Campylobacter jejuni and C. coli in Finnish poultry production

Päivikki Perko-Mäkelä

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.
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 SmaI and KpnI patterns, 18 different PFGE types were identified.

Thirty-five Finnish C. jejuni strains with five SmaI/SacII 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, HaeIII ribotypes, and HS serotypes. The discriminatory power of PFGE, AFLP and ribotyping with HaeIII 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 KpnI 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 been 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.
Acknowledgements

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List of original publications

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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

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cfu</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra acetic acid</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>flaA</td>
<td>flagellin A gene</td>
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<tr>
<td>HACCP</td>
<td>hazard analysis and critical control points</td>
</tr>
<tr>
<td>HS</td>
<td>heat-stable</td>
</tr>
<tr>
<td>HL</td>
<td>heal-labile</td>
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<tr>
<td>mCCDA</td>
<td>modified charcoal cefoperazone deoxycholate agar</td>
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<tr>
<td>MLST</td>
<td>multilocus sequence typing</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
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<tr>
<td>SVR</td>
<td>short variable region</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>ST</td>
<td>sequence type</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighted pair group method using arithmetic averages</td>
</tr>
<tr>
<td>VBNC</td>
<td>viable but non-cultivable</td>
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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/jy/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 37°C, but for the thermophilic species C. jejuni, C. coli, C. lari and C. upsaliensis the optimum temperature is 42°C. 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/rokiteneuvonta/elainlajikohtaiset_rokotteet/siipikarjaroakotteet/). 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

- Imported grandparent stock: 18 weeks rearing period (including 12 weeks quarantine), 42 weeks brooding period
- Hatchery: 21 days
- Breeder stock: 18 weeks rearing period, 42 weeks brooding period
- Slaughter: Three slaughterhouses specialized in broilers
- Commercial broiler flock: Rearing period 35-39 days
- Broiler meat products: Sold as fresh, marinated pieces and packaged in modified gas atmosphere

Figure 3  Turkey meat production chain in Finland

- Imported parent stock: 29 weeks rearing period (including 12 weeks quarantine), 29 weeks brooding period
- Hatchery: 28 days
- Commercial turkey flock: Rearing period; females 14 days, males 18 days
- Turkey meat products: Sold as fresh, marinated pieces and packaged in modified gas atmosphere
- Slaughter: One slaughterhouses specialized in turkeys
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/Lihaasetus.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 54-56°C. 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 4°C as soon as possible. In Finland, broiler slaughterhouses use air chilling to chill the carcasses (2°C 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).
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 54-56°C. Evisceration and cleaning is performed manually. Turkey carcasses are chilled in a water tank at 2°C for five minutes before hanging them for 24 hours in a refrigerated room at 2°C. 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](image)

**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 (Lynngstad 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.
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). Berndtson 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 $3.6 \log_{10} \text{cfu/ml}$. 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 $6.5 \log_{10} \text{cfu per carcass}$) and after evisceration 66.7% of the samples were positive (mean count of $6.0 \log_{10} \text{cfu per carcass}$) (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 $5.3 \log_{10} \text{cfu Campylobacter}$ and carcasses from low prevalence flocks had an average of $2.3 \log_{10} \text{cfu Campylobacter}$. In broiler meat, contamination levels have even been over $4 \log_{10} \text{cfu per meat sample}$ (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 $7 \log_{10} \text{cfu/g}$ from caecum, from $0.5$ to $3.5 \log_{10} \text{cfu/g}$ from neck skin and the levels of turkey meat samples ranged from $0.1$ to $1.9 \log_{10} \text{cfu/g}$ (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 25°C, 37°C and 42°C, indoxyl acetate hydrolysis, and production of H₂S (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 *SmaI* 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% O₂, 10% CO₂, 85% N₂), 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'-GGATGA CAC TTT TCG GAG C-3' and 16S rRNA-campR2 5'-GGCTTC 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'-CGAG 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 MgCl₂ 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% H₂O₂), 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 CG C-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 NH₄- Buffer (Mg²⁺ free), 4.0 μl of MgCl₂ (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 TPProfessional 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 SmaI or KpnI restriction enzymes (I), with SmaI and SacII restriction enzymes (II), or with KpnI 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 25 s for 19 h (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 Smal bands they were considered as different patterns and named as S1, S2 and so on. If they had five or more different bands in KpnI 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 Smal and SacII pattern was designated as a PFGE type in study II. If strains had one to five differing fragments in their Smal and SacII 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 HaeIII (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

<table>
<thead>
<tr>
<th>Month</th>
<th>No. of flocks</th>
<th>No. of positive flocks</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>May</td>
<td>227</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>June</td>
<td>224</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>July</td>
<td>230</td>
<td>16</td>
<td>7.0</td>
</tr>
<tr>
<td>August</td>
<td>220</td>
<td>10</td>
<td>4.5</td>
</tr>
<tr>
<td>September</td>
<td>231</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td>Total</td>
<td>1132</td>
<td>33</td>
<td>2.9</td>
</tr>
</tbody>
</table>

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

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

* Eleven samples after washing and disinfection
Each *Campylobacter*-positive turkey farm had its own flock-related PFGE type when analyzed with *KpnI* 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. *SmaI* enzyme identified 14 different patterns and *KpnI* enzyme identified 15 different patterns. Together there were 18 different genotypes. The most common HS serotype, 6,7, was associated with *SmaI* genotype S2 (4/7) and three of the isolates were not digested with *SmaI*. All S2 isolates had highly similar patterns when digested with *KpnI*. Furthermore, serotype 6,7 isolates which were not digested with *SmaI* had identical *KpnI* 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 *SmaI* and *SacII* 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 *HaeIII* 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

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Month of isolation</th>
<th>Farm</th>
<th>No. of birds in the flock</th>
<th>No.of Campylobacter positive houses/total no.of houses</th>
<th>Serotype (Penner)</th>
<th>Smal I pattern</th>
<th>Kpn I pattern</th>
<th>Combined</th>
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<tr>
<td>1831</td>
<td>May</td>
<td>A</td>
<td>15500</td>
<td>1/2</td>
<td>4,13,16,43,50</td>
<td>S3</td>
<td>K6</td>
<td>C6</td>
</tr>
<tr>
<td>1959</td>
<td>June</td>
<td>B</td>
<td>37500</td>
<td>1/1</td>
<td>6,7</td>
<td>S2</td>
<td>K3</td>
<td>C3</td>
</tr>
<tr>
<td>2059</td>
<td>June</td>
<td>C</td>
<td>15000</td>
<td>1/1</td>
<td>27</td>
<td>S2</td>
<td>K4</td>
<td>C4</td>
</tr>
<tr>
<td>2165</td>
<td>July</td>
<td>D</td>
<td>30000</td>
<td>1/1</td>
<td>12</td>
<td>S1</td>
<td>K1</td>
<td>C1</td>
</tr>
<tr>
<td>2166</td>
<td>July</td>
<td>E</td>
<td>15000</td>
<td>1/2</td>
<td>NS</td>
<td>S2</td>
<td>K5</td>
<td>C5</td>
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<tr>
<td>2186</td>
<td>July</td>
<td>B</td>
<td>3000</td>
<td>4/4</td>
<td>6,7</td>
<td>S2</td>
<td>K3</td>
<td>C3</td>
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<td>K3</td>
<td>C3</td>
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<td>S2</td>
<td>K3</td>
<td>C3</td>
</tr>
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<td>6,7</td>
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<td>S2</td>
<td>K3</td>
<td>C3</td>
</tr>
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<td>K6</td>
<td>C7</td>
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<td>K8</td>
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<td>K1</td>
<td>C1</td>
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<td>4,13,16,43,50</td>
<td>S5</td>
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<td>C8</td>
</tr>
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<td>ND</td>
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<td>K4</td>
<td>C4</td>
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<td>K2</td>
<td>C2</td>
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<tr>
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<td>K</td>
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<td>1/1</td>
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<td>S11</td>
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<td>C15</td>
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<td>S6</td>
<td>K9</td>
<td>C10</td>
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<td>1/1</td>
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<td>S2</td>
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<td>C5</td>
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<td>S6</td>
<td>K9</td>
<td>C10</td>
</tr>
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<td>NS</td>
<td>S2</td>
<td>K4</td>
<td>C4</td>
</tr>
<tr>
<td>2447</td>
<td>Aug.</td>
<td>O</td>
<td>15000</td>
<td>2/2</td>
<td>C. coli</td>
<td>S14</td>
<td>K15</td>
<td>C18</td>
</tr>
<tr>
<td>2448</td>
<td>Aug.</td>
<td>O</td>
<td>15000</td>
<td>C. coli</td>
<td>S14</td>
<td>K15</td>
<td>C18</td>
<td></td>
</tr>
<tr>
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<td>Aug.</td>
<td>P</td>
<td>15000</td>
<td>1/1</td>
<td>6,7</td>
<td>UD</td>
<td>K8</td>
<td>C9</td>
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<td>2450</td>
<td>Aug.</td>
<td>Q</td>
<td>30000</td>
<td>1/2</td>
<td>6,7</td>
<td>UD</td>
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<td>C9</td>
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<tr>
<td>2458</td>
<td>Aug.</td>
<td>B</td>
<td>44000</td>
<td>1/1</td>
<td>27</td>
<td>S2</td>
<td>K4</td>
<td>C4</td>
</tr>
<tr>
<td>2538</td>
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<td>30000</td>
<td>2/2</td>
<td>NS</td>
<td>S12</td>
<td>K13</td>
<td>C16</td>
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<td>2/2</td>
<td>ND</td>
<td>S8</td>
<td>K11</td>
<td>C12</td>
</tr>
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<td>ND</td>
<td>ND</td>
<td>S1</td>
<td>K1</td>
<td>C1</td>
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<tr>
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<td>J</td>
<td>7000</td>
<td>1/1</td>
<td>5</td>
<td>S9</td>
<td>K12</td>
<td>C13</td>
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<tr>
<td>2899</td>
<td>Sept.</td>
<td>T</td>
<td>27000</td>
<td>1/2</td>
<td>NS</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>2946</td>
<td>Sept.</td>
<td>T</td>
<td>8000</td>
<td>1/5</td>
<td>11</td>
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<td>K12</td>
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<tr>
<td>2965</td>
<td>Sept.</td>
<td>V</td>
<td>15500</td>
<td>1/3</td>
<td>NS</td>
<td>S13</td>
<td>K14</td>
<td>C17</td>
</tr>
</tbody>
</table>

NS, nonserotypable; ND, not done; UD, undigested
Table 4  
*C. jejuni* strains, their sources, PFGE patterns, ribotypes, AFLP types and HS serotypes

<table>
<thead>
<tr>
<th>Strain (n = 35)</th>
<th>Source data a</th>
<th>PFGE pattern (Smal/SacII)</th>
<th>Ribotype (HaeIII)</th>
<th>AFLP type</th>
<th>Combined genotype</th>
<th>Serotype (HS)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>5423F</td>
<td>Patient, Pori, 98-07</td>
<td>I/K</td>
<td>A</td>
<td>AF1</td>
<td>G1</td>
<td>12</td>
</tr>
<tr>
<td>4593</td>
<td>Chicken, retail shop, Helsinki, producent A, 98-08</td>
<td>I/K</td>
<td>A</td>
<td>AF1</td>
<td>G1</td>
<td>12</td>
</tr>
<tr>
<td>4772</td>
<td>Chicken, retail shop, Helsinki, producent B, 98-08</td>
<td>I/K</td>
<td>A</td>
<td>AF1</td>
<td>G1</td>
<td>12</td>
</tr>
<tr>
<td>FB3886</td>
<td>Patient, Helsinki, 98-07</td>
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<td>A</td>
<td>AF1</td>
<td>G1</td>
<td>1,44</td>
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<tr>
<td>FB4287</td>
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<td>A</td>
<td>AF1</td>
<td>G1</td>
<td>1,44</td>
</tr>
<tr>
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<td>Chicken fecal sample, 98-07</td>
<td>I/Kc</td>
<td>B</td>
<td>AF1</td>
<td>G2</td>
<td>57</td>
</tr>
<tr>
<td>5768</td>
<td>Chicken, retail shop, Helsinki, producent C, 98-09</td>
<td>I/K</td>
<td>A</td>
<td>AF1</td>
<td>G1</td>
<td>12</td>
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<tr>
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<td>AF2</td>
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<td>A</td>
<td>AF3</td>
<td>G4</td>
<td>6,7</td>
</tr>
<tr>
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<td>Chicken, fecal sample 98-11</td>
<td>IV</td>
<td>C</td>
<td>AF4</td>
<td>G5</td>
<td>1,44</td>
</tr>
<tr>
<td>37A</td>
<td>Chicken, fecal sample 98-11</td>
<td>I/Kc</td>
<td>B</td>
<td>AF4</td>
<td>G6</td>
<td>57</td>
</tr>
<tr>
<td>28A</td>
<td>Chicken, fecal sample 98-08</td>
<td>I/Ka</td>
<td>Aa</td>
<td>AF5</td>
<td>G7</td>
<td>27</td>
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<tr>
<td>BK116</td>
<td>Chicken, retail shop, Helsinki, producent C, 97-08</td>
<td>I/K</td>
<td>A</td>
<td>AF5</td>
<td>G8</td>
<td>27</td>
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<tr>
<td>5862</td>
<td>Chicken, retail shop, Helsinki, 98-09</td>
<td>VII</td>
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</table>

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.
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>
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<th>No. of isolates</th>
<th>Flock</th>
<th>Date of slaughter</th>
<th>Faecal droppings at farm (f)</th>
<th>Faecal material from transport crates (tc)</th>
<th>Water from defeathering machine (wd)</th>
<th>Content of cecum (cc)</th>
<th>Neck skin (ns)</th>
<th>Environment (evisceration room) (ee)</th>
<th>Boots in evisceration room (be)</th>
<th>Chilling water (cw)</th>
<th>Environment (chilling room) (ech)</th>
<th>Environment (cutting room) (ecu)</th>
<th>Meat cuts (mc)</th>
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<tbody>
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</tr>
</tbody>
</table>

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 SVR alleles
6 Numbers indicate flaA alleles

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

<table>
<thead>
<tr>
<th>Faecal droppings at farm</th>
<th>Faecal material from transport crates</th>
<th>Water from defeathering machine</th>
<th>Content of cecum</th>
<th>Neck skin</th>
<th>Environment (evisceration room)</th>
<th>Boots in evisceration room</th>
<th>Chilling water</th>
<th>Environment (chilling room)</th>
<th>Environment (cutting room)</th>
<th>Meat cuts</th>
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$^1$ 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 (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).

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 (54-56°C), drying of the carcass skin during air chilling (24 hours at 2°C), 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 \textit{C. jejuni} subtype is present in the majority of \textit{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 \textit{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. \textit{Campylobacter}-negative flocks, B2 and F1, became contaminated during processing by the same subtypes of \textit{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 \textit{C. jejuni} from the equipment surfaces after cleaning and disinfection in three out of four slaughterhouses visited. It is possible that \textit{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 \textit{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 \textit{Campylobacter} types disappears due to environmental stress factors, the selection of pre-existing genotypes could be detected. In study III, two \textit{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 reliably identified with phenotypic methods. The same uncertainty 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 KpnI 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 KpnI 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


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