-scale genome rearrangements. These mechanisms may contribute to the observed small variation in the number and size of fragments, as was noted in all selected genotypes with otherwise-similar PFGE or AFLP patterns. This minor genomic variability, however, may lead to overestimation of genetic diversity, as recently shown for *Helicobacter pylori* with in silico comparison of PFGE patterns of two *H. pylori* strains with known whole genome sequences. Minor sequence variation was mainly caused by silent nucleotide variation in genes which accounted for the most verified differences in the PFGE patterns of two *H. pylori* strains J199 and 26995 (1). We have shown earlier that at least certain *C. jejuni* strains may change

their genotypes after experimental infections in chickens (12) and Wassenaar et al. (32) noted genomic changes in a set of highly related strains from a batch of meat. The present selection of strains may represent natural variation occurring in a genetic lineage after isolation from various hosts.

In conclusion, our study on selected *C. jejuni* strains isolated during the same time period from humans and chickens indicates that five predominant Finnish genotypes shared PFGE, AFLP, and ribotypes and formed genetic lineages which seemed to persist for 1 year. PFGE and AFLP analyses were shown to have a high level of discriminatory power, although in some cases AFLP was able to further distinguish strains with identical PFGE patterns. In one case AFLP patterns of the strains were highly similar, but PFGE patterns showed differences. Ribotyping allotted the strains into the same genotyping groups as PFGE and AFLP. Identical serotypes were distributed among different genotypes, suggesting that serotyping alone cannot be used for strain identification. In epidemiological studies combined serotyping and genotyping could provide the most relevant data for the identification of strains.

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REFERENCES

1. **Alm, R. A., L.-S. L. Ling, D. Moir, D. T. King, et al.** 1999. Genomic sequence comparison of two unrelated isolates of the gastric pathogen *Helicobacter pylori*. Nature **397:**176–180.
2. **Aschenbacher, M., and J.-C. Piffaretti.** 1989. Population genetics of human and animal enteric *Campylobacter* strains. Infect. Immun. **57:**1432–1437.
3. **Berndtson, E., U. Emanuelsson, M.-L. Danielsson-Tham, and A. Engvall.** 1996. One year epidemiological study of campylobacters in eighteen Swedish chicken farms. Prevent. Vet. Med. **26:**167–185.
4. **de Boer, P., B. Duim, A. Rigter, J. van der Plas, W. F. Jacobs-Reitsma, and J. A. Wagenaar.** 2000. Computer-assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. **38:**1940–1946.
5. **Duim, B., T. Wassenaar, A. Rigter, and J. Wagenaar.** 1999. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl. Environ. Microbiol. **65:**2369–2375.
6. **Fitzgerald, C., R. J. Owen, and J. Stanley.** 1996. Comprehensive ribotyping scheme for heat-stable serotypes of *Campylobacter jejuni*. J. Clin. Microbiol. **34:**265–269.
7. **Frost, J. A., A. N. Oza, R. T. Thwaites, and B. Rowe.** 1998. Serotyping scheme for *Campylobacter jejuni* and *Campylobacter coli* based on direct agglutination of heat-stable antigens. J. Clin. Microbiol. **36:**335–339.
8. **Gibson, J. R., K. Sutherland, and R. J. Owen.** 1994. Inhibition of DNase activity in PFGE analysis of *Campylobacter jejuni*. Lett. Appl. Microbiol. **19:**357–358.
9. **Gibson, J. R., C. Fitzgerald, and R. J. Owen.** 1995. Comparison of PFGE, ribotyping and phage-typing in the epidemiological analysis of *Campylobacter jejuni* serotype H2 infections. Epidemiol. Infect. **11:**215–225.
10. **Gibson, J., E. Lorenz, and R. J. Owen.** 1997. Lineages within *Campylobacter jejuni* defined by numerical analysis of pulsed-field gel electrophoretic DNA profiles. J. Med. Microbiol. **46:**157–163.
11. **Hänninen, M.-L., S. Pajarre, M.-L. Klossner, and H. Rautelin.** 1998. Typing of *Campylobacter jejuni* isolates in Finland by pulsed-field gel electrophoresis. J. Clin. Microbiol. **36:**1787–1789.
12. **Hänninen, M.-L., M. Hakkinen, and H. Rautelin.** 1999. Genomic stability of related macrorestriction patterns of *Campylobacter jejuni* studied by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. **65:**2272–2275.
13. **Hänninen, M.-L., S. Salmi, L. Mattila, R. Taipalinen, and A. Siitonen.** 1995. Association of *Aeromonas* spp. with traveller's diarrhoea in Finland. J. Med. Microbiol. **42:**26–31.
14. **Hänninen, M.-L., P. Perko-Mäkelä, A. Pitkala, and H. Rautelin.** 2000. A three-year study of *Campylobacter jejuni* genotypes in humans with domestically acquired infections and in chicken samples from the Helsinki area. J. Clin. Microbiol. **38:**1998–2000.
15. **Harrington, C. S., F. M. Carter, and P. E. Carter.** 1997. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*. Implications for the flagellin gene typing scheme. J. Clin. Microbiol. **35:**2386–2392.
16. **Harrington, C. S., F. M. Thomson-Carter, and P. E. Carter.** 1999. Molecular epidemiological investigation of an outbreak of *Campylobacter jejuni* identifies a dominant clonal line within Scottish serotype HS55 populations. Epidemiol. Infect. **122:**367–375.
17. **Jackson, C. J., A. F. Fox, D. M. Jones, D. R. A. Wareing, and D. N. Hutchinson.** 1998. Association between heat-stable (O) and heat-labile (HL) serogroup antigens of *Campylobacter jejuni*: evidence for interstrain relationships within three O/HL serovars. J. Clin. Microbiol. **36:**2223–2228.
18. **Jacobs-Reitsma, W. F., A. W. van de Giessen, N. M. Bolder, and R. W. A. W. Mulder.** 1995. Epidemiology of *Campylobacter* spp. at two Dutch broiler farms. Epidemiol. Infect. **114:**413–421.
19. **Kapperud, G., E. Skjerve, N. H. Bean, S. M. Ostroff, and J. Lassen.** 1992. Risk factors for sporadic *Campylobacter* infections: results of a case-control study in southern Norway. J. Clin. Microbiol. **30:**3117–3121.
20. **Maslow, J. N., A. M. Slutsky, and R. D. Arbeit.** 1993. Application of pulsed-field gel electrophoresis to molecular epidemiology, p. 563–572. *In* D. H. Pershing, T. F. Smith, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
21. **Moller Nielsen, E., J. Engberg, and M. Madsen.** 1997. Distribution of serotypes of *Campylobacter jejuni* and *C. coli* from Danish patients, poultry, cattle and swine. FEMS Immunol. Med. Microbiol. **19:**47–56.
22. **Nachamkin, I.** 1997. *Campylobacter jejuni*, p. 159–170. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. American Society for Microbiology, Washington, D.C.
23. **Owen, R. J., E. Slater, D. Telford, T. Donovan, and M. Barnham.** 1997. Subtypes of *Campylobacter jejuni* from sporadic cases of diarrhoeal disease at different locations in England are highly diverse. Eur. J. Epidemiol. **13:**837–840.
24. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, et al.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665–668.
25. **Patton, C. M., I. K. Wachsmuth, G. M. Evins, J. A. Keilbauch, B. D. Plikaytis, N. Troup, L. Tomkins, and H. Lior.** 1991. Evaluation of ten methods to distinguish epidemic-associated *Campylobacter* strains. J. Clin. Microbiol. **29:**680–688.
26. **Rautelin, H., and M.-L. Hänninen.** 1999. Commercial test for serotyping heat-stable antigens of *Campylobacter jejuni* as compared with genotyping with pulsed-field gel electrophoresis. J. Med. Microbiol. **48:**617–621.
27. **Skirrow, M. B.** 1994. Diseases due to *Campylobacter, Helicobacter* and related bacteria. J. Comp. Pathol. **111:**113–149.
28. **Stanley, K. N., J. S. Wallace, J. E. Currie, P. J. Diggle, and K. Jones.** 1998. The seasonal variation of thermophilic campylobacters in beef cattle, dairy cattle and calves. J. Appl. Microbiol. **85:**472–480.
29. **Tauxe, R. V.** 1992. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 9–19. *In* I. Nachamkin, M. J. Blaser, and L. S. Tomkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
30. **Vauterin, L. A., and P. Vauterin.** 1992. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur. Microbiol. **1:**37–41.
31. **Wang, Y., and D. E. Taylor.** 1990. Natural transformation in *Campylobacter* species. J. Bacteriol. **172:**949–945.
32. **Wassenaar, T. M., B. Geilhausen, and D. G. Newell.** 1998. Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. Appl. Environ. Microbiol. **65:**1816–1821.
33. **Yuki, N., and T. Miyatake.** 1998. Guillain-Barre syndrome and Miller-Fisher's syndrome following *Campylobacter jejuni* infection. Ann. N. Y. Acad. Sci. **845:**330–340.

III

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A longitudinal study of *Campylobacter* distribution in a turkey production chain

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Abstract

Background: *Campylobacter* is the most common cause of bacterial enteritis worldwide. Handling and eating of contaminated poultry meat has considered as one of the risk factors for human campylobacteriosis.*Campylobacter* contamination can occur at all stages of a poultry production cycle. The objective of this study was to determine the occurrence of *Campylobacter* during a complete turkey production cycle which lasts for 1,5 years of time. For detection of *Campylobacter*, a conventional culture method was compared with a PCR method. *Campylobacter* isolates from different types of samples have been identified to the species level by a multiplex PCR assay.

Methods: Samples (N = 456) were regularly collected from one turkey parent flock, the hatchery, six different commercial turkey farms and from 11 different stages at the slaughterhouse. For the detection of *Campylobacter*, a conventional culture and a PCR method were used. *Campylobacter* isolates (n = 143) were identified to species level by a multiplex PCR assay.

Results: No *Campylobacter* were detected in either the samples from the turkey parent flock or from hatchery samples using the culture method. PCR detected *Campylobacter* DNA in five faecal samples and one fluff and eggshell sample. Six flocks out of 12 commercial turkey flocks where found negative at the farm level but only two were negative at the slaughterhouse.

Conclusion: During the brooding period *Campylobacter* might have contact with the birds without spreading of the contamination within the flock. Contamination of working surfaces and equipment during slaughter of a *Campylobacter* positive turkey flock can persist and lead to possible contamination of negative flocks even after the end of the day's cleaning and desinfection. Reduction of contamination at farm by a high level of biosecurity control and hygiene may be one of the most efficient ways to reduce the amount of contaminated poultry meat in Finland. Due to the low numbers of *Campylobacter* in the Finnish turkey production chain, enrichment PCR seems to be the optimal detection method here.

Background

Campylobacter is the most common cause of bacterial enteritis worldwide. Commonly recognized risk factors are drinking surface water or water from private wells, swimming in natural waters, and drinking unpasteurised milk [1-5]. However, meat and especially the handling and consumption of undercooked poultry meat are considered as main risk factors for human campylobacteriosis [6-8].

Campylobacter contamination can occur at all stages of a poultry production cycle. In studies concerning vertical transmission, *C. jejuni* has been found on both outer and inner egg shell surfaces [9,10] and in the reproductive tract of laying and broiler breeder hens [11,12]. Hiet et al. [13] have shown the presence of *Campylobacter* DNA in fluff and eggshell samples. In contrast, Petersen et al. [14] and Herman et al. [15] reported no findings of *Campylobacter* from different samples collected in the hatchery e.g. incubator content, swab samples from hatchery machinery and floors and yolk sacs of diseased or dead chicks. Despite these observations, there is no clear evidence that vertical transmission or horizontal hatchery transmission does occur [14,16].

Many studies have provided strong evidence that the farm environment serves as a reservoir for the *Campylobacter* colonising poultry flocks. Dogs and other farm animals, wild birds, flies and untreated water may play a role in transmission of *Campylobacter* [17-21]. The prevalence of *Campylobacter* in broiler flocks varies in the different areas. Nordic countries like Finland, Sweden, Norway and Iceland have reported relatively low prevalences of 2,9%, 27%, 18% and 27,5% respectively [22-24]. In contrast, studies from other countries showed much higher occurrences of *Campylobacter* at the farm level, for example, 87.5% in the USA [25] and 42.7% in France [26]. Limited work has been carried out on investigating the prevalence of *Campylobacter* in the turkey production chain. Cox et al. [27] showed positive findings of 77% in male and 80% in female turkeys at 15 weeks of age. Other studies reported 48% and more than 80% of positive turkey flocks at the time of slaughter [28,29].

In spite of current cleaning and disinfection procedures, transport crates may be contaminated with *Campylobacter*, which may in turn contaminate birds during transport from the farm to the slaughterhouse [30,31]. During the slaughter process, contamination of the poultry carcasses and the equipment with *Campylobacter* occurs during defeathering, evisceration and the chilling processes [25,32]. Air also is found as a potential source of contamination at the slaughterhouse [33]. Contamination of turkey carcasses with *Campylobacter* at slaughter has been reported with levels of between 35% and 91.7% [34-37].

The aim of this study was to determine the occurrence of *Campylobacter* during one total turkey production cycle of 1,5 years time period, starting from imported parents (day-old chicks) to slaughter. For detection of *Campylobacter* at all stages of the production chain, a conventional culture method was compared with a PCR method. *Campylobacter* isolates from different types of samples have been identified to species level by a multiplex PCR assay.

Materials and methods

Study population and turkey production cycle

Between April 2005 and October 2006, one total turkey production cycle was studied. One cycle was defined as follows: Day-old parent chicks are imported from the UK. They are kept in parent rearing farms for 28 weeks. Before they start laying, the turkeys are transported to brooding farms, where they stay for 24 weeks. All the eggs they lay at the brooding farm are hatched in one hatchery. Day-old turkey chicks are transported to commercial farms. Turkey females and males are reared in the same house but separated by various types of walls. Following the slaughter of the females at 13–15 weeks, the males are allowed to use the entire house. Males are slaughtered at an age of 17–18 weeks.

At the parent rearing farm, the flock size was 2,700 and at the brooding farms the flock size was 2,300. Hatchery capacity was 900,000 poults per year. The size of the commercial farms varied from 6,000 to 18,000 birds per cycle. The slaughterhouse slaughtered only turkeys and the capacity was 3,500–5,000 birds/day. Only one flock was slaughtered per day.

Collection and transport of the samples

All samples were collected on each occasion within 2 h, placed in a cool box and transported immediately to the laboratory, where they were processed within 2–4 h. Processing varied depending on the type of samples. Table 1 presents types and numbers of samples taken during this study.

For transporting swab samples from the farms and the slaughterhouse, each swab was put into a tube containing 37 g l^{-1} Brain Heart Infusion Broth (LabM, Lancashire, UK) with 5% calf blood and 0.5% agar (Scharlau-Chemie, Barcelona, Spain) and stored at 4°C. In the laboratory, the swabs were placed into tubes containing 3 ml physiological saline (0.85% NaCl, w/v) and left to stand for five to 10 min to suspend bacteria before further processing.

Table 1: Places of sampling, types and numbers of samples taken during one total turkey production cycle

Place of sampling	Type of samples	Number of samples (n)
Farm		
- Parent rearing farm	Paper liners, swabs from faecal droppings	80
- Parent brooding farm	Swab samples from droppings	70
- Rearing farm	Swab samples from droppings	360
Hatchery	Eggshells and fluff	30
Slaughterhouse	Caecal samples	120
	Environmental samples (swabs, water, faecal material)	336
	Neck skin samples	120
	Meat samples	60
Total number of samples (N)		456

Sterile gauze swabs (10 ×10 cm) were used to collect samples from the surfaces at the slaughter and meat-cutting departments. Before use, they were pre-moistened in Bolton selective enrichment Broth (Oxoid CM0983, Hampshire, UK) without supplement, placed in a sterile jar and stored at 4°C.

At the slaughterhouse, all environmental, neck skin and caecal samples were collected during the slaughtering process. At the same time, swab samples were collected from the transportation crates after disinfection and from the rubber boots of the workers in the evisceration room. Gauze samples were taken from different surfaces of the evisceration and cutting room and from the floor of the chilling room. All meat samples and environmental samples from the meat-cutting department were taken on the day of processing.

Process water samples of one litre were collected during the slaughter of each flock concerned from the defeathering machine and the chilling tank, respectively, into sterile plastic bottles.

Samples

Faecal samples from parent rearing, brooding and commercial farms

At the first time of sampling in the parent rearing farm, ten samples were taken from the chick transportation bed including paper liners and faecal droppings. Thereafter ten swab samples were collected from fresh faecal droppings once every month over a period of seven months. After transfer of the birds to the brooding farm, ten swab samples were taken from fresh faecal droppings once every month, over a period of seven months. One swab was put into one transport tube. For enrichment, five swabs were pooled together to create two subsamples.

One to two weeks prior to the slaughter of females and males, 20 swab samples were taken from fresh faecal droppings at six rearing farms. The farms were randomly coded A to F. Five swabs were pooled together to create four subsamples. For enrichment, these four samples were pooled together.

Hatchery samples

Ten samples containing eggshell and fluff were taken three times over a period of three weeks and collected into separate plastic bags. In the laboratory, 20 g of each sample were measured into 180 ml Bolton selective enrichment broth (Oxoid CM0983, Hampshire, UK) with selective supplement (Oxoid SR0183) and 5% lysed horse blood for enrichment. In addition, 1 g was put into 10 ml physiological saline (0.85% NaCl) and left to stand for 10 min.

Caecal samples at the slaughterhouse

Ten caeca were taken at the evisceration line during the slaughter of each flock in question. Five caeca at a time were placed into one transport container. In the laboratory, each caecum was opened aseptically and swab samples from each caecum were taken. Five swabs were pooled to create two subsamples.

Environmental samples at the slaughterhouse

A total of 336 environmental samples were collected, consisting of swab, water, and faecal samples. The various sampling methods are described below:

A total of 180 gauzes were pre-moistened in Bolton broth (without supplement) and the different surfaces were wiped vigorously for 30 s. Gauzes were placed into a jars with 50 ml Bolton broth, without supplement. In the laboratory, 50 ml Bolton Broth with supplement was added to jars and mixed. The water samples were filtered in the laboratory through 0.45 μm filters (Fennolab, Vantaa, Finland) and four to eight filters were placed into 15–20 ml Bolton Broth (with supplement). Twenty-four litres of water were collected.

Faecal material from the transport crates was collected into a plastic bag. In the laboratory, 5 g of the material were placed into 45 ml Bolton broth (with supplement). A total of 12 faecal material samples were collected. Swab samples were collected as described earlier from transport crates (after disinfection) and from rubber boots in the evisceration room. Five swabs were pooled to create one sample.

Neck skin and meat samples at the slaughterhouse

Ten samples of neck skin were collected during the slaughter of each flock concerned. Furthermore, five meat samples consisting of a variety of cuts were collected separately into plastic bags from the meat-cutting department. In the laboratory, 25 g of neck skin (2 pooled samples of five times 5 g each) or meat (five separate samples 5 g each) were aseptically transferred into a Stomacher® 400 bag (Seward BA6041, Worthing, UK) containing 225 ml Bolton broth (with supplement) and shaken manually for 3 min.

Culture method for detection of Campylobacter

All samples were tested by both direct plating and enrichment culture. Direct plating and isolation after enrichment was done on modified charcoal cefoperazone deoxycholate agar plate (mCCDA) (Oxoid CM739) supplemented with SR 155 (Oxoid). Plates were incubated at 42 ± 1°C for 48 ± 4 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2), generated by CampyGen™ (Oxoid CN0035). For enrichment, Bolton selective enrichment broth (Oxoid CM0983) with selective supplement (Oxoid SR0183) and 5% lysed horse blood was used and incubated at 42 ± 1°C for 22 ± 2 h under microaerobic conditions generated by CampyGen™ (Oxoid). The same enrichment and plating procedure was used for all samples described above.

PCR method for detection of Campylobacter

For PCR, aliquots of 1 ml saline or Bolton broth, respectively, were collected from all samples both directly and after enrichment and centrifuged at 13,000 rpm for 8 min at room temperature. The supernatant was removed carefully and the pellet frozen at -80°C. DNA isolation from the frozen pellet was carried out using a DNA isolation kit, MagneSil® KF Genomic System (Promega MD1460, Madison, WI, USA), with a Dynal MPC®-S magnetic stand (Dynal Biotech, Oslo, Norway) as described in Katzav et al. [38]. The detection of *Campylobacter* spp. in the samples was based on amplification of the 16S rRNA gene [39] using a set of oligonucleotide primers: C412F 5'-GGA TGA CAC TTT TCG GAG C-3' and 16S rRNA-campR2 5'-GGC TTC ATG CTC TCG AGT T-3' as described by Linton et al 1996 and Lund et al. [40], respectively. The internal amplification control (IAC) was prepared by isolating genomic DNA from the bacterium *Yersinia ruckeri* which is the causative agent of enteric redmouth disease in salmonid fish species [41]. This bacterium is not found naturally in chickens. For detection of the internal control, the primers Yers F8 5'-CGA GGA GGA AGG GTT AAG TG-3' and Yers R10 5'-AAG GCA CCA AGG CAT CTC TG-3' slightly modified from Gibello et al. [41] and slightly modified were used. All the primers were synthesised by Oligomer Oy (Helsinki, Finland). The PCR conditions used in the present study are described by Lund et al. [40] with a few modifications. Briefly, the PCR amplification was performed in 50 μl volumes containing 5 μl of the DNA, 25 μl of a PCR master mix (Promega, Madison, WI, USA), 1 μl of a 25 mM $MgCl_2$ solution, 0.5 μl of a 10 mg ml^{-1} BSA solution (New England Biolabs, Ipswich, MA, USA), 20 pmol of each of the *Campylobacter* primers and 5 pmol of each of the internal control primers and 10 pg of genomic Yersinia ruckeri DNA primers. The PCR was performed in a Peltier Thermal Cycler (PTC-200; MJ Research Inc., Watertown, MA, USA) and the conditions were one cycle of 95°C for 2 minutes, 58°C for 1 minutes, 72°C for 1 minute, followed by 34 cycles of 95°C for 15 seconds, 58°C for 40 seconds and 72°C for 40 seconds. The last elongation step lasted 5 minutes. The PCR product was loaded onto a 2% agarose gel (1.35% SeaKem® LE Agarose and 0.65% NuSieve® GTG Agarose, Cambrex Bio Science, Rockland, ME, USA) containing 0.1 g ml^{-1} ethidium bromide. A DNA molecular weight marker 100 bp low ladder (P1473, Sigma-Aldrich, Saint Louis, MO, USA) was included in each gel. The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA). The PCR reaction for each sample was performed twice and considered positive if the primer set gave a distinct band of the right size (857 bp). Samples with no internal control band were run again using a tenfold dilution of DNA.

For sequencing of bands visible on the gel, PCR fragments was purified from the gel using an Qiaquick PCR purification kit (Qiagen GmbH Hilden, Germany) and sent for sequencing at DNA technology (Århus, Denmark) using the same primers for sequencing as used for the PCR. The homology of the sequenced PCR fragments to other *Campylobacter* sequences was determined using BLAST Sequence alignments.

Identification of Campylobacter spp. isolates

Up to three *Campylobacter*-like colonies from each positive sample from rearing farms and slaughterhouse were selected, subcultured on mCCDA agar without supplement and incubated as described above. Identification to genus level was performed according to the method of the National Committee of Food Analyses [42]. To test their ability to grow in air, the colonies were streaked out onto blood plates (CASO agar, Casein-Peptone Soymeal-Peptone, Merck, Darmstadt, Germany with 5% bovine blood) and incubated aerobically at 37°C for up to three days.

For identification to species level, a multiplex PCR assay and two sets of primers based on the method described

by Vandamme et al. [43] were used. The isolates were cultured on mCCDA agar without supplement and mixed with 20 μl of water and kept for 10 min at 100° C. The first primer set was *C. coli* specific, COL1 (5'-AG GCA AGG GAG CCT TTA ATC-3') and COL2 (5'-TAT CCC TAT CTA CAA ATT CGC-3') and the second set *C. jejuni* specific, JUN3 (5'-CA TCT TCC CTA GTC AAG CCT-3') and JUN4 (5'-AAG ATA TGG CTC TAG CAA GAC 3'). All primers were synthesised by Oligomer Oy (Helsinki, Finland). PCR amplification was performed in 25 μl volumes containing 3 μl of template, 12.5 μl of a PCR master mix (Promega, Madison, WI, USA), 1.5 μl of water and 20 pmol of each primer. PCR was performed in a Peltier Thermal Cycler (PTC-200; MJ Research Inc., Watertown, MA, USA) and the conditions were according to Vandamme et al. [43]. A DNA molecular weight marker 100 bp low ladder (P1473, Sigma-Aldrich, Saint Louis, MO, USA) was included in each gel. The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA).

Data management and calculations

For data management and calculations Microsoft® Excel 97 SR 2 was used. The level of agreement according to precision was expressed as the kappa statistic, defined as the proportion of potential agreement beyond chance exhibited by two tests. Diagnostic specificity was calculated as: $d/(b + d)$ where d is the number of samples negative both by PCR and by culture and b is the number of samples positive by PCR, but negative by culture. The level of agreement between two tests was calculated as: $(a + d)/n$, where a is the number of samples positive both by PCR and by culture, d is the number of samples negative by both methods and n is the total number of samples under examination [44,45].

Results

None of the 150 samples from the turkey parent flock, collected during the rearing and brooding period, and of the 30 samples from the hatchery were *Campylobacter* positive either by direct culture or culture following enrichment. However, using the PCR method, five samples from the parent flock in the brooding farm and one sample from the hatchery were *Campylobacter* positive. The PCR products from these samples were sequenced and identified as *C. jejuni*.

Table 2 shows the number of positive faecal samples in the six commercial farms (A-F) studied by culture and PCR method. Three farms (A, C and E) were found to be colonised with *Campylobacter* prior to slaughter. At farms A and E, both females and males were found positive. From farm C, only samples from the females were found *Campylobacter* positive whereas the males were negative at the first sampling. After transport of the females from farm C to the slaughterhouse, the male flock also became colonised with *Campylobacter*. No *Campylobacter* were found in the three other farms (B, D and F) either by direct and enrichment culture or by PCR method.

Table 3 provides details of the percentage of *Campylobacter* in the flocks at slaughter and at meat cutting. At the slaughterhouse, *Campylobacter* was isolated from at least one sample in 10 out of the 12 flocks studied. However, from two female flocks of the farms B and D no *Campylobacter* was detected. The female flock of farm B was *Campylobacter* negative also by PCR method, but

Table 2: *Campylobacter* colonisation in Finnish turkey rearing farms one to two weeks prior to slaughter and comparison of the conventional culture and PCR method for the detection of *Campylobacter*

Sampling month	Farm	Direct culture		Enrichment culture		PCR		PCR after enrichment	
		Female	Male	Female	Male	Female	Male	Female	Male
July	A1[1], A2[2]	4/4[3]	3/4	1/1	1/1	3/4	2/4	ND[4]	ND
August	A2		3/4		1/1		3/4		1/1
August	B1, B2	0/4	0/4	0/1	0/1	0/4	0/4	ND	ND
August	B2		0/4		0/1		0/4		ND
August	C1, C2	4/4	0/4	1/1	0/1	4/4	0/4	ND	ND
September	C2		3/4		1/1		4/4		1/1
August	D1, D2	0/4	0/4	0/1	0/1	0/4	0/4	ND	ND
September	D2		0/4		0/1		0/4		0/1
August	E1, E2	1/4	1/4	1/1	1/1	1/4	2/4	ND	ND
September	E2		1/4		1/1		0/4		1/1
September	F1, F2	0/4	0/4	0/1	0/1	0/4	0/4	0/1	0/1
October	F2		0/4		0/1		0/4		0/1

[1]Number one after the capital indicates female turkeys.
[2]Number two after the capital indicates male turkeys.
[3]Number of positive/number examined.
[4]ND. Not determined.

Table 3: Prevalence of *Campylobacter* in turkey flocks during slaughter and meat cutting detected by culture and/or PCR method

Farm	Processing plant No. of positive/no. examined (%)				Meat samples No. of positive/no. examined (%)			
	Female		Male		Female		Male	
	Culture[1]	PCR[1]	Culture[1]	PCR[1]	Culture[1]	PCR[1]	Culture[1]	PCR[1]
A	9/11 (82)	7/11[2] (64)	10/12 (83)	11/12 (92)	2/5 (40)	0/5 (0)	1/5 (20)	1/5 (20)
B	0/12 (0)	0/12[2] (0)	6/12 (50)	1/12[2] (8)	0/5 (0)	0/5 (0)	0/5 (0)	1/5 (20)
C	12/12 (100)	12/12 (100)	10/12 (83)	10/12 (83)	4/5 (80)	2/5 (40)	3/5 (60)	2/5 (40)
D	0/12 (0)	3/12 (25)	9/12 (75)	10/12 (83)	0/5 (0)	0/5 (0)	5/5 (100)	5/5 (100)
E	5/12 (42)	6/12 (50)	10/12 (83)	10/12 (83)	0/5 (0)	2/5 (40)	2/5 (40)	3/5 (60)
F	2/12 (17)	3/12 (25)	1/12 (8)	4/12 (33)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

[1]No. of samples tested positive by direct and/or enrichment method.
[2]PCR not performed after enrichment.

PCR was not performed after enrichment from the samples of this flock. Generally, the percentage of *Campylobacter* of the samples taken during the slaughter process was higher than of those taken during the cutting process. In contrast, the meat samples of the males from farm D were all positive for *Campylobacter*, while only 75% to 83% the slaughter samples were positive.

Table 4 shows the number of *Campylobacter* positive samples taken at the processing plan. When using enrichment culture for *Campylobacter* determination, the highest percentage of positive samples was found in the environmental samples from the evisceration room (75%). Also faecal material collected from the transport crates (67%), the chilling water (67%) and the neck skins (62.5%) had high isolation rates after enrichment. Following enrichment, higher percentages of positive samples were observed among neck skin samples (62.5%) than among the caecal samples (33%). Environmental samples from the chilling- and cutting room were all negative by direct culture and direct PCR. However, following enrichment, 50% and 42% of the same samples from the chilling room, and 56% and 56% from the cutting room were found positive for *Campylobacter* by culture and PCR, respectively. Also water samples from the defeathering machine, neck skin samples, swab samples from the rubber boots of the workers in the evisceration room and meat cutting samples showed a higher percentage of *Campylobacter* using PCR after enrichment (Table 4).

A total of 143 *Campylobacter* isolates from samples taken from the commercial farms and the slaughterhouse were identified as *Campylobacter* spp. by PCR. When species

Table 4: Occurrence of *Campylobacter* in samples at different stages and the environment of the slaughter and meat cutting departments detected by culture and PCR method

	Direct Culture	Enrichment culture	PCR	PCR after enrichment
	No. of positive/ no. examined (%)	No. of positive/ no. examined (%)	No. of positive/ no. examined (%)	No. of positive/ no. examined (%)
Transportation crates	1/11* (9)	1/11* (9)	1/11* (9)	1/9* (11)
Faecal material from transportation crates	7/12 (58)	8/12 (67)	7/12 (58)	7/9 (78)
Water from defeathering machine	0/12 (0)	5/12 (42)	3/12 (25)	5/9 (56)
Caecal material	9/24 (37.5)	8/24 (33)	8/24 (33)	8/18 (44)
Neck skin	2/24 (8)	15/24 (62.5)	6/24 (25)	12/18 (67)
Environment (evisceration room)	6/12 (50)	9/12 (75)	7/12 (58)	9/9 (100)
Rubber Boots (evisceration room)	3/12 (25)	6/12 (50)	3/12 (25)	5/9 (56)
Chilling water	3/12 (25)	8/12 (67)	3/12 (25)	7/9 (78)
Environment (chilling room)	0/12 (0)	6/12 (50)	0/12 (0)	5/9 (56)
Environment (meat cutting room)	0/12 (0)	5/12 (42)	0/12 (0)	5/9 (56)
Meat samples	0/60 (0)	17/60 (28)	4/60 (7)	13/45 (29)

*Eleven samples after washing and disinfection.

identification was performed using the multiplex PCR method, 105 isolates were identified as *C. jejuni* and none as *C. coli*. Thirty-eight isolates were not identified as either *C. jejuni* or *C. coli* by the multiplex PCR method. Thirty-four of these isolates originated from different slaughterhouse samples from both female and male flocks from farm C.

The diagnostic specificity for the comparison of direct PCR to direct culture was 0.88 with a level of agreement of 0.88 and for the comparison of both methods by selective enrichment was 0.88 with a level of agreement of 0.92.

Discussion

Campylobacter contamination may occur at all stages of a turkey production cycle. In the present study, *Campylobacter* DNA was detected by PCR from five faecal samples collected during the brooding period. It is likely that the brooding flock had been in contact with *Campylobacter* but the infection had not spread within the flock. Self-limitation of colonisation and detection of antibodies against *C. jejuni* without colonisation of the bacterium has previously been described [17].

Detection of *Campylobacter* DNA by PCR in one fluff and eggshell sample supports the findings of Hiett et al. [13]. The bacterium was not isolated either from the present brooding flocks or from the hatchery and it is not possible to determine whether it is alive or dead. Thus, no further conclusions can be made on vertical transmission based on the present study.

The risk for *Campylobacter* contamination is high when strict biosecurity barriers are loosened and a poultry flock may come in contact with the environment via people and equipment on the farm. The possibility of compromising biosecurity during partial depopulation or "thinning" has yielded conflicting data. Several authors have demonstrated that the catching team can introduce the bacterium into the house, and therefore, partial depopulation has been considered a risk factor for *Campylobacter* colonisation [46-48]. In contrast, it has also been demonstrated that it does not necessarily influence *Campylobacter* colonisation in the flock [49]. At Finnish turkey farms, the flocks are usually divided and females and males are reared in separate groups but in the same house. Females are slaughtered two to four weeks before the males. After the turkey females have been slaughtered, the males can use the area where the females have been. This area could be seen as a risk for contamination since the personnel catching the turkeys can break the hygiene barriers during collection of the female birds. In this study, three flocks were *Campylobacter* negative before slaughter of the females and remained negative when testing the males two to three weeks later. Hansson et al. [50] found no differences in the presence of *Campylobacter* in the environment between producers who frequently or rarely deliver *Campylobacter* positive slaughter batches. Thus, our results could be explained by good hygiene control of the catching equipment and personnel in the negative farms.

The slaughter process was found to be a risk factor for the *Campylobacter* contamination of turkey products. The number of *Campylobacter* positive samples within a flock at slaughter varied between 0 and 94% in this study. High variation in the turkey flocks at the slaughterhouse has also been demonstrated previously [35,37]. Since enrichment was needed to recover the bacteria, it seems that some processing steps like the scalding and defeathering process had an adverse effect on the bacteria. This study found more positive neck skin samples than caecal samples (Table 3). Neck skins are mentioned as good indicators of *Campylobacter* contamination at the slaughterhouse [32]. Hansson et al. [31] found more *Campylobacter* from neck skin samples than from cloacal samples and concluded that if cloacal samples were negative, the neck skin samples might have been contaminated from the slaughterhouse environment. This may also explain the results of the present study.

Evisceration is a critical stage where bacteria can be spread in poultry processing. This fact is confirmed by this study, showing samples from the evisceration room and rubber boots to be 50 to 100% *Campylobacter* positive. It has been shown that contamination at the slaughterhouse cannot be avoided when a *Campylobacter* positive poultry flock is processed [15]. Allen et al. [51] isolated *Campylobacter* at a slaughterhouse from aerosols, particles and droplets in the hanging, plucking and evisceration areas also during the processing of a *Campylobacter* negative flock. In this study, all slaughtered birds originated from the same flock and only one flock per day was slaughtered. Thus, cross-contamination from another, potentially positive, flock slaughtered earlier the same day was not possible. However, in this study there is also evidence that contamination at a slaughterhouse can withstand cleaning and disinfection. Flocks B2, D2, F1 and F2 were *Campylobacter* negative at the farm level, caecum culture-negative at slaughter, but tested positive during the slaughter process. Peyrat *et al*. [52] also recovered *C. jejuni* from the equipment surfaces after cleaning and disinfection in three out of four slaughterhouses visited. It is possible that *Campylobacter*, as well as other bacteria, persist on surfaces in poultry-processing facilities forming a biofilm [53-55]. Thus, the

release of the bacterium from such biofilms may also contaminate products which touch the surface of the processing equipment.

In the slaughterhouse studied here, the turkey carcasses were chilled by placing them first in a water tank for five minutes before hanging them for 24 hours in a room at 2°C. More positive samples from the chilling water than from the chilling room environment were observed, suggesting the chilling water as being a source of carcass contamination. Extended air-chilling might lead to drying of the carcass surface and the environment of the chilling room resulting in a reduction of *Campylobacter* [51,56,57]. In this study, the occurrence of *Campylobacter* in the samples taken during the meat cutting process was lower than of those taken during the slaughter process. In the present slaughterhouse, the meat was cut the day after slaughter. It is known that certain subpopulations of *Campylobacter* are able to survive environmental stress like the scalding- and chilling process and remain in the final meat products [58]. However, the low rate of *Campylobacter* in the final meat products found in the present study (28%) is reflected by the low findings in poultry products at the Finnish retail level with reported numbers of 12% and 21%, respectively [38,59].

Of the 143 *Campylobacter* spp. isolates, 105 (73%) were identified as *C. jejuni*, none as *C. coli*, so 38 (26%) remained unidentified to the species level. It is known that the majority of the *Campylobacter* found in raw poultry are *C. jejuni* [37,57,60]. Takahashi et al. [61] found both *C. jejuni* and *C. coli* in farm samples, *C. jejuni* at all stages of the processing line. However, they did not find *C. coli* anymore after defeathering and speculated lower numbers of *C. coli* in poultry faeces to be the reason. Certain *C. jejuni* strains might be more stress-resistant and overgrow possible *C. coli* strains in the same samples [58].

As the high level of agreement between the different detection methods shows, there were no significant differences between the conventional culture and the PCR method in the samples analysed in this study. However, the need for enrichment in this study for the detection of *Campylobacter* at certain processing steps, also when performing PCR, might indicate low numbers of *Campylobacter* at the farm and slaughterhouse level. Thus, a combination of enrichment and PCR assay seems to be the optimal method for detection of *Campylobacter* in this situation.

Conclusion

The presence of *Campylobacter* DNA from the brooding flock and hatchery sample shows that they have been in contact with *Campylobacter*, but for unknown reasons the contamination has not been spread. The present study also shows that during the processing of a *Campylobacter* positive turkey flock, working surfaces and equipment at the slaughterhouse can become contaminated, leading to possible contamination of negative flocks, even if slaughtered on following days. Persistence of *Campylobacter* on surfaces emphasises the need for efficient cleaning and disinfection of the processing facilities. However, the need for enrichment in this study for detection of *Campylobacter* at certain processing steps, also when performing PCR, might indicate low numbers of *Campylobacter* at the farm and the slaughterhouse level. Since complete elimination of thermophilic *Campylobacter* from the turkey production chain does not seem feasible, reduction of contamination at the farm level by a high level of biosecurity control and hygiene may be one of the most efficient ways to reduce the amount of contaminated poultry meat in Finland.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PPM, UL, MK, PI and M-LH participated in the discussion on the study design. PPM, UL, MK and PI participated in the collection of samples, analysis and interpretation of the data. PPM, UL, MK and PI carried out the microbiological analyses of the samples. Analysis and interpretation of the PCR were carried out by PPM. Analysis and interpretation of the sequencing were carried out by ML. PPM and UL wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Jacobs-Reitsma WF: ***Campylobacter* in the food supply.** *Campylobacter* Washington DC: ASM: Nachamkin I, Blaser MJ 22000, 467–481.
2. Pattison M: **Practical intervention strategies for *Campylobacter*.** *Symp Ser Soc Appl Microbiol* 2001, **30:**121–125.
3. Park SF: **The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens.** *Int J Food Microbiol* 2002, **74:**177–188.
4. Hänninen M-L, Haajanen H, Pummi T, Wermundsen K, Katila M-L, Sarkkinen H, Miettinen I and Rautelin H: **Detection and typing of *Campylobacter jejuni* and *Campylobacter* coli and analysis of indicator organisms in three waterborne outbreaks in Finland.** *Appl Environ Microbiol* 2003, **69:**1391–1396.

5. Schönberg-Norio D, Takkinen J, Hänninen M-L, Katila M-L, Kaukoranta S-S, Mattila L and Rautelin H: **Swimming and *Campylobacter* infections.** *Emerg Infect Dis* 2004, **10:**1474–1477.
6. Evans MR, Ribeiro CD and Salmon RL: **Hazards of healthy living: Bottled water and salad vegetables as risk factors for *Campylobacter* infection.** *Emerg Infect Dis* 2003, **9:**1219–1225.
7. Potter RC, Kaneene JB and Hall WN: **Risk factors for sporadic *Campylobacter jejuni* infections in Rural Michigan: a prospective case-control study.** *Am J Publ Health* 2003, **93:**2118–2123.
8. Luber P and Bartelt E: **Enumeration of *Campylobacter* spp. on the surface and within chicken breast fillets.** *J Appl Microbiol* 2007, **102:**313–318.
9. Doyle MP: **Association of *Campylobacter jejuni* with laying hens and eggs.** *Appl Environ Microbiol* 1984, **47:**533–536.
10. Shanker S, Lee A and Sorrell TC: ***Campylobacter jejuni* in broilers: the role of vertical transmission.** *J Hyg* 1986, **96:**153–159.
11. Jacobs-Reitsma WF: **Aspects of epidemiology of *Campylobacter* in poultry.** *Vet Q* 1997, **19(3):**113–117.
12. Buhr RJ, Cox NA, Stern NJ, Musgrove MT, Wilson JL and Hiett KL: **Recovery of *Campylobacter* from segments of the reproductive tract of broiler breeder hens.** *Avian Dis* 2002, **46:**919–924.
13. Hiet KL, Cox NA and Stern NJ: **Direct polymerase chain reaction detection of *Campylobacter* spp. in poultry hatchery samples.** *Avian Dis* 2002, **46:**219–223.
14. Petersen L, Nielsen EM and On SLW: **Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks.** *Vet Microbiol* 2001, **82:**141–154.
15. Herman L, Heyndrick M, Grijspeerdt K, Vandekerchove D, Rollier I and De Zutter L: **Routes for *Campylobacter* contamination of poultry meat: epidemiological study from hatchery to slaughterhouse.** *Epidemiol Infect* 2003, **131:**1169–1180.
16. Callicot KA, Friðriksdóttir V, Reiersen J, Lowman R, Bisaillon J-R, Gunnarsson E, Berndtson E, Hiett KL, Needleman DS and Stern NJ: **Lack of evidence for vertical transmission of *Campylobacter* spp. in chickens.** *Appl Environ Microbiol* 2006, **9:**5794–5798.
17. Newell DG and Fearnley C: **Sources of *Campylobacter* colonization in broiler chickens.** *Appl Environ Microbiol* 2003, **69:**4343–4351.
18. Hald B, Skovgård H, Bang DD, Petersen K, Dybdahl J, Jespersen JB and Madsen M: **Flies and *Campylobacter* infection of broiler flocks.** *Emerg Infect Dis* 2004, **10(8):**1490–1492.
19. Johnsen G, Kruse H and Hofshagen M: **Genetic diversity and description of transmission routes for *Campylobacter* on broiler farms by amplified-fragment length polymorphism.** *J Appl Microbiol* 2006, **10:**1130–1139.
20. Bull SA, Allen VM, Dominique G, Jørgensen F, Frost JA, Ure R, Whyte R, Tinker D, Corry JEL, Gillard-King J and Humphrey TJ: **Sources of *Campylobacter* spp. colonizing housed broiler flocks during rearing.** *Appl Environ Microbiol* 2006, **1:**645–652.
21. Hald B, Sommer HM and Skovgård H: **Use of fly screens to reduce *Campylobacter* spp. introduction in broiler houses.** *Emerg Infect Dis* 2007, **13:**1951–1953.
22. Berndtson E, Emanuelson U, Engvall A and Danielsson-Tham ML: **A 1-year epidemiological study of *Campylobacters* in 18 Swedish chicken farms.** *Prev Vet Med* 1996, **26:**167–185.
23. Perko-Mäkelä P, Hakkinen M, Honkanen-Buzalski T and Hänninen M-L: **Prevalence of *Campylobacter* in chicken flocks during the summer of 1999 in Finland.** *Epidemiol Infect* 2000, **129:**187–192.
24. Guerin MT, Wayne M, Reiersen J, Berke O, McEvan SA, Bisaillon J-R and Lowman R: **A farm-level study of risk factors associated with the colonization of broiler flocks with *Campylobacter* spp. in Iceland, 2001–2004.** *Acta Vet Scand* 2007, **49:**18.
25. Stern NJ, Fedorka-Cray P, Bailey JS, Cox NA, Craven SE, Hiett KL, Musgrove MT, Ladely S, Cosby D and Mead GC: **Distribution of *Campylobacter* spp. in selected U.S. poultry production and processing operations.** *J Food Prot* 2001, **64:**1705–1710.
26. Refrégier-Petton J, Rose N, Denis M and Salvat G: **Risk factors for *Campylobacter* spp. contamination in French broiler-chicken flocks at the end of the rearing period.** *Prev Vet Med* 2001, **50:**89–100.
27. Cox NA, Stern NJ, Craven SE, Berrang ME and Musgrove MT: **Prevalence of *Campylobacter* and *Salmonella* in the cecal droppings of turkeys during production.** *J Appl Poultry Res* 2000, **9:**542–545.
28. Wallace JS, Stanley KN and Jones K: **The colonization of turkeys by thermophilic campylobacters.** *J Appl Microbiol* 1998, **85:**224–230.
29. Borck B: ***Campylobacter* spp. in the Danish turkey production.** *PhD Thesis* Danish Veterinary Institute and Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University; 2003.
30. Slader J, Dominique G, Jorgensen F, McAlpine K, Owen RJ, Bolton FJ and Humprey TJ: **Impact of transport crate reuse and of catching and processing on *Campylobacter* and *Salmonella* contamination of broiler chickens.** *Appl Environ Microbiol* 2002, **68:**713–719.
31. Hansson I, Ederoth M, Andersson L, Vågsholm I and Olsson Engvall E: **Transmission of *Campylobacter* spp. to chickens during transport to slaughter.** *J Appl Microbiol* 2005, **99:**1149–1157.
32. Berndtson E, Danielsson-Tham ML and Engvall A: ***Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process.** *Int J Food Microbiol* 1986, **32:**35–47.
33. Posch J, Feierl G, Wuest G, Sixl W, Schmidt S, Haas DU, Reinthaler FF and Marth E: **Transmission of *Campylobacter* spp. in a poultry slaughterhouse and genetic characterisation of the isolates by pulsed-field gel electrophoresis.** *Brit Poult Sci* 2006, **3:**286–293.
34. Logue CM, Sherwood JS, Elijah LM, Olah PA and Docker MR: **The incidence of *Campylobacter* spp. on processed turkey from processing plants in the Midwestern United States.** *J Appl Microbiol* 2003, **95:**234–241.
35. Borck B and Pedersen K: **Pulsed-field gel electroforesis types of *Campylobacter* spp. in Danish turkeys before and after slaughter.** *Int J Food Microbiol* 2005, **101:**63–72.
36. Arsenault J, Letellier A, Quessy S, Morin J-P and Boulianne M: **Prevalence and risk factors for *Salmonella* and *Campylobacter* spp. carcass contamination in turkeys slaughtered in Quebec, Canada.** *J Food Prot* 2007, **6:**1350–1359.
37. Atanassova V, Reich F, Beckmann L and Klein G: **Prevalence of *Campylobacter* spp. in turkey meat from a slaughterhouse and in turkey meat retail products.** *FEMS Immunol Med Microbiol* 2007, **49:**141–145.
38. Katzav M, Isohanni P, Lund M, Hakkinen M and Lyhs U: **PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products.** *Food Microbiol* 2008, **25:**908–914.
39. Linton D, Owen RJ and Stanley J: **Rapid identification by PCR of the genus *Campylobacter* and five *Campylobacter* species enteropathogenic for man and animals.** *Res Microbiol* 1996, **147:**707–718.
40. Lund M, Nordentoft S, Pedersen K and Madsen M: **Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR.** *J Clin Microbiol* 2004, **42:**5125–5132.
41. Gibello MM, Blanco MA, Moreno MT, Cutuli A, Domenech L, Domínguez C and Fernández-Garayzábal JF: **Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout.** *Appl Environ Microbiol* 1999, **65:**346–350.
42. National Committee of Food Analyses: **Thermotolerant *Campylobacter.*** *Detection, semi-quantitative and quantitative determination in foods and drinking water. NCFA method. no. 119* 32007.
43. Vandamme P, Van Doorn L-J, Al Rashid ST, Quint WGV, Plas Van Der J, Chan VL and On SLW: ***Campylobacter hyoilei* Alderton et *al.* 1995 and *Campylobacter coli* Véron and Chatelain 1973 are subjective synonyms.** *Int J Syst Bacteriol* 1997, **47:**1055–1060.
44. Smith RD: *Veterinary clinical epidemiology* Florida: CRC Press Inc; 1995.
45. Martin SW, Meek AH and Willberg P: *Veterinary epidemiology, principles and methods* Iowa: Iowa State University Press; 1997.
46. Hald B, Rattenborg E and Madsen M: **Role of batch depletion of broiler houses on the occurrence of *Campylobacter* spp. in chicken flocks.** *Lett Appl Microbiol* 2001, **4:**253–256.
47. Jacobs-Reitsma W, Wilpshaar E, Gussinklo B, Wagenaar J and Stegeman A: **Epidemiological investigations into the colonisation of Dutch broiler flocks with *Campylobacter*.** *Int J Med Microbiol* 2001, **291:**42–43.
48. Anonymous: **Interactions to control *Campylobacter* in the broiler production.** *Report of an international expert consultation. Copenhagen, Denmark, 26–27 November 2007Rosenquist H, Boysen L, Borck B I* http://www.food.dtu.dk.
49. Russa AD, Bouma A, Vernooij JCM, Jacobs-Reitsma W and Stegeman JA: **No association between partial depopulation and *Campylobacter* spp. colonization of Dutch broiler flocks.** *Lett Appl Microbiol* 2005, **41:**280–285.
50. Hansson I, Vågsholm I, Svensson L and Olsson Engvall E: **Correlations between *Campylobacter* spp. prevalence in the environment and broiler flocks.** *J Appl Microbiol* 2007, **103:**640–649.

51. Allen VM, Bull SA, Corry JEL, Dominigue GJ, Jørgensen F, Frost JA, Whyte R, Conzales A, Elviss N and Humphrey TJ: ***Campylobacter* spp. contamination of chicken carcasses during processing in relation to flock colonisation.** *Int J Food Microbiol* 2007, **113:**54–61.
52. Peyrat MB, Soumet C, Maris P and Sanders P: **Phenotypes and genotypes of *Campylobacter* strains isolated after cleaning and disinfection in poultry slaughterhouses.** *Vet Microbiol* 2008, **128:**313–326.
53. Jeffrey JS, Tonooka KH and Lozanot J: **Prevalence of *Campylobacter* spp. from skin, crop, and intestine of commercial broiler chicken carcasses at processing.** *Poult Sci* 2001, **80:**1390–1392.
54. Cools I, Uyttendaele M, Cerpentier J, D'Haese E, Nelis HJ and Debevere J: **Persistence of *Campylobacter jejuni* on surfaces in a processing environment and on cutting boards.** *Lett Appl Microbiol* 2005, **40:**418–423.
55. Sanders SQ, Boothieet DH, Frank JF and Arnold JW: **Culture and detection of *Campylobacter jejuni* within mixed microbial populations of biofilms on stainless steel.** *J Food Prot* 2007, **70 (6):**1379–1385.
56. Sanchez MX, Fluckey WM, Brashears MM and McKee SR: **Microbial profile and antibiotic susceptibility of Campylobacter spp. and Salmonella spp. in broilers processed in air-chilled and immersion-chilled environments..** *J Food Prot* 2001, **65:**948–956.
57. Klein G, Reich F, Beckman L and Atanassova V: **Quantification of thermophilic *Campylobacter* spp. in broilers during meat processing.** *Antonie van Leeuwenhoek* 2007, **92:**267–273.
58. Alter T, Gaull F, Froeb A and Fehlhaber K: **Distribution of *Campylobacter jejuni* strains at different stages of a turkey slaughter line.** *Food Microbiol* 2005, **22:**345–351.
59. Hänninen M-L, Perko-Mäkelä P, Pitkälä A and Rautelin H: **A three-year study of *Campylobacter jejuni* genotypes in humans with domestically acquired infections and in chicken samples from the Helsinki area.** *J Clin Microbiol* 2000, **38:**1998–2000.
60. Hamedy A, Alter T, Schlichting D, Ludewig M and Fehlhaber K: **Quantitative detection of *Campylobacter* spp. on turkey carcasses and turkey meat.** *Fleischwirtsch* 2000, **87:**121–124.
61. Takahashi R, Shahada F, Chuma T and Okamoto K: **Analysis of *Campylobacter* spp. contamination in broilers from the farm to the final meat cuts by using restriction fragment length polymorphism of the polymerase chain reaction products.** *Int J Food Microbiol* 2006, **110:**240–245.

ORIGINAL ARTICLE

Distribution of *Campylobacter jejuni* isolates from Turkey Farms and Different Stages at Slaughter Using Pulsed-Field Gel Electrophoresis and *flaA*-Short Variable Region Sequencing

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Impacts

- During processing, *Campylobacter*-negative poultry flocks may become contaminated by the same subtypes/clones of *Campylobacter jejuni* introduced into the slaughterhouse on preceeding days by positive flocks.
- Proper and efficient cleaning and disinfection of the slaughter (or processing) environment is necessary to reduce the risk of cross-contamination, especially in countries with a low prevalence of *Campylobacter* spp.
- To assess the relatedness and distribution of *Campylobacter* strains, a combination of two different genotyping methods (PFGE analysis and *flaA*-short variable region sequencing) was used.

Keywords:
Campylobacter; turkey; slaughterhouse; PFGE; *flaA*-SVR typing; cross-contamination

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Summary

The aim of this study was to assess the diversity of thermotolerant *Campylobacter* spp. isolated from turkey flocks at six rearing farms 1–2 weeks prior to slaughter (360 faecal swab samples) and from 11 different stages at the slaughterhouse (636 caecal, environmental, neck skin and meat samples). A total of 121 *Campylobacter* isolates were identified to species level using a multiplex PCR assay and were typed by pulsed-field gel electrophoresis (PFGE) and *flaA*-short variable region (SVR) sequencing. All *Campylobacter* isolates were identified as *Campylobacter jejuni*. PFGE analysis with *Kpn*I restriction enzyme resulted in 11 PFGE types (I–XI) and *flaA* SVR typing yielded in nine *flaA*-SVR alleles. The *Campylobacter*-positive turkey flocks A, C and E were colonized by a limited number of *Campylobacter* clones at the farm and slaughter. The present study confirms the traceability of flock-specific strains (PFGE types I, V and IX; *flaA* types 21, 36 and 161) from the farm along the entire processing line to meat cuts. It seems that stress factors such as high temperature of the defeathering water (54–56°C), drying of the carcass skin during air chilling (24 h at 2°C), and oxygen in the air could not eliminate *Campylobacter* completely. *Campylobacter*-negative flocks became contaminated during processing by the same subtypes of *Campylobacter* introduced into the slaughter house by preceeding positive flocks even if they were slaughtered on subsequent days. Proper and efficient cleaning and disinfection of slaughter and processing premises are needed to avoid cross-contamination, especially in countries with a low prevalence of *Campylobacter* spp. The majority of *flaA* SVR alleles displayed a distinct association with a specific PFGE type. However, a linear relationship for all strains among both typing methods could not be established. To specify genetic relatedness of strains, a combination of different genotyping methods, is needed.

Introduction

Campylobacter is the most common cause of bacterial enteritis worldwide. Commonly recognized risk factors are drinking surface water or water from private wells, swimming in natural waters, drinking of unpasteurized milk and especially the handling and consumption of undercooked poultry meat (Pattison, 2001; Park, 2002; Evans et al., 2003; Hänninen et al., 2003; Schönberg-Norio et al., 2004; Luber and Bartelt, 2007; Jacobs-Reitsma et al., 2008).

The slaughter process is known to be a risk factor for *Campylobacter* contamination of poultry products (Rasschaert et al., 2006; Perko-Mäkelä et al., 2009). To prevent cross-contamination at slaughter and to produce *Campylobacter*-free fresh poultry meat, separate processing of positive and negative poultry flocks, for example, logistic or scheduled slaughter, is applied (Nauta et al., 2005; Katsma et al., 2007). To test the *Campylobacter* status of a flock on arrival at the slaughterhouse, Polymerase Chain Reaction (PCR) is routinely used in Denmark (Lund et al., 2003). The reliability of negative results depends strongly on the length of the time between testing and slaughter (Katsma et al., 2007).

Different molecular typing methods are available for the differentiation of thermotolerant *Campylobacter* spp. for better understanding of their epidemiology and source tracking. Pulsed-field gel electrophoresis (PFGE) is a reproducible and highly discriminatory technique, and has been applied extensively for confirmation of the epidemiological link between *Campylobacter* isolates from positive poultry flocks at farm level and different stages of slaughter up to the end-products (Borck and Pedersen, 2005; Höök et al., 2005; Lienau et al., 2007). *FlaA*-SVR (short variable region, SVR) sequencing is another commonly used, cost-effective, and well-accepted sequence-based typing method for *Campylobacter* with low levels of non-typeability (Newell et al., 2001; Dingle et al., 2005; Meinersmann et al., 2005; Rasschaert et al., 2006; Pittenger et al., 2009).

The aim of this study was to follow the survival and diversity of thermotolerant *Campylobacter* spp. isolated from six turkey rearing farms and at different stages of the slaughter process of turkey flocks using PFGE and *flaA*-SVR sequencing.

Material and Methods

Sampling and isolation

Between July 2006 and October 2006, 1–2 weeks prior to the slaughter a total of 360 swab samples from fresh faecal droppings of turkey flocks at six rearing farms (A–F, male and female birds) were sampled (Perko-Mäkelä et al., 2009). Number 1 after the capital letter indicates female turkeys. Number 2 after the capital letter indicates male turkeys (Table 1). Of 20 samples per flock five swabs were pooled together to create four subsamples. For enrichment, these four samples were pooled together (Perko-Mäkelä et al., 2009). The size of the commercial farms varied from 6000 to 18 000 birds per cycle. Turkey females and males are reared in the same house but separated by partitions. Following the slaughter of the females at 13–15 weeks, the males are allowed to use the entire house until slaughtered at an age of 17–18 weeks. The house is cleaned after slaughtering of the males. During the slaughter of these flocks, 636 different samples were taken from 11 different stages at the slaughterhouse and processed as detailed previously described by Perko-Mäkelä et al. (2009). All flocks were slaughtered and sampled at the same slaughterhouse. A total of 240 caeca and neck skin samples (120 each) were taken during the slaughter of each flock in question. The environmental samples (total 336) consisted of swab, water, and faecal samples and were taken during the slaughter process. Cotton swab samples were collected from the transportation crates after disinfection (25 cm^2 per surface) and from the rubber boots of the workers in the evisceration room. For transporting, each swab was put into a tube containing 37 g l^{-1} Brain Heart Infusion Broth (LabM, Lancashire, UK) with 5% calf blood and 0.5% agar (Scharlau-Chemie, Barcelona, Spain). Sterile gauze swabs were used to collect samples from different work surfaces of the evisceration and meat-cutting room and from the floor of the chilling room (25 cm^2 per surface wiped vigorously for 30 s). For transporting, gauzes were placed into a jar with 50 ml Bolton broth (Oxoid CM0983, Hampshire, UK). Process water samples of 1 l were collected during the slaughter of each flock concerned from the water running trough the defeathering machine and the chilling tank, respectively. Faecal material (5 g each sample) from the transport crates were collected after the birds were taken out for slaughter. A total of 60 meat-cutting samples were taken in the meat-cutting department consisting of five separate subsamples (5 g each) pooled to 25 g to create one sample.

All samples were tested for thermotolerant *Campylobacter* spp. by direct plating and enrichment culture, and cultured on a modified charcoal cefoperazone deoxycholate agar plate (mCCDA) (Oxoid CM739) supplemented with SR 155 (Oxoid). For enrichment, Bolton-selective enrichment broth (Oxoid CM0983) with selective supplement (Oxoid SR0183) and 5% lysed horse blood was used and incubated (42 ± 1°C for 22 ± 2 h) under microaerobic conditions generated by CampyGen™ (Oxoid CN0035). All plates were also incubated in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2), generated by

Table 1. PFGE types* and *flaA*-SVR alleles† identified among *C. jejuni* isolates from Finnish turkey rearing farms and at different stages of the slaughter line

				Slaughterhouse stages											
Farm-ID	No. isolates	Flock	Date of slaughter	Faecal droppings at farm (f)	Transport crates (tc)	Faecal material from transport crates (ft)	Water from defeathering machine (wd)	Content of caecum (cc)	Neck skin (ns)	Environment (evisceration room) (ee)	Boots in evisceration room (be)	Chilling water (cw)	Environment (chilling room) (ech)	Environment (cutting room) (ecu)	Meat cuts (mc)
A	14	A1‡,§	8/18/2006	I/21	I/161	I/21	–	I/21	I/161	I/21	I/161	–	–	–	I, II/21
	21	A2‡,¶	9/12/2006	I/21	–	I/21, 161	I/21, 252	I/161	I/36	I/161	I/161	I/161	I/36	I/15	I/15
B	–	B1	8/10/2006	–	–	–	–	–	–	–	–	–	–	–	–
	5	B2	8/22/2006	–	–	I/21	–	–	I/21	–	–	–	I/21	–	–
C	23	C1	8/25/2006	IX/21, 36	IX/36	IX/21	–	IX/21, 36	IX/21, 36	Not typeable/21	IX/21	IX/21, 36	IX/36	–	IX/36
	19	C2	9/20/2006	IX/36		IX/36		IX/36	IX/36, 70	IX/36	IX, XI/22, 36	IX/36	IX/36	–	IX, XI/22, 36
D	–	D1	8/29/2006	–	–	–	–	–	–	–	–	–	–	–	–
	14	D2	10/3/2006	–	–	V, IX/36, 161	IX/36	–	IV/36	IX/36	IX/36	III, V/36	VIII/72	–	VI, VII/36, 508
E	7	E1	9/1/2006	V/161	–	V/161	–	–	V/161	V/161	–	V/161	–	–	–
	15	E2	9/26/2006	V/161	–	V/161	V/161	V/161	V/161	V/161	V/161	V/161	V/161	–	–
F	2	F1	9/22/2006	–	–	IX/36	–	–	–	IX/36	–	–	–	–	–
	1	F2	10/23/2006	–	–	V/36	–	–	–		–	–	–	–	–

*Roman numerals indicate PFGE types.
†Numbers indicate *flaA* alleles.
‡Letter indicate farm.
§Number 1 indicates female turkeys.
¶Number 2 indicates male turkeys.

CampyGen™ (42 ± 1°C for 48 ± 4 h) (Perko-Mäkelä et al., 2009). Two to three presumptive colonies from each positive sample were isolated to detect *Campylobacter* spp. and subcultured on mCCDA agar (without supplement) three times prior genotyping. One single *Campylobacter* isolate was further used for genotyping. All strains were frozen at −80°C in Brucella Broth (Scharlau Chemie 02-042, Barcelona, Spain) with 15% (v/v) glycerol solution.

Identification and typing of Campylobacter spp. isolates

Identification to genus level

A total of 143 isolates obtained from turkey flocks at farms (22 isolates) and during slaughter (121 isolates) were identified to genus level according to the method of the National Committee of Food Analyses (2007) including Gram staining (cell morphology), motility of the cells, catalase test (3% H_2O_2), oxidase test (Oxoid BR0064) and hippurate hydrolysis test (1% hippurate solution and ninhydrin reagent). To test their ability to grow in air, the colonies were streaked out onto blood plates (CASO agar, Casein-Peptone Soymeal-Peptone, Merck, Darmstadt, Germany with 5% bovine blood) and incubated aerobically at +37°C for up to 3 days. Twenty-two isolates were not recovered for further identification.

For PCR, DNA isolation from the frozen pellet was carried out using a DNA isolation kit, MagneSil® KF Genomic System (Promega MD1460, Madison, WI, USA), with a Dynal MPC®-S magnetic stand (Dynal Biotech, Oslo, Norway) as described in Katzav et al. (2008). The detection of *Campylobacter* spp. in the samples was based on amplification of the 16S rRNA gene (Linton et al., 1996) using a set of oligonucleotide primers: C412F 5′-GGA TGA CAC TTT TCG GAG C-3′ and 16S rRNA-campR2 5′-GGC TTC ATG CTC TCG AGT T-3′ as described by Linton et al. (1996) and Lund et al. (2004), respectively. The internal amplification control (IAC) was prepared by isolating genomic DNA from the unrelated bacterium *Yersinia ruckeri,* which is the causative agent of enteric redmouth disease in salmonid fish species (Gibello et al., 1999). This bacterium is not found naturally in poultry. For detection of the internal control, the primers Yers F8 5′-CGA GGA GGA AGG GTT AAG TG-3′ and Yers R10 5′-AAG GCA CCA AGG CAT CTC TG-3′ slightly modified from Gibello et al. (1999) were used**.** All the primers were synthesized by Oligomer Oy (Helsinki, Finland). The PCR conditions used in the present study are described by Lund et al. (2004) with a few modifications. Briefly, PCR amplification was performed in 50 μl volumes containing 5 μl of the DNA, 25 μl of a PCR master mix (Promega), 1 μl of a 25 mM $MgCl_2$ solution, 0.5 μl of a 10 mg ml^{-1} BSA solution (New England Biolabs, Ipswich, MA, USA), 20 pmol of each of the

Campylobacter primers and 5 pmol of each of the internal control primers and 10 pg of genomic *Yersinia ruckeri* DNA primers. PCR was performed in a Peltier Thermal Cycler (PTC-200; MJ Research Inc., Watertown, MA, USA) and the conditions were one cycle of 95°C for 2 min, 58°C for 1 min, 72°C for 1 min, followed by 34 cycles each of 95°C for 15 s, 58°C for 40 s and 72°C for 40 s. The last elongation step (72°C) lasted for 5 min. The PCR product was loaded onto a 2% agarose gel (1.35% SeaKem® LE Agarose and 0.65% NuSieve® GTG Agarose, Cambrex Bio Science, Rockland, ME, USA) containing 0.1 g ml^{-1} ethidium bromide. A DNA molecular weight marker 100 bp low ladder (P1473; Sigma-Aldrich, St Louis, MO, USA) was included in each gel. The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA). The PCR reaction for each sample was performed twice and considered positive if the primer set gave a distinct 857 bp band. Samples with no internal control band were run again using a 10-fold dilution of DNA.

Identification to species level

For identification to species level, a multiplex PCR assay based on the method described by Wang et al. (2002) was used. Primers used were 23SF (5′-TAT ACC GGT AAG GAG TGC TGG AG-3′) and 23SR (5′-ATC AAT TAA CCT TCG AGC AC CG-3′) for *Campylobacter* spp. (size 650 bp), CJF (5′-ACT TCT TTA TTG CTT GCT GC-3′) and CJR (5′-GCC ACA ACA AGT AAA GAA GC-3′) for *C. jejuni* (size 323 bp), CCF (5′-GTA AAA CCA AAG CTT ATC GTG-3′) and CCR (5′-TCC AGC AAT GTG TGC AAT G-3′) for *C. coli* (size 126 bp) (Wang et al., 2002). All primers were synthesized by TIB MOLBIOL GmbH (Berlin, Germany). PCR amplification was performed in 25 μl volumes containing 2.5 μl of template DNA, 2.5 μl of 10 × NH_4-buffer (Mg^{2+} free), 4.0 μl of $MgCl_2$ (50 mM), 1.5 μl of dNTP-Mix (10 mM), 1.25 U of *Taq* DNA polymerase (all Bioline GmbH Luckenwalde, Germany), 0.5 μM of *C. jejuni* primers, 1 μM of *C. coli* primers and 0.2 μM of 23S rRNA primers. The volume was adjusted with sterile distilled water to give 25 μl. PCR was performed in a TProfessional Basic Thermal Cycler (Biometra, Göttingen, Germany) and the conditions were according to Wang et al. (2002). A DNA molecular weight marker (Hyperladder IV, Bioline) was included in each gel (2% agarose gel). The gel was photographed under UV light (Alpha DigiDoc; Alpha Innotech, San Leandro, CA, USA).

Pulsed-field gel electrophoresis

A total of 121 *Campylobacter* isolates originating from farms (15 isolates) and the slaughterhouse (106 isolates) were typed by PFGE based on the method of Maslow et al. (1993). Of these isolates, 40 (33%) were obtained from direct culture and 81 (67%) from enrichment. Of the 121 strains, five were not typeable. Briefly, the isolates were grown on Brucella blood agar (1–2 days at 37°C) in a microaerobic atmosphere. The bacterial cells were harvested and treated with formaldehyde (to inactivate endogenous nuclease) and mercaptoethanol. The bacteria were embedded in 1% low-melting-point agarose plugs (InCert Agarose, Cambrex Bio Science, Rockland, ME, USA). After DNA purification, 1-mm slices of the agar plugs were digested 16 h with *Kpn*I restriction enzyme (New England Biolabs, Hertfordshire, UK), as described by the manufacturer. The DNA fragments were separated with Gene Navigator (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in a 1% agarose gel (SeaKem Gold Agarose, Cambrex Bio Science) in 0.5 × TBE buffer (45 mmol of Tris, 45 mmol of boric acid, 1 mmol of EDTA) at 200 V. Fragments were separated with a ramped pulse from 1 to 25 s for 19 h. Lambda Ladder PFG marker was used as a standard molecular weight marker in all gels.

A computer program (BioNumerics, version 5.1; Applied Maths, Sint-Martens-Latem, Belgium) was used to identify the clusters of closely related and identical patterns. The gels were analysed using band matching with UPGMA clustering using the Dice coefficient and 1% tolerance. PFGE clusters were defined at a similarity level of 90%. Clusters were assigned a Roman numeral (I–XI).

flaA-SVR sequencing

A total of 121 *C. jejuni* isolates originating from farms (15 isolates) and the slaughterhouse (106 isolates) were typed by *flaA*-SVR sequencing. Of these isolates, 40 (33%) were obtained from direct culture and 81 (67%) from enrichment. Typing was performed by amplifying the *flaA* SVR, followed by sequencing of the PCR product. The *flaA*-SVR was amplified using primers FLA4F (5′-GGA TTT CGT ATT AAC ACA AAT GGT GC-3′) and FLA625RU (5′-CAA GWC CTG TTC CWA CTG AAG-3′) as described previously (Nachamkin et al., 1993). PCR products were cleaned by using MiniElute PCR Purification Kit (Qiagen, Hilden, Germany). Sequence data were obtained using a 3730 DNA Analyzer (Applied Biosystems). The nucleotide region between primers FlaA242FU and FlaA625RU was used for allelic comparisons. Forward and reverse sequence results were confirmed by assembling them in Accelrys Gene v2.5 (Accelrys Inc., San Diego, CA, USA). The nucleotide sequences were compared with the *C. jejuni* FlaA database (http://pubmlst.org/campylobacter/flaA/) and allele numbers were assigned accordingly. Confirmed sequences were aligned using BioNumerics v5.1 (Applied Maths).

Calculation of the discriminating power of the genotyping methods

The Simpson's index of diversity (Hunter and Gaston, 1988) was used to calculate the discriminating power of both genotyping methods.

Results

All 121 *Campylobacter* isolates were identified as *C. jejuni* using the multiplex PCR method by Wang et al. (2002). Table 1 summarizes the PFGE types and *flaA* SVR alleles of *C. jejuni* strains from the turkey rearing farms and at different stages of the slaughter process. PFGE analysis with *Kpn*I restriction enzyme resulted in 11 PFGE types (I–XI) ($D = 0.7295$) and *flaA* SVR typing yielded nine *flaA* SVR alleles among the *C. jejuni* tested in this study ($D = 0.7098$).

Figure 1 shows PFGE patterns of isolated *C. jejuni* strains and the UPGMA clustering based on the similarity of the patterns. Eleven distinct major clusters were defined at a similarity level of 95%. A1 to F2 indicates flock identification; the small letters at the end represent the isolation stage at slaughter, using the abbreviations defined in Table 1.

The flocks from farms A, C and E were found to be colonized with *Campylobacter* prior to slaughter. Based on the fresh fecal droppings of each flock, the *Campylobacter* detection rates were 100% (A1) and 75% (A2) at farm A, 100% (C1) and 75% (C2) at farm C and 25% (E1) and 25% (E2) at farm E (Perko-Mäkelä et al., 2009). Each farm had its own flock-related PFGE type, farm A PFGE type I, farm C PFGE type IX and farm E PFGE type V which were found at farm level and at different slaughter stages (Table 1, Fig. 2). The flocks of farms B (1 and 2), D (1 and 2) and F (1 and 2) were *Campylobacter*-negative at rearing time. Female flocks B1 and D1 remained *Campylobacter*-negative during the slaughter process. From the flocks B2, F1 and F2, *Campylobacter* were isolated during slaughtering and the strains shared the PFGE types I (B2), IX (F1) and VI (F2), respectively (Table 1). The male flock D2 was *Campylobacter*-negative at the farm. Isolates of *Campylobacter* positive samples of this flock obtained from eight different slaughter stages and from the meat cuttings formed a heterogeneous group of seven PFGE types (III–IX) (Table 1).

Isolates belonging to PFGE type I were also found from slaughterhouse samples (faecal material from the transport crates, the neck skins and the environment of the chilling room) of flock B2 (Fig. 1). This flock, slaughtered 3 days after flock A1, was *Campylobacter*-negative at the farm. Also Flock F1 was *Campylobacter*-negative at farm level, but *Campylobacter* strains were isolated from the faecal material from the transport crates and the environment of the evisceration room during the slaughter process. These isolates shared PFGE type IX, which was mainly found in isolates of flock C2. Flock F1 was slaughtered 1 day after flock C2 (Fig. 1).

At the nucleotide level, the most prominent *flaA*-SVR alleles detected were *flaA* allele 36 (33.1%), *flaA* allele 161 (28.1%) and *flaA* allele 21 (24.8%). The dominant *flaA* SVR type 36 was found in different samples of flocks C1, C2, D2 and F1. Whereas flocks C1 and C2 carried that genotype already at farm level, flocks D2 and F1 tested *Campylobacter*-negative at farm level. In these two flocks, *flaA* type 36 was detectable from faecal material from the transport crates and the evisceration room. These results correlate with the PFGE data. The *flaA* SVR type 36 was additionally detectable in faecal material of the transport crates from flock F2.

Flocks E1 and E2 carried exclusively *flaA* allele 161 strains. Strains with *flaA* allele 21 dominated flocks A (in combination with *flaA* allele 161), B2 and C1 (in combination with *flaA* allele 36). The highest diversity of *flaA* alleles (36, 72, 161, 508) was detected in the strains isolated from flock D2. *FlaA* alleles 15 and 508 only appeared at the processing facilities (meat cutting rooms).

Discussion

In this study, three flocks (B, D and F) were *Campylobacter*-negative and three flocks were *Campylobacter* positive (A, C and E) before slaughter. Altogether, three PFGE types and three *flaA* SVR types were detectable in all flocks before slaughter. At the end of the slaughter and processing line (meat cuts), six distinct PFGE types and five *flaA* SVR types were identified among all meat cut samples.

PFGE types I, V and IX dominated in the positive farms and were also detected at individual slaughter stages (Table 1, Fig. 2). Both flocks B2 and F1 were *Campylobacter*-negative at farm level and caecum culture-negative at slaughter. However, *Campylobacter* strains sharing PFGE type I (B2) and IX (F1), respectively, were isolated from certain stages of the slaughter process (Fig. 1). Flock B2 was slaughtered 3 days (weekend) after flock A1. Flock F1 was slaughtered 1 day after flock C2. In the present slaughterhouse, only one turkey flock was slaughtered every sampling day. Cleaning and disinfection procedures were performed daily. In a study characterizing pheno- and genotypes of *Campylobacter* after cleaning and disinfection, Peyrat et al. (2009) concluded that *C. jejuni* and *C. coli* can survive overnight on the surfaces of slaughterhouse equipment after cleaning and certain genotypes may be particularly adapted to survive cleaning and disinfection. In the present study, *Campylobacter*-negative flocks became contaminated during processing

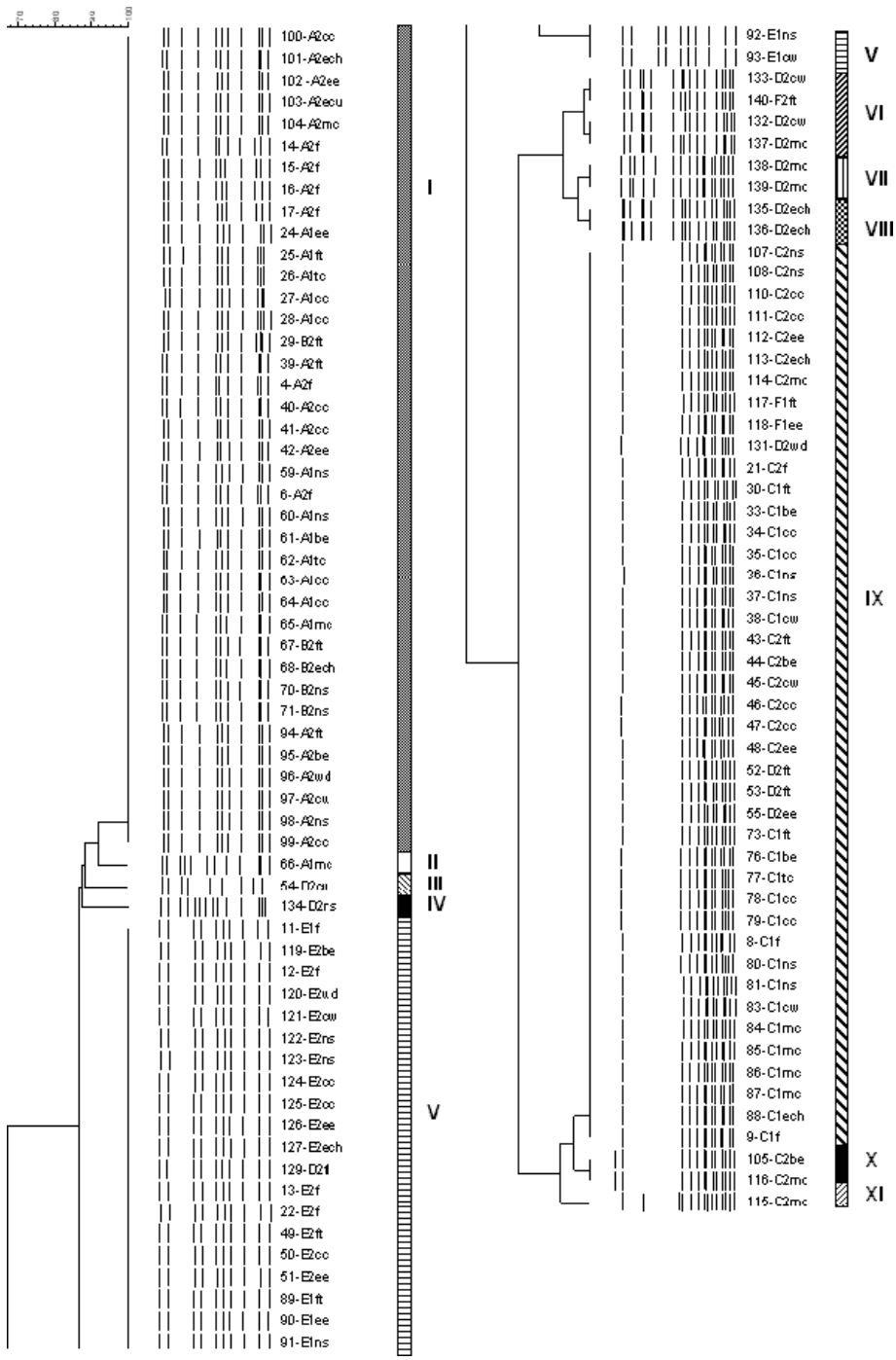

Fig. 1. PFGE pattern of isolated *C. jejuni* strains from Finnish turkey rearing farms and at different stages of the slaughter line (A1–F2, flock identification; small letters at the end represent isolation stage; abbreviations used in this figure are defined in Table 1).

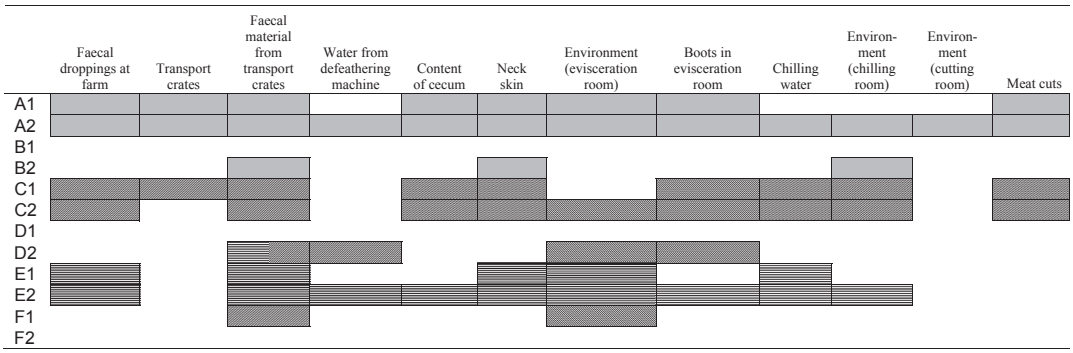

Fig. 2. Sequential spread of the dominant *Campylobacter* PFGE types isolated from Finnish turkey rearing farms and at different stages of the slaughter line. The shading pattern in each box is related to a different PFGE type. The same pattern means the same PFGE type. PFGE type I (■), PFGE type IX (▨), PFGE type V (▤).

by the same subtypes of *Campylobacter* introduced into the slaughterhouse by positive flocks even if slaughtered on following days. In the Nordic countries like Finland, Sweden, Norway and Iceland a low prevalence (2.9–27.5%) of *Campylobacter* carriage in poultry flocks has been reported (Kapperud et al., 1993; Engvall, 1999; Perko-Mäkelä et al., 2000; Johnsen et al., 2006; Guerin et al., 2007). Nonetheless, in this study, besides the low number of positive flocks (six of 12), the detection rate of *Campylobacter* within these flocks has been 25–100% from direct samples and 100% after enrichment (Perko-Mäkelä et al., 2009). However, cross-contamination of carcasses from poultry, coming from different flocks but slaughtered at same slaughterhouse, seems to be unavoidable with the present slaughter logistics. Thus, a single flock infected with *Campylobacter* may constitute a contamination risk for *Campylobacter*-negative flocks. Perko-Mäkelä et al. (2009) studying the occurrence of *Campylobacter* during one total turkey production cycle found the evisceration and the chilling water (detection rate of 100% and 78%, respectively by PCR after enrichment) as critical stages during the slaughter process where spread of bacteria can lead to carcass contamination. The neck skins, mentioned as good indicators of *Campylobacter* contamination at the slaughterhouse, had an isolation rate of 67% by PCR method after enrichment. To reduce the cross-contamination of *Campylobacter*-negative flocks with persistent clones during the slaughter process, hygiene measures are needed. Proper and efficient cleaning and disinfection of slaughter and processing premises is needed to avoid cross-contamination, especially in countries with a low prevalence of *Campylobacter* spp. Even though risk assessments generally regard logistic slaughter as non-effective, it is an additional control option for countries with a very low prevalence of *Campylobacter* in poultry flocks (Rosenquist et al., 2003; Nauta et al., 2005; Johannessen et al., 2007).

In the present study, the *Campylobacter* positive turkey flocks A, C and E are colonized by a limited number of *Campylobacter* clones at the farm and slaughter level (1–3 PFGE types and 3–5 *flaA* SVR alleles). Different studies performed in several countries, like the Netherlands with a flock size of 20 000 to 30 000 birds (Jacobs-Reitsma et al., 1995), United Kingdom (flock sizes not given, Shreeve et al., 2000), Denmark (flock sizes not given, Borck and Pedersen, 2005) or Switzerland with a flock sizes between 3800 and 14 720 birds (Ring et al., 2005) indicate that poultry flocks are mainly colonized by 1–2 genotypes. Nonetheless, some authors detected multiple subtypes in single poultry flocks, like Hiett et al. (2002) in Arkansas and California, USA, Lienau et al. (2007) in Germany and De Cesare et al. (2008) in Italy (in all studies flock sizes not given). In this study, post-transport isolates of flock D2 formed a heterogeneous group of seven PFGE types (II–VIII) or four *flaA* alleles (36, 72, 161, 508), respectively. However, only one isolate per positive sample has been typed and it is possible that other minor subtypes are also present. It has been further discussed that the cultural isolation method might preferentially select for certain strains (Newell et al., 2001).

The present study confirms the traceability of flock-specific strains (PFGE types I, V and IX; *flaA* types 21, 36 and 161) from the farm along the entire processing line to the end-products (meat cuts). Clonal dominance of certain types has also been reported by other authors (Newell et al., 2001; Borck and Pedersen, 2005; Lienau et al., 2007). These findings indicate a resistance of certain *Campylobacter* clones to environmental and technological stresses (Hänninen et al., 2001; Alter et al., 2005; Callicott et al. 2008; Hunter et al., 2009). The three domi-

nant PFGE types I, V and IX could not be detected from the transport crates (except A1 and C1), from the water of the defeathering machine (except flock A2, D2 and E2) or from the cutting room (except flock A2). However, clones of flocks A (I21) and C (IX36) have been found in the meat cuts and partly in the environment of the cutting room. It seems that stress factors such as high temperature of the defeathering water (54–56°C), drying of the carcass skin during air chilling (24 h at 2°C), and the oxygen in the air could not eliminate *Campylobacter* completely. Alter et al. (2005) confirmed a significant decrease of *Campylobacter*-positive poultry carcasses after the final chilling period.

In the European Union, most poultry processing plants use air chilling whereas in the United States ice-water immersion is common. According to the EU regulation No. 853/2004 (Anonymous, 2004), after inspection and evisceration, slaughtered poultry must be cleaned and chilled to not more than 4°C as soon as possible. In the slaughterhouse studied here, the turkey carcasses were chilled by placing them first in a water tank of 2°C for 5 min before hanging them for 24 h in a room at 2°C. Comparative studies on the effect of air chilling (2°C) or ice-water immersion (2°C) on the *Campylobacter* load on carcasses reported similar or moderately higher reduction rates by immersion chilling compared to air chilling (Rosenquist et al., 2006; Berrang et al., 2008).

All strains recovered in this study were identified as *C. jejuni*. It is well established that the majority of the *Campylobacter* found in poultry and raw poultry meat are *C. jejuni* (Atanassova et al., 2007; Hamedy et al., 2007; Klein et al., 2007). Nonetheless, several authors reported high *C. coli* prevalence in turkeys before and at slaughter (Logue et al., 2003; Smith et al., 2004; Lee et al., 2005; Wesley at al., 2005). Wright et al. (2008) reported prevalence among 15 turkey flocks in Eastern North Carolina ranging from 31 to 86% for *C. jejuni* and 0 to 67% for *C. coli*. Takahashi et al. (2006) found both *C. jejuni* and *C. coli* in broiler farm samples, *C. jejuni* at all stages of the processing line, but *C. coli* was not detected after defeathering. Of 209 *Campylobacter* isolates from broiler caeca and carcasses De Cesare et al. (2008) identified 155 (74.2%) as *C. coli* and 54 (25.8%) as *C. jejuni*. It might be that in poultry faeces there are lower numbers of *C. coli* which perhaps grow slower or might be overgrown by faster-growing and more stress-resistant *C. jejuni* strains (Alter et al., 2005). In contrast, Wesley et al. (2005) demonstrated a shift in *C. jejuni* and *C. coli* population in turkeys associated with pre-slaughter events. These authors reported an increase of *C. coli* prevalence in cloacal swabs after transportation of turkeys compared with on-farm sampling. They speculate that feed withdrawal, crating and transportation might have favoured the survival and replication of *C. coli* rather than *C. jejuni*. It is also possible that a difference in recovery rate of these two species by the used selective isolation media might have biased our results (Rivoal et al., 1999).

In this study, we applied PFGE using *Kpn*I restriction enzyme, a macro-restriction method in combination with *flaA*-SVR sequencing. The *flaA* SVR typing differentiated the isolates into nine different sequence types and PFGE differentiated into 11 clusters. We found that PFGE had a slightly better discriminatory power of 0.7295 compared with 0.7098 for *fla*A SVR typing. These results are consistent with other studies investigating the discriminatory powers of different typing methods that showed a higher discriminatory power of PFGE compared to *flaA* SVR typing (Miller et al., 2010).

The diversity in PGFE banding patterns is most likely caused by genomic rearrangements. These genetic changes may have occurred in the bacterial population in the intestine of individual birds. In addition, there is evidence that instability and related changes in the macrorestriction profiles may occur when *in vitro* stress factors influence *Campylobacter*, for example, during isolation and extensive subculturing (Wassenaar et al., 1998; Höök et al., 2005). Even though only minimal subculturing was performed in our study, such *in vitro* induced genetic changes cannot be ruled out completely. The majority of *flaA* SVR alleles displayed a distinct association with a specific PFGE type. Nonetheless, a linear relationship for all strains among both typing methods could not be established. The *flaA* SVR method alone cannot track recombinant effects and is by itself poorly suited for the investigation of the molecular epidemiology of *Campylobacter* strains (Levesque et al., 2008). Thus, a combination of different genotyping methods is needed to specify genetic relatedness of strains.

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References

Alter, T., F. Gaull, A. Froeb, and K. Fehlhaber, 2005: Distribution of *Campylobacter jejuni* strains at different stages of a turkey slaughter line. *Food Microbiol.* 22, 345–351.

Anonymous, 2004: Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. Off. J. Eur. Union L 139/55.

Atanassova, V., F. Reich, L. Beckmann, and G. Klein, 2007: Prevalence of *Campylobacter* spp. in turkey meat from a slaughterhouse and in turkey meat retail products. *FEMS. Immunol. Med. Microbiol.* 49, 141–145.

Berrang, M. E., R. J. Meinersmann, D. P. Smith, and H. Zhuang, 2008: The effect of chilling in cold air or ice water on the microbiological quality of broiler carcasses and the population of *Campylobacter*. *Poult. Sci.* 87, 992–998.

Borck, B., and K. Pedersen, 2005: Pulsed-field gel electrophoresis types of *Campylobacter* spp. in Danish turkeys before and after slaughter. *Int. J. Food Microbiol.* 101, 63–72.

Calliott, K. A., H. Harðardóttir, F. Georgsson, J. Reiersen, V. Friðriksdóttir, E. Gunnarsson, P. Michel, J.-R. Bisaillon, K. G. Kristinsson, H. Briem, K. L. Hiett, D. S. Needleman, and N. J. Stern, 2008: Broiler *Campylobacter* contamination and human campylobacteriosis in Iceland. *Appl. Environ. Microbiol.* 74, 6483–6494.

De Cesare, A., A. Parisi, V. Bondioli, G. Normanno, and G. Manfreda, 2008: Genotypic and phenotypic diversity within three *Campylobacter* populations isolated from broiler caeca and carcasses. *Poult. Sci.* 87, 2152–2159.

Dingle, K. E., F. M. Colles, D. Falush, and M. C. Maiden, 2005: Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. *J. Clin. Microbiol.* 43, 340–347.

Engvall, A., 1999: Surveillance and control of *Salmonella* and *Campylobacter* in Swedish commercial poultry. COST Action 97, Commission of the European Union, Lelystad, the Netherlands.

Evans, M. R., C. D. Ribeiro, and R. L. Salmon, 2003: Hazards of healthy living: bottled water and salad vegetables as risk factors for *Campylobacter* infection. *Emerg. Infect. Dis.* 9, 1219–1225.

Gibello, M. M., M. A. Blanco, M. T. Moreno, A. Cutuli, L. Domenech, C. Domínguez, and J. F. Fernández-Garayzábal, 1999: Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. *Appl. Environ. Microbiol.* 65, 346–350.

Guerin, M. T., M. Wayne, J. Reiersen, O. Berke, S. A. McEvan, J.-R. Bisaillon, and R. Lowman, 2007: A farm-level study of risk factors associated with the colonization of broiler flocks with *Campylobacter* spp. in Iceland, 2001–2004. *Acta Vet. Scand.* 49, 18.

Hamedy, A., M. Ludewig, K. Fehlhaber, T. Alter, and D. Schlichting, 2007: Quantitative detection of *Campylobacter* spp. on turkey carcasses and turkey meat. *Fleischwirtschaft* 87, 121–124.

Hänninen, M.-L., P. Perko-Mäkelä, H. Rautelin, B. Duim, and J. A. Wagenaar, 2001: Genomic relatedness within five common Finnish *Campylobacter jejuni* pulsed-field gel electrophoresis genotypes studied by amplified fragment length polymorphism analysis, ribotyping, and serotyping, Appl. *Environ. Microbiol.* 67, 1581–1586.

Hänninen, M.-L., H. Haajanen, T. Pummi, K. Wermundsen, M.-L. Katila, H. Sarkkinen, I. Miettinen, and H. Rautelin, 2003: Detection and typing of *Campylobacter jejuni* and *Campylobacter coli* and analysis of indicator organisms in three waterborne outbreaks in Finland. *Appl. Environ. Microbiol.* 69, 1391–1396.

Hiett, K. L., N. J. Stern, P. Fedorka-Cray, N. A. Cox, M. T. Musgrove, and S. Ladely, 2002: Molecular subtype analysis of *Campylobacter* spp. from Arkansas and California poultry operations. *Appl. Environ. Microbiol.* 68, 6220–6236.

Höök, H., M. A. Fattah, H. Ericsson, I. Vågsholm, and M.-L. Danielsson-Tham, 2005: Genotype dynamics of *Campylobacter jejuni* in a broiler flock. *Vet. Microbiol.* 106, 109–117.

Hunter, S. M., M. E. Berrang, R. J. Meinersmann, and M. A. Harrison, 2009: Genetic diversity of *Campylobacter* on broiler carcasses collected preevisceration and postchill in 17 U.S. poultry processing plants. *J. Food Prot.* 72, 49–54.

Hunter, P. R., and M. A. Gaston, 1988: Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26, 2465–2466.

Jacobs-Reitsma, W. F., U. Lyhs, and J. Wagenaar, 2008: *Campylobacter* in the food supply. In: Nachamkin, I., C. M. Szymanski, and M. J. Blaser (eds), Campylobacter, 3rd edn, pp. 627–644. ASM, Washington, DC.

Jacobs-Reitsma, W. F., A. W. van de Giessen, N. M. Bolder, and R. W. Mulder, 1995: Epidemiology of *Campylobacter* spp. at two Dutch broiler farms. *Epidemiol. Infect.* 114, 413–421.

Johnsen, G., H. Kruse, and M. Hofshagen, 2006: Genotyping of *Campylobacter jejuni* from broiler carcasses and slaughterhouse environment by amplified fragment length polymorphism. *Poult. Sci.* 85, 2278–2284.

Johannessen, G. S., G. Johnsen, M. Okland, K. S. Cudjoe, and M. Hofshagen, 2007: Enumeration of thermotolerant *Campylobacter* spp. from poultry carcasses at the end of the slaughter-line. *Lett. Appl. Microbiol.* 44, 92–97.

Kapperud, G., E. Skjerve, L. Vik, K. Hauge, A. Lysaker, and I. Aalmen, 1993: Epidemiological investigation of risk factors for *Campylobacter* colonization in Norwegian broiler flocks. *Epidemiol. Infect.* 111, 245–255.

Katsma, W. E. A., A. A. De Koeijer, W. F. Jacobs-Reitsma, M.-J. Mangen, and J. A. Wagenaar, 2007: Assessing interventions to reduce the risk of *Campylobacter* prevalence in broilers. *Risk Anal.* 27, 863–867.

Katzav, M., P. Isohanni, M. Lund, M. Hakkinen, and U. Lyhs, 2008: PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products. *Food Microbiol.* 25, 908–914.

Klein, G., F. Reich, L. Beckman, and V. Atanassova, 2007: Quantification of thermophilic *Campylobacter* spp. in broilers during meat processing. *Antonie van Leeuwenhoek* 92, 267–273.

Lee, B. C., N. Reimers, H. J. Barners, C. D'Lima, D. Carver, and S. Kathariou, 2005: Strain persistence and fluctuation of multiple-antibiotic resistant *Campylobacter coli* colonizing turkeys over successive production cycles. *Foodborne Pathog. Dis.* 2, 102–110.

Lévesque, S., E. Frost, R. D. Arbeit, and S. Michaud, 2008: Multilocus sequence typing of *Campylobacter jejuni* isolates from humans, chickens, raw milk, and environmental water in Quebec, Canada. *J. Clin. Microbiol.* 46, 3404–3411.

Lienau, J.-A., L. Ellerbroek, and G. Klein, 2007: Tracing flock-related *Campylobacter* clones from broiler farms trough slaughter to retail products by pulsed-field gel electrophoresis. *J. Food Prot.* 3, 536–542.

Linton, D., R. J. Owen, and J. Stanley, 1996: Rapid identification by PCR of the genus *Campylobacter* and five *Campylobacter* species enteropathogenic for man and animals. *Res. Microbiol.* 147, 707–718.

Logue, C. M., J. S. Sherwood, L. M. Elijah, P. A. Olah, and M. R. Dockter, 2003: The incidence of *Campylobacter* spp. on processed turkey from processing plants in the midwestern United States. *J. Appl. Microbiol.* 95, 234–241.

Luber, P., and E. Bartelt, 2007: Enumeration of *Campylobacter* spp. on the surface and within chicken breast fillets. *J. Appl. Microbiol.* 102, 313–318.

Lund, M., S. Nordentoft, K. Pedersen, and M. Madsen, 2004: Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. *J. Clin. Microbiol.* 42, 5125–5132.

Lund, M., A. Weldderkopp, M. Waino, S. Nordentoft, D. D. Bang, K. Pedersen, and M. Madsen, 2003: Evaluation of PCR for detection of *Campylobacter* in a national broiler surveillance programme in Denmark. *J. Appl. Microbiol.* 94, 929–935.

Maslow, J., A. Slutsky, and R. Arbeit, 1993: The application of pulsed field gel electrophoresis to molecular epidemiology. In: Persing, H., T. Smith, F. Tenover, and T. White (eds), Diagnostic Molecular Microbiology: Principles and Applications, pp. 563–572. American Society for Microbiology, Washington, DC.

Meinersmann, R. J., R. W. Phillips, K. L. Hiett, and P. Fedorka-Cray, 2005: Differentiation of *Campylobacter* populations as demonstrated by flagellin short variable region sequences. *Appl. Environ. Microbiol.* 71, 6368–6374.

Miller, R. S., W. G. Miller, M. Behringer, H. Hariharan, V. Matthew, and O. A. Oyarzabal, 2010: DNA identification and characterization of *Campylobacter jejuni* and *Campylobacter coli* isolated from caecal samples of chickens in Grenada. *J. Appl. Microbiol.* 108, 1041–1049.

Nachamkin, I., K. Bohachick, and C. M. Patton, 1993: Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* 31, 1531–1536.

National Committee of Food Analyses, 2007: Thermotolerant *Campylobacter*. Detection, Semi-Quantitative and Quantitative Determination in Foods and Drinking Water. NCFA Method. No. 119, 3rd edn.

Nauta, M., I. van der Fels-Klerx, and A. Havelaar, 2005: A poultry-processing model for quantitative microbiological risk assessment. *Risk Anal.* 25, 85–97.

Newell, D. G., J. E. Shreeve, M. Toszeghy, G. Domingue, S. Bull, T. Humphrey, and G. Mead, 2001: Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. *Appl. Environ. Microbiol.* 67, 2636–2640.

Park, S. F., 2002: The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int. J. Food Microbiol.* 74, 177–188.

Pattison, M., 2001: Practical intervention strategies for *Campylobacter*. *Symp. Ser. Soc. Appl. Microbiol.* 30, 121–125.

Pittenger, L. G., M. D. Englen, C. T. Parker, J. G. Frye, B. Quiñones, S. T. Horn, I. Son, P. J. Fedorka-Cray, and M. A. Harrison, 2009: Genotyping *Campylobacter jejuni* by comparative genome indexing: an evaluation with pulsed-field gel electrophoresis and *flaA* SVR sequencing. *Foodborne Pathog. Dis.* 6, 337–349.

Perko-Mäkelä, P., M. Hakkinen, T. Honkanen-Buzalski, and M.-L. Hänninen, 2000: Prevalence of *Campylobacter* in chicken flocks during the summer of 1999 in Finland. *Epidemiol. Infect.* 129, 187–192.

Perko-Mäkelä, P., P. Isohanni, M. Katzav, M. Lund, M.-L. Hänninen, and U. Lyhs, 2009: A longitudinal study of *Campylobacter* distribution in a turkey production chain. *Acta Vet. Scand.* 51, 18–28.

Peyrat, M. B., C. Soumet, P. Maris, and P. Sanders, 2009: Phenotypes and genotypes of *Campylobacter* strains isolated after cleaning and disinfection in poultry slaughterhouses. *Vet. Microbiol.* 128, 313–326.

Rasschaert, G., K. Houf, J. Van Hende, and L. De Zutter, 2006: *Campylobacter* contamination during poultry slaughter in Belgium. *J. Food Prot.* 69, 27–33.

Rivoal, K., M. Denis, G. Salvat, P. Colin, and G. Ermel, 1999: Molecular characterization of the diversity of *Campylobacter* spp. isolates collected from a poultry slaughterhouse: analysis of cross-contamination. *Lett. Appl. Microbiol.* 29, 370–374.

Rosenquist, H., N. L. Nielsen, H. M. Sommer, B. Norrung, and B. B. Christensen, 2003: Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. *Int. J. Food Microbiol.* 83, 87–103.

Rosenquist, H., H. M. Sommer, N. L. Nielsen, and B. B. Christensen, 2006: The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. *Int. J. Food Microbiol.* 108, 226–232.

Ring, M., M. A. Zychowska, and R. Stephan, 2005: Dynamics of *Campylobacter* spp. spread investigated in 14 broiler flocks in Switzerland. *Avian Dis.* 49, 390–396.

Schönberg-Norio, D., J. Takkinen, M.-L. Hänninen, M.-L. Katila, S.-S. Kaukoranta, L. Mattila, and H. Rautelin, 2004:

Swimming and *Campylobacter* infections. *Emerg. Inf. Dis.* 10, 1474–1477.

Shreeve, J. E., M. Toszeghy, M. Pattison, and D. G. Newell, 2000: Sequential spread of *Campylobacter* infection in a multiple broiler house. *Avian Dis.* 44, 983–988.

Smith, K., N. Reimers, H. J. Barnes, B. C. Lee, R. Siletzky, and S. Kathariou, 2004: *Campylobacter* colonization of sibling turkey flocks reared under different management conditions. *J. Food Prot.* 67, 1463–1468.

Takahashi, R., F. Shahada, T. Chuma, and K. Okamoto, 2006: Analysis of *Campylobacter* spp. contamination in broilers from the farm to the final meat cuts by using restriction fragment length polymorphism of the polymerase chain reaction products. *Int. J. Food Microbiol.* 110, 240–245.

Wang, G, . C. G. Clark, T. M. Taylor, C. Pucknell, C. Barton, L. Price, D. L. Woodward, and F. G. Rodgers, 2002: Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *J. Clin. Microbiol.* 40, 4744–4747.

Wassenaar, T. M., B. Geilhausen, and D. G. Newell, 1998: Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. *Appl. Environ. Microbiol.* 64, 1816–1821.

Wesley, I. V., W. T. Muraoka, D. W. Trampel, and H. S. Hurd, 2005: Effect of preslaughter events on prevalence of *Campylobacter jejuni* and *Campylobacter coli* in market-weight turkeys. *Appl. Environ. Microbiol.* 71, 2824–2831.

Wright, S. L., D. K. Carver, R. M. Siletzky, S. Romine, W. E. M. Morrow, and S. Kathariou, 2008: Longitudinal study of prevalence of *Campylobacter jejuni* and *Campylobacter coli* from turkeys and swine grown in close proximity. *J. Food Prot.* 71, 1791–1796.

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