Campylobacter Epidemiology – Insights from Subtyping by Pulsed-Field Gel Electrophoresis

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Abstract

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Campylobacter jejuni and *Campylobacter coli* are frequent worldwide causes of food-borne gastroenteritis. Poultry is regarded as the most important infection source. In temperate countries, a marked seasonal variation of human campylobacteriosis is seen, with most cases occurring during the warmer season. In this PhD project, pulsed-field gel electrophoresis, PFGE, was used to explore the genetic and epidemiological relationships among both campylobacters colonising broilers and campylobacters causing sporadic infection in humans.

In a commercial broiler flock, a subsequent addition of genotypes occurred during rearing, with two types found at two weeks of age and six types on the day before slaughter. Two new types were found in the slaughter samples. In two-thirds of the individual birds sampled the day before slaughter, more than one type were found.

In two separate studies, *Campylobacter* isolates from humans infected in Sweden were characterised by PFGE. Although a large variation in distinct restriction patterns was found, most isolates could be sorted into clusters based on pattern similarities. Isolates in some clusters and subclusters were significantly more seasonally concentrated than other isolates.

A sequenced strain of *C. jejuni*, NCTC 11168, was used as a reference strain and molecular size marker for PFGE. Using a *Campylobacter* strain for this purpose may be advantageous compared with using commercially available molecular markers, as the more sample-like DNA migration patterns facilitate and refine interpretation and analysis.

In conclusion, PFGE was found to be a useful tool for investigating *Campylobacter* epidemiology in both broilers and humans. The results show that multiple genotypes of *C. jejuni* may be present in a commercial broiler flock during rearing and even in gastrointestinal tracts of individual birds. Both recurring environmental exposure and genetic changes within the population may explain the genotype diversity. Although a large number of genotypes may be found among *Campylobacter* strains infecting humans, a large proportion of these may be genetically related. It seems that certain *Campylobacter* genotypes infect humans in the regions studied during a short period of the year, and that these genotypes account for a substantial proportion of the cases during the season (summer and early autumn) with highest campylobacteriosis incidence.

Keywords: *Campylobacter*, *Campylobacter jejuni*, epidemiology, seasons, gastrointestinal diseases, humans, chickens, genotyping, pulsed-field gel electrophoresis

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Sammanfattning

Campylobacter jejuni och *Campylobacter coli* är vanliga orsaker till matförgiftning över hela världen. En vanlig smittkälla är fjäderfä, i synnerhet slaktkyckling. I de flesta tempererade länder varierar förekomsten av human campylobakterios kraftigt med årstiden. Flest fall inträffar under den varma årstiden. I detta doktorandprojekt användes pulsfältsgelelektrofores, PFGE, för att undersöka genetiska och epidemiologiska samband dels bland *Campylobacter* som koloniserar kycklingar, dels bland *Campylobacter* som orsakar sporadisk infektion hos människor.

Den genotypiska diversiteten och dynamiken hos *Campylobacter* följdes under uppfödningen av en slaktkycklingflock. Under uppfödningen tillkom alltfler genotyper, med två olika typer funna vid två veckors ålder och sex typer dagen före slakt. Ytterligare två nya typer hittades i prover från slakten av flocken. Två tredjedelar av de provtagna fåglarna hade mer än en genotyp i mag-tarmkanalen. Inga tecken sågs på att någon typ konkurrerade ut någon annan under uppfödningen av flocken.

I två separata studier karakteriserades *Campylobacter*-isolat från människor som smittats i Sverige. Ett stort antal olika restriktionsmönster hittades, men många typer liknade varandra och kunde på grundval av mönsterlikhet sorteras in i olika kluster. De två största klustren i varje studie inkluderade omkring två tredjedelar av isolaten. Isolat i några kluster och subkluster uppträdde signifikant mer koncentrerat under året än andra isolat. Restriktionsmönstren för de typer som var mer koncentrerade till sommaren stämde överens mellan de två studierna. Grupper av isolat som var relaterade i typ och tid, möjligen representerande mindre utbrott, identifierades. Isolat från barn tenderade att vara av mer ovanliga typer än isolat från vuxna.

En sekvenserad stam av *C. jejuni*, NCTC 11168, användes som referensstam och storleksmarkör för PFGE. Användning av en sekvenserad stam av *Campylobacter* för detta ändamål kan ha fördelar jämfört med användning av kommersiellt tillgängliga markörer, då vandringsmönstret för DNA från den sekvenserade stammen är mer likt vandringsmönstret för DNA från andra *Campylobacter*. På så vis underlättas och förfinas tolkning och analys av PFGE-profilerna.

PFGE visade sig vara ett användbart verktyg för att undersöka epidemilogin för *Campylobacter* hos både slaktkyckling och människor. Resultaten visar att flera genotyper kan finnas samtidigt i en kommersiellt uppfödd slaktkycklingflock och även i mag-tarmkanalen hos enskilda fåglar. Såväl upprepad miljöexponering som genetiska förändringar inom populationen kan förklara denna diversitet. Även om människor infekteras av många olika genotyper av *Campylobacter* så kan en stor del av dessa vara genetiskt relaterade. Det verkar som om vissa genotyper av *Campylobacter* i de studerade områdena uppträder koncentrerat under en kort period av året. Dessa genotyper kan ligga bakom en stor del av fallen under sommar och tidig höst, det vill säga den period då incidensen av campylobakterios är som högst.

Contents

Appendix

Papers I–IV

The present thesis is based on the following papers, which will be referred to by their roman numerals.

I. Höök, H., Abdel Fattah, M., Ericsson, H., Vågsholm, I. & Danielsson-Tham, M.-L. 2005. Genotype dynamics of *Campylobacter jejuni* in a broiler flock. *Veterinary Microbiology 106***,** 109–117.

II. Höök, H, Ericsson, H., Vågsholm, I. & Danielsson-Tham, M.-L. A sequenced *Campylobacter jejuni* strain as reference and molecular size marker for pulsed-field gel electrophoresis. In manuscript.

III. Höök, H., Ekegren, M.-B., Ericsson, H., Vågsholm, I. & Danielsson-Tham, M.-L. 2004. Genetic and epidemiological relationships among *Campylobacter* isolates from humans. *Scandinavian Journal of Infectious Diseases 36***,** 435–442.

IV. Höök, H., Ericsson, H., Gustavsson, O., Vågsholm, I. & Danielsson-Tham, M.-L. Seasonal distribution of *Campylobacter* genotypes from humans in Sweden. In manuscript.

Abbreviations

AFLP	amplified fragment length polymorphism
bp	base pairs
C.	*Campylobacter*
CMO	County Medical Officer
DNA	deoxyribonucleic acid
GC content	content of guanine (G) and cytosine (C) in DNA
kbp	kilobase pairs
LPS	lipopolysaccharide
LPSN	List of Prokaryotic Names with Standing in Nomenclature
MLST	multilocus sequence typing
MRP	macrorestriction profiling
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RAPD	random amplified polymorphic DNA
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SMI	The Swedish Institute for Infectious Disease Control
TMAO	trimethylamine-N-oxide hydrochloride
VBNC	viable but non-culturable campylobacters

Introduction

The genus *Campylobacter* is of great importance in human medicine and food safety, moreover, species in this genus are classical veterinary pathogens. Since McFadyean and Stockman (1913) first isolated the organism from aborted ovine foetuses at the beginning of the 20th century, and Smith and Taylor (1919) proposed the name *Vibrio fetus* for the bacterium, a huge amount of knowledge has been discovered about this fascinating organism. Present knowledge now includes the complete genome sequencing of *Campylobacter jejuni* (Parkhill *et al.*, 2000), the most important human pathogen among campylobacters. Despite this, many details regarding the pathogenicity of the organism and the epidemiology of human campylobacteriosis remain unknown.

Taxonomy and morphology

The genus Campylobacter

In 1963, Sebald and Véron proposed that *Vibrio fetus* and *Vibrio bubulus*, based on a considerably lower GC content than other vibrios, should form a new genus, *Campylobacter* (from Greek *καμπυλος (kampulos)* = curved and *βαχτηρς (baktron)* = rod) (Sebald & Véron, 1963). Ten years later, Véron and Chatelain (1973), designated and described the neotype strain for the type species, *Campylobacter fetus*, of the new genus. They also proposed the transfer of *Vibrio jejuni* and *Vibrio coli* to the genus *Campylobacter*.

Since then, the genus *Campylobacter* has undergone several revisions, and species originally assigned to *Campylobacter* have formed new genera, the most notable being *Helicobacter* and *Arcobacter* (Goodwin *et al.*, 1989; Vandamme *et al.*, 1991). The genera *Campylobacter*, *Arcobacter* and *Sulfurospirillum* belong to the family *Campylobacteraceae* (Vandamme & De Ley, 1991; On, 2000), which, together with some other genera and unnamed *Campylobacter*-like organisms, form a separate phylogenetic branch, known as rRNA superfamily VI, within the class *Proteobacteria* (Vandamme *et al.*, 1991). According to the current version of the List of Prokaryotic Names with Standing in Nomenclature, LPSN (Euzéby, 2005), the genus *Campylobacter* consists of 17 species, whereof four are further divided into two subspecies each (Figure 1). Of these 21 taxons, 15 were first described in 1981 or later.

According to the LPSN, *Campylobacter sputorum* is divided into two subspecies: *C. sputorum* subsp. *sputorum* and *C. sputorum* subsp. *bubulus* (Euzéby, 2005). However, On *et al.* (1998b) proposed that the infrasubspecific divisions of *C. sputorum* should be revised to include three biovars of the species, based on their ability to produce catalase and urease: *C. sputorum* bv. sputorum (catalase- and urease-negative), *C. sputorum* bv. faecalis (catalase-positive, urease-negative) and *C. sputorum* bv. paraureolyticus (catalase-negative, urease-positive). They recommended that strains previously assigned to *C. sputorum* subsp. *bubulus* should be redesignated as bv. sputorum (Roop *et al.*, 1985; On *et*

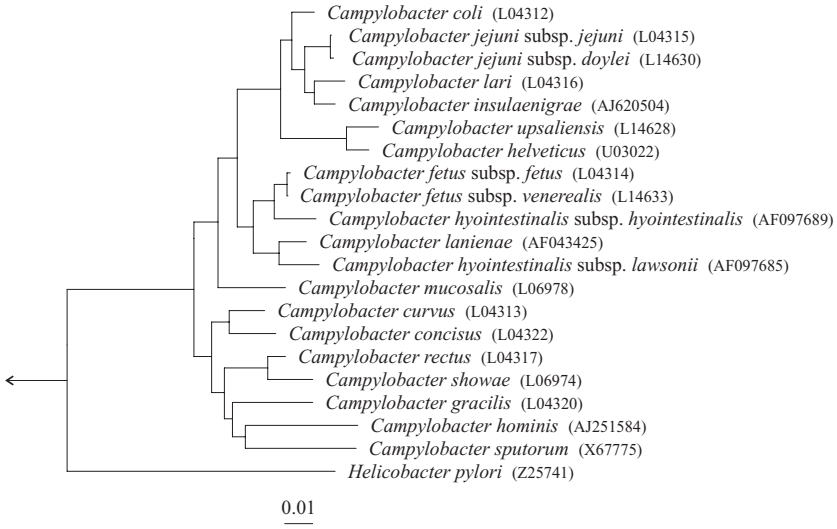

Figure 1. Phylogenetic tree showing all currently approved *Campylobacter* species and subspecies. The tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) from a distance matrix that was corrected for multiple substitutions at single locations by the two-parameter method (Kimura, 1980). Prealigned 16S rRNA sequences were obtained from the Ribosomal Database Project, RDP-II (http://rdp.cme.msu.edu/, Cole *et al.*, 2005), with the exception of the sequence of *Campylobacter insulaenigrae* which was obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and manually aligned. All strains used were type strains. Accession numbers in GenBank are shown in parentheses. *Helicobacter pylori*, a species of a related genus, is also shown in the tree for comparison. *Escherichia coli* (M35018) and *Pasteurella multocida* (X80725) were used as an outgroup. (K.-E. Johansson & H. Höök, 2005, unpublished).

al., 1998b). The Subcommittee on the Taxonomy of *Campylobacter* and Related Bacteria approved this revised biovar nomenclature (Vandamme & On, 2001).

Morphology and biochemical characteristics

Campylobacters are Gram-negative, non-spore-forming, slender, comma or spiral shaped rods, 0.2 to 0.5 μm wide and up to 8 μm long (Smibert, 1984). Cells exposed to oxygen or in old cultures may become coccoid (Ng *et al.*, 1985). The bacterium is motile in a characteristic corkscrew-like manner by means of a single unsheathed flagellum at one or both poles (Smibert, 1984). Most campylobacters are microaerophilic and require an oxygen concentration of 3–15% and a carbon dioxide concentration of 3–5%, but some species are anaerobic. Campylobacters are unable to utilise carbohydrates, instead they obtain energy from amino acids or tricarboxylic acid cycle intermediates (Smibert, 1984). They are oxidase-positive.

Genetic properties

For most *Campylobacter* species, including thermophilic campylobacters, the DNA GC content is 30–36%, although for the entire genus it varies between 29%

and 46% (Vandamme & De Ley, 1991). Since publishing of the complete genome sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000), genetic knowledge regarding this organism has increased rapidly. The chromosome of the sequenced strain is small (1,641,481 base pairs) compared to other prokaryotes, with a low GC content (30.6%), and an unusually high percentage protein coding sequences (Parkhill *et al.*, 2000). In contrast to other sequenced prokaryotes, almost no insertion sequences or phage-associated sequences have been found, and very few repetitive sequences (Parkhill *et al.*, 2000). Another property is the apparent lack of operon organisation of the genes (Parkhill *et al.*, 2000). An important finding is the occurrence of hypervariable sequences, found mostly in genes coding for biosynthesis or surface structure modification, and closely linked genes (Parkhill *et al.*, 2000). The hypervariable regions of the genome may be important for the adaptation and survival of *C. jejuni* in different environments, and for its pathogenic potential (Parkhill *et al.*, 2000; Linton, Karlyshev & Wren, 2001).

Campylobacter jejuni *and related species*

DNA:rRNA hybridisation has demonstrated that *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis* form a separate rRNA subcluster within rRNA cluster I (constituting the genus *Campylobacter*) in rRNA superfamily VI (Vandamme *et al.*, 1991). *Campylobacter helveticus* is a later described species which shows close DNA homology with *C. upsaliensis* (Stanley *et al.*, 1992). Recently, *Campylobacter insulaenigrae*, a new distinct species most closely related to *C. lari* and *C. jejuni*, was described (Foster *et al.*, 2004). *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* are often referred to as thermophilic or thermotolerant campylobacters (Skirrow, 1994). This refers to the fact that most strains of the named species, together with *C. helveticus*, prefer a slightly higher growth temperature than other campylobacters (Doyle & Roman, 1981). However, this terminology is not completely clear-cut, as neither *C. jejuni* subsp. *doylei* nor the recently described and genetically related *C. insulaenigrae* grows at 42°C (Vandamme & Goossens, 1992; Foster *et al.*, 2004). Moreover, several other *Campylobacter* species grow at 42°C (Vandamme & Goossens, 1992), and consequently may be called thermotolerant campylobacters. It is notable that the described use of "thermophilic" and "thermotolerant" is appropriate only in the strict context of campylobacters, as thermophilic/thermotolerant as general microbiological terms refer to microorganisms that thrive in or tolerate temperatures above 50°C. In this regard, all campylobacters belong to the mesophilic organisms.

As one of the leading bacterial causes of human enteritis worldwide, *C. jejuni* is the *Campylobacter* species that has been most extensively studied. The organism was first isolated from cattle with infectious diarrhoea by Jones, Orcutt and Little (1931), who named it *Vibrio jejuni*. Unfortunately, the original strains were lost, and the species was redefined when it was transferred to the new genus *Campylobacter* (Véron & Chatelain, 1973). *C. jejuni* is further divided into two subspecies: *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. Subsp. *doylei* differs from subsp. *jejuni* in that it does not reduce nitrate or grow at 42°C (Vandamme & Goossens, 1992). Hereafter in this thesis, *C. jejuni* refers to *C. jejuni* subsp. *jejuni*.

The second most common *Campylobacter* species isolated from humans is *C. coli*. The organism was first isolated from swine with swine dysentery (Doyle, 1944), and is the primary *Campylobacter* species found in swine (Pearce *et al.*, 2003; Guévremont, Higgins & Quessy, 2004). As in the case of *C. jejuni*, the original strains were lost and the current type strain is from a later date (Véron & Chatelain, 1973). *C. coli* differs phenotypically from *C. jejuni* primarily by its inability to hydrolyse hippurate (Vandamme & Goossens, 1992).

C. lari differs from other thermophilic campylobacters in its resistance to nalidixic acid and ability to grow anaerobically in the presence of trimethylamine-N-oxide hydrochloride, TMAO (Benjamin *et al.*, 1983). It was first isolated in 1976 from human faeces, but most strains are found in wild birds (Benjamin *et al.*, 1983; Waldenström *et al.*, 2002).

During the 1980s, several authors reported isolation of catalase-negative or weakly positive thermotolerant campylobacters from dogs (Sandstedt & Wierup, 1981; Sandstedt, Ursing & Walder, 1983), cats (Fox *et al.*, 1989) and humans (Steele, Sangster & Lanser, 1985; Patton *et al.*, 1989). The organisms were called CNW (catalase-negative or weakly positive) campylobacters until 1991, when the new species *C. upsaliensis* was proposed (Sandstedt & Ursing, 1991).

Growth and survival

Microaerobic atmosphere

An optimal microaerophilic atmosphere for growth of campylobacters is one containing 5–7% oxygen, 10–15% carbon dioxide and 65–85% nitrogen or hydrogen (Kiggins & Plastridge, 1956; Butzler & Skirrow, 1979). This can be achieved by evacuating anaerobic jars and filling them with the above gas mixture (Luechtefeld *et al.*, 1982) or by using commercially available gas envelopes (Wang *et al.*, 1982). A candle extinction jar is a cheap and simple alternative, although it gives a slightly higher oxygen pressure (Luechtefeld *et al.*, 1982; Wang *et al.*, 1982).

Growth temperature and pH

All campylobacters grow well at 37°C. *C. jejuni* has been found to grow between 30°C and 45°C, with an optimal temperature range of 42°C to 45°C, and a pH range of 5.8 to 8.0 (Doyle & Roman, 1981; Gill & Harris, 1982; Kelana & Griffiths, 2003). No growth occurs below pH 4.9, under otherwise optimal conditions (Doyle & Roman, 1981).

Survival in different environments

As thermophilic campylobacters do not multiply at normal room temperature, the role of *C. jejuni* as a food-borne pathogen is associated with its ability to survive in food during storage and handling. The temperature seems to be a key factor for the survival of *C. jejuni*, and one that determines which effect other adverse conditions will have on the decrease in viable cells. In cattle slurry, *C. jejuni*

survives 41 days at 4°C and 7.7 days at 37°C (Paluszak & Olszewska, 2000). Low pH (pH 3.0 to 4.5) results in a reduction in viable cells even in a growth-supporting medium, but the inactivation is much faster at higher temperatures. At 4°C and pH 4.5, more than 4 days is required for a 3-$\log_{10}$ decrease in the number of cells (Doyle & Roman, 1981). *C. jejuni* has been shown to survive for days or weeks in refrigerated foodstuffs (Zhao, Doyle & Berg, 2000). The number of *C. jejuni* on beef decreased during the first week of frozen storage and remained thereafter constant (Moorhead & Dykes, 2002). *C. jejuni* has been shown to survive in frozen chicken for more than 12 months (Beuchat, 1987). Garlic has been shown to have an inhibiting effect on *C. jejuni* survival in butter (Zhao *et al.*, 2000).

Recently, *C. jejuni* was shown to be infective for the protozoan *Acanthamoeba polyphaga*, and to survive for longer periods when cocultured with amoebae (Axelsson-Olsson *et al.*, 2005). Protozoan hosts may play a role in the survival of campylobacters in the aquatic environment, although this remains to be elucidated.

Viable but non-culturable campylobacters (VBNC)

Campylobacters may go into a viable but non-culturable stage (Rollins & Colwell, 1986). VBNC, viable but non-culturable campylobacters, typically show a coccoid form with intact cell membranes (Rollins & Colwell, 1986). However, the coccoid form is not necessarily associated with non-culturability. The role of VBNC as infectious agents is still debated. In a recent study, VBNC were unable to revert to the viable *Campylobacter* form and colonise chicken gut with normal caecal flora (Ziprin & Harvey, 2004).

Isolation and identification

A great variety of selective media and procedures for the isolation of campylobacters exists. Since species differ in their resistance to antibiotics and other selective agents, no single medium is sufficient for the isolation of all *Campylobacter* spp. In most cases, selective media are necessary to enable isolation of the relatively slow-growing campylobacters in samples with a competing normal microbiological flora, for example faeces or food (Butzler & Skirrow, 1979). A prerequisite for recognising the frequent appearance of *C. jejuni* as a human pathogen, and a starting point for the growing interest in *Campylobacter* was when Skirrow (1977) developed a selective agar for *C. jejuni* and *C. coli* isolation from faeces. The Skirrow agar is based on blood agar supplemented with trimethroprim, polymyxin B and vancomycin. Many other media have since been developed according to the same principle: suppression of other microflora by the addition of various selective agents. Both blood-containing media, e.g. Butzler agar (Lauwers, De Boeck & Butzler, 1978) and Campy-BAP (Blaser *et al.*, 1980), and charcoal-based media, e.g. mCCDA agar (Hutchinson & Bolton, 1984), have been shown to be effective for the isolation of campylobacters from human and animal faeces. Preston agar (Bolton & Robertson, 1982) was developed for *Campylobacter* isolation from faeces as well as environmental samples. The membrane filtration technique utilises the fact that campylobacters,

in contrast to most other bacteria, easily pass through filters with a pore size of 0.45 µm (Dekeyser *et al.*, 1972; Mégraud & Elharrif, 1985; Bolton, Hutchinson & Parker, 1988; Moreno *et al.*, 1993). Filtration techniques are especially suitable for the isolation of *C. upsaliensis*, as this species is sensitive to most antibiotics used in other *Campylobacter* media (Bolton *et al.*, 1988; Goossens *et al.*, 1990). A pore size of 0.65 µm enabled a higher isolation rate of *C. jejuni* and *C. coli* (Bolton *et al.*, 1988). However, selective media with a high rate of isolation of *C. upsaliensis* as well as other thermophilic campylobacters have been developed (Aspinall *et al.*, 1993, 1996).

Recently, increased interest in *Campylobacter* species other than *C. jejuni* and *C. coli* as causes of human enteritis has occasionally resulted in the recommendation to routinely incubate specimens at 37°C instead of 42°C. This is aimed at increasing the probability of isolation of these other species, without any significant decrease in the isolation rate of *C. jejuni* and *C. coli.* However, a large British study with four participating laboratories found 42°C to be the optimal temperature for the isolation of *C. jejuni*, and that incubation at 37°C significantly reduced the isolation rate of this species (Gee *et al.*, 2002). The incubation temperature (37°C or 42°C) had no effect on the isolation rates of *Campylobacter* from foodstuffs cultured on Preston agar after an enrichment step (Scates, Moran & Madden, 2003). However, each temperature was found to select for certain *C. jejuni* genotypes, which led to the recommendation to use both temperatures to detect the widest range of genotypes.

Species identification

Phenotypic tests used to differentiate between different *Campylobacter* species include growth at 25°C and 42°C or 43°C, catalase production, nitrate and nitrite reduction, H_2 requirement for microaerophilic growth, indoxyl acetate hydrolysis, growth in the presence of 3.5% NaCl, 1% glycine and 0.1% TMAO, and susceptibility to specific antibiotics such as nalidixic acid and cephalotin (Skirrow & Benjamin, 1980; Vandamme & De Ley, 1991). *C. jejuni* is the only *Campylobacter* species that hydrolyses hippurate (*C. jejuni* subsp. *doylei* may vary in its reaction) (Vandamme & Goossens, 1992). Therefore, hippurate hydrolysis (Hwang & Ederer, 1975) has become the most widely used test to identify *C. jejuni*, and especially to differentiate it from the phenotypically and genotypically similar *C. coli* (Walder, Sandstedt & Ursing, 1983; Lior, 1984). In addition to observed variability in hippurase reaction (Morris *et al.*, 1985), some strains of *C. jejuni* have eventually been shown to be hippurase-negative (Totten *et al.*, 1987; Fermér & Engvall, 1999). This indicates the need for alternative or additional tests.

A number of PCR (polymerase chain reaction)-based methods for identifying thermophilic campylobacters have been developed. PCR of the hippuricase gene identifies *C. jejuni* with higher sensitivity than the hippurate hydrolysis test (Linton *et al.*, 1997). Other PCRs detect and differentiate all thermophilic species (Fermér & Engvall, 1999).

Reservoirs

Campylobacter spp. are found on mucous membranes of the reproductive and gastrointestinal tracts and in oral cavities in a great variety of animals. Although the principal organ system in which bacteria are found is typical for most *Campylobacter* species, few species seem to be strictly host species-specific. *C. jejuni*, the leading cause of human campylobacteriosis, is frequently found in both birds and mammals. Although *C. jejuni* (as well as the classical causative agent of "vibrionic abortion", *C. fetus* subsp. *fetus*) causes abortion in sheep (Diker & Istanbulluoglu, 1986; Hedstrom *et al.*, 1987), and has occasionally been reported in association with various diseases in cattle (Welsh, 1984), poultry (Stephens, On & Gibson, 1998), mink (Hunter *et al.*, 1986), goats (Anderson *et al.*, 1983), horses (Atherton & Ricketts, 1980; Hong & Donahue, 1989) and dogs (Davies, Gebhart & Meric, 1984; Misawa *et al.*, 2002), animals often carry the bacteria without any visible signs of disease or other harm.

Birds

As the temperature optimum for thermophilic campylobacters corresponds to the body temperature of birds rather than mammals, they seem to be well adapted to the avian gut, and birds have been suggested as the natural hosts for these organisms (Newell & Wagenaar, 2000). Campylobacters have been found in a great variety of bird species, both domesticated and wild. Among domesticated birds, a high prevalence of *C. jejuni* and *C. coli* is often found in broiler chickens (Wedderkopp, Rattenborg & Madsen, 2000; Hansson *et al.*, 2004), as well as in older birds such as broiler breeder flocks and egg-laying hens (Doyle, 1984; Cox *et al.*, 2002b). Furthermore, *Campylobacter* colonisation is common in turkeys (Wallace, Stanley & Jones, 1998; Cox *et al.*, 2000; Borck, 2003), geese (Aydin, Atabay & Akan, 2001), ducks (Savill *et al.*, 2003), ostriches and quails (Minakshi, Dogra & Ayyagari, 1988; Oyarzabal, Conner & Hoerr, 1995).

Campylobacters have been isolated from a wide range of wild bird species (Luechtefeld *et al.*, 1980; Fricker & Metcalfe, 1984; Petersen *et al.*, 2001a; Broman *et al.*, 2004). However, they are unevenly distributed among species, and the feeding behaviour of birds has been shown to influence the *Campylobacter* colonisation rate (Waldenström *et al.*, 2002). *Campylobacter* species found in wild birds include the most common human pathogens *C. jejuni* and *C. coli*. Moreover, a substantial proportion of isolates are identified as *C. lari*. Wild birds have been suggested to be important reservoirs for campylobacters infecting broilers and humans. However, comparisons of *C. jejuni* subtypes from wild birds with subtypes from humans and chickens reveal only a few common subtypes (Petersen *et al.*, 2001a; Broman *et al.*, 2002; Broman, 2003).

Other animals

C. jejuni and *C. coli* are often found in faeces from food-producing animals such as cattle, sheep and swine (Rosef *et al.*, 1983; Stanley *et al.*, 1998; Nielsen, 2002; Inglis, Kalischuk & Busz, 2003; Sasaki *et al.*, 2003; Stanley & Jones, 2003; Guévremont *et al.*, 2004). In cattle and sheep, *C. jejuni* is the most frequently

isolated species, with only a small proportion of *C. coli* found. In swine, the ratio between the two species is the opposite, with *C. coli* accounting for the great majority of isolates (Rosef *et al.*, 1983; Guévremont *et al.*, 2004). A recent study reported the novel species *Campylobacter lanianae* being the most frequently found *Campylobacter* species in cattle (Inglis *et al.*, 2003). Horses and goats seem to be more rare carriers of *Campylobacter* spp. (Rosef *et al.*, 1983).

Dogs and cats, both with and without diarrhoea, are frequent carriers of campylobacters (Moreno *et al.*, 1993; Hald & Madsen, 1997; Sandberg *et al.*, 2002; Engvall *et al.*, 2003; Hald *et al.*, 2004). *C. upsaliensis* is the most frequently isolated species, but *C. jejuni* and *C. coli* account for a substantial proportion of the isolates. Also *C. helveticus* can be isolated from dogs and cats (Stanley *et al.*, 1992; Moser *et al.*, 2001).

Few studies have addressed the possible occurrence of campylobacters in wild mammals. However, *C. jejuni* and *C. coli* have been isolated from various wild mammals such as the hare, hedgehog, squirrel, deer, badger, fox, rodents and seal (Fernie & Park, 1977; Rosef *et al.*, 1983; Petersen *et al.*, 2001a).

Campylobacter in foods

Poultry products

The occurrence of *Campylobacter* in all parts of the broiler production chain is well documented all over the world. The *Campylobacter* prevalence in fresh and frozen poultry meat for human consumption varies from 7% to 83% in different countries and investigations (Park *et al.*, 1981; Aho & Hirn, 1988; Willis & Murray, 1997; Madden, Moran & Scates, 1998; Osano & Arimi, 1999; Uyttendaele, De Troy & Debevere, 1999; Kramer *et al.*, 2000; Shih, 2000). Roasted chicken or other poultry products ready for consumption have sometimes been reported to be contaminated by campylobacters (Quinones-Ramirez *et al.*, 2000). Cross-contamination from raw poultry products due to poor hygiene practices is a suspected cause of this (Quinones-Ramirez *et al.*, 2000), although inadequate cooking may contribute.

Other meats

In an investigation in Northern Ireland, no evidence of *Campylobacter* contamination was found on beef carcasses or retail beef or pork (Madden *et al.*, 1998). Likewise, a Japanese investigation did not demonstrate any campylobacters on fresh meat from cattle or swine (Ono & Yamamoto, 1999). In contrast, *Campylobacter* contamination of bovine, porcine and ovine liver was found in Britain (Moore & Madden, 1998; Kramer *et al.*, 2000). Lamb from halal butchers in Britain was found to be contaminated with *C. jejuni* and *C. coli* (Little *et al.*, 1999). Investigations where all lamb carcasses were *Campylobacter*-negative have been reported in Spain (Sierra *et al.*, 1995) and Northern Ireland (Madden *et al.*, 1998).

Hence, although campylobacters are frequently isolated from food-producing animals other than poultry, the prevalence in food products from these animals seems to be low. A possible cause of this difference may be the difference in slaughter procedures between poultry and larger animals.

Other foods

A high prevalence (6 %) of *Campylobacter* in unpasteurized milk on sale to the public has been demonstrated in Britain (Humphrey & Hart, 1988). *C. jejuni* and *C. coli* have been found in shellfish (Reinhard *et al.*, 1996; Wilson & Moore, 1996). There are few studies on the *Campylobacter* occurrence in food of non-animal origin. In Canada, 1.6% to 3.3% of various vegetables on the retail market were demonstrated to be contaminated with *Campylobacter* (Park & Sanders, 1992).

Campylobacteriosis in humans

Spiral-shaped bacteria in faeces from children with enteritis were described by Escherich already in 1886 (Escherich, 1886). However, for many years after 1906, the year of the first successful isolation of the organism that would later become *Campylobacter* (McFadyean & Stockman, 1913), diseases caused by these organisms were of exclusively veterinary concern. Campylobacters were first isolated from humans during a milk-borne enteritis outbreak in 1938 (Levy, 1946), and around 1960 a number of "related vibrios", apparently what we now know as *C. jejuni* and *C. coli*, were isolated from the blood of humans with diarrhoea (King, 1957, 1962). Nevertheless, it was not until the breakthrough of the culture of campylobacters from faeces in the 1970s (Cooper & Slee, 1971; Dekeyser *et al.*, 1972; Butzler *et al.*, 1973) that the human pathogenic potential of *Campylobacter* was universally recognised. After Skirrow's medium for isolation of campylobacters from faeces was described (Skirrow, 1977), diarrhoea-associated occurrence of campylobacters in humans was reported from many countries, and during the 1980s the reported campylobacteriosis incidence increased rapidly.

The vast majority of campylobacteriosis cases in humans are gastrointestinal infections. The incubation time is usually 2 to 5 days, but may vary from 1 to 11 days (Black *et al.*, 1988; Skirrow, 1994). Sudden onset of diarrhoea, which may be watery or bloody, is the most common symptom. Other symptoms are abdominal cramps, fever, myalgia, headache, nausea and vomiting. The onset of fever often precedes the onset of diarrhoea by 12 to 24 hours (Black *et al.*, 1988). The illness is most often self-limiting, with symptoms diminishing after a few days up to two weeks. Faecal excretion of campylobacters usually continue for two to three weeks (Skirrow, 1994).

Experimental studies have shown that an ingestion dose of 500 to 800 organisms may be sufficient to cause illness (Robinson, 1981; Black *et al.*, 1988), although the attack rate was higher among volunteers given higher doses (Black *et al.*,

1988). The dose-response relationship of *Campylobacter* infections has recently been reconsidered (Teunis *et al.*, 2005).

A few percent of patients develop reactive arthritis as a sequel to *Campylobacter* enteritis. The interval between the preceding infection and arthritis onset is up to four weeks. Individuals with human lymphocyte antigen B27 (HLA-B27) are more often affected (Peterson, 1994). A more infrequent sequel is the acute immune-mediated inflammation of peripheral nerves known as Guillain-Barré syndrome (Nachamkin, 2001).

Extra-intestinal manifestations of *Campylobacter* infection in humans are rare. Transient bacteraemia may be present during the course of an enteric infection, but is uncommon (Black *et al.*, 1988). Systemic illness in association with *Campylobacter* occurrence in blood is rarely seen (Skirrow *et al.*, 1993), and most often affects immunocompromised individuals (Söderström, Schalen & Walder, 1991). However, there are reports of *Campylobacter* septicaemia in immunocompetent patients (Söderström *et al.*, 1991; Krause *et al.*, 2002). *C. lari* seems to be unproportionally common in *Campylobacter* septicaemia, with regard to its otherwise rare appearance as a human pathogen (Skirrow *et al.*, 1993). Other reported manifestations, with or without preceding enteritis, are spontaneous abortion (Simor *et al.*, 1986; Selander *et al.*, 1993), meningitis (Herve *et al.*, 2004), myocarditis (Wanby & Olsen, 2001; Westling & Evengard, 2001; Cunningham & Lee, 2003) and cellulitis (Ichiyama *et al.*, 1998; Briedis *et al.*, 2002; Cone *et al.*, 2003; Monselise *et al.*, 2004).

Epidemiology of human infections

Campylobacteriosis incidence

C. jejuni and *C. coli* are common worldwide causes of human gastroenteritis, in developed as well as developing countries (Pebody, Ryan & Wall, 1997; Coker *et al.*, 2002). The reported incidence varies between countries, probably due to differences in surveillance systems as well as real differences in incidence. In 2004, 69 cases per 100,000 inhabitants were reported in Sweden, whereof 35% were reported to be domestically infected (Swedish Institute for Infectious Disease Control, 2005a). The yearly incidence during the past eight years is shown in Figure 2. The situation in other Scandinavian countries is similar, with a yearly incidence of about 50, 80 and 75 cases per 100,000 inhabitants in Norway, Denmark and Finland, respectively (European Commission, 2005). However, the proportion of infections acquired within the country is considerably higher in Denmark than Sweden, whereas a higher proportion of cases in Norway is acquired abroad. Hence, considering only domestic cases in each country reveals larger differences between countries.

Especially mild *Campylobacter* infections can be expected to be substantially underreported. Some efforts to estimate the true campylobacteriosis incidence have been made. Mead *et al.* (1999) assumed the quota reported:unreported cases of campylobacteriosis in the United States to be 1:38, based on estimates from salmonellosis data. Others estimate the true incidence to be five to eight times

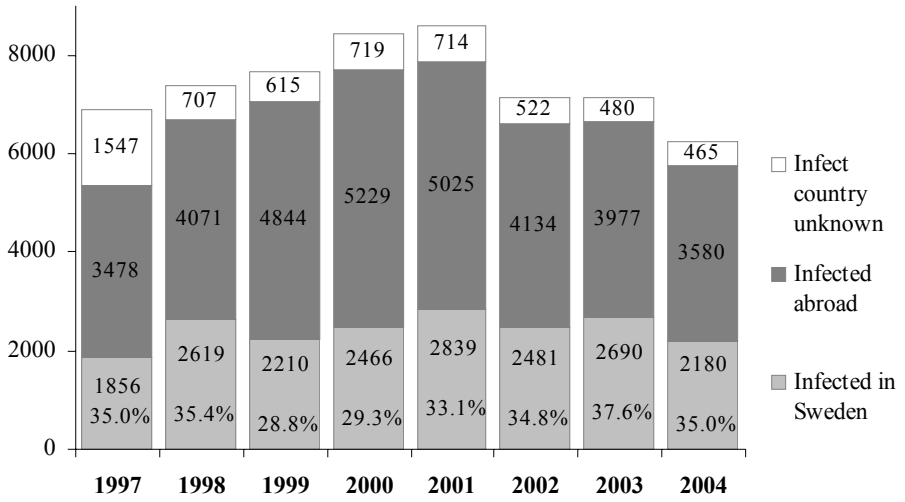

Figure 2. Human campylobacteriosis cases in Sweden, reported according to the Communicable Disease Act. The number of cases of each category is indicated on the bars. For cases reported as infected in Sweden, the proportions of the total number of cases are shown. The data were obtained from the official statistics on communicable diseases in Sweden (http://gis.smittskyddsinstitutet.se/mapapp/build/22-141000/Disease_eng.html).

higher than the reported incidence (Wheeler *et al.*, 1999; de Wit *et al.*, 2000; MAFF, 2000).

Outbreaks

Most information on infection sources and transmission routes for human campylobacteriosis is derived from outbreak investigations. However, outbreaks are relatively rare events for this pathogen, most infections appearing to be sporadic (Pebody *et al.*, 1997).

Large outbreaks of campylobacteriosis, with hundreds or thousands of cases, have been associated with contaminated water (Mentzing, 1981; Andersson, deJong & Studahl, 1997; Furtado *et al.*, 1998; Melby *et al.*, 2000; Kuusi *et al.*, 2004; Kuusi *et al.*, 2005). A mixture of *Campylobacter* and other agents, for example *Escherichia coli* (Bopp *et al.*, 2003), *Shigella sonnei* (Maurer & Sturchler, 2000), norovirus/small round viruses (Maurer & Sturchler, 2000), *Cryptosporidium* or *Giardia*, is sometimes found during waterborne outbreaks of gastroenteritis.

Unpasteurized milk is a well documented cause of campylobacteriosis outbreaks (Robinson & Jones, 1981; Finch & Blake, 1985; Evans *et al.*, 1996; Kálmán *et al.*, 2000; Lehner *et al.*, 2000). Faecal contamination of raw milk may be the most common transmission pathway (Potter *et al.*, 1983; Birkhead *et al.*, 1988), but direct udder excretion of campylobacters has also been reported (Hutchinson *et al.*, 1985; Orr *et al.*, 1995).

Many outbreaks have been related to the consumption of poultry (Istre *et al.*, 1984; Rosenfield *et al.*, 1985; Murphy *et al.*, 1995; Evans *et al.*, 1998; Pearson *et*

al., 2000; Allerberger *et al.*, 2003). More unusual food vehicles associated with campylobacteriosis outbreaks include garlic butter (Zhao *et al.*, 2000) and lettuce (Graves, Bradley & Crutcher, 1998). Cross-contamination, primarily from raw chicken, was suggested as a probable transmission route in these outbreaks.

Sporadic infections

Poultry and poultry products are considered to be common infection sources of sporadic campylobacteriosis (Newell & Wagenaar, 2000). Epidemiological evidence for this assumption has been gathered in many case-control studies, in which poultry consumption is found to be a significant risk factor for campylobacteriosis (Norkrans & Svedhem, 1982; Oosterom *et al.*, 1984; Harris, Weiss & Nolan, 1986; Deming *et al.*, 1987; Kapperud *et al.*, 1992; Ikram *et al.*, 1994; Eberhart-Phillips *et al.*, 1997; Neal & Slack, 1997; Studahl & Andersson, 2000; Effler *et al.*, 2001). The sudden drop in *Campylobacter* infections when poultry products were withdrawn from the market during the dioxin scare in Belgium in June 1999 gives additional support to this hypothesis (Vellinga & Van Loock, 2002). In Iceland, the human campylobacteriosis incidence decreased when *Campylobacter*-positive broiler carcasses were frozen prior to distribution (Stern *et al.*, 2003). The estimated aetiological fraction of cases due to chicken consumption varies between 10% and 50% in different studies (Harris *et al.*, 1986; Neal & Slack, 1997; Neimann *et al.*, 1998; Effler *et al.*, 2001; Vellinga & Van Loock, 2002).

Other suggested transmission modes identified as risk factors in case-control studies include drinking or handling raw/contaminated milk (Southern, Smith & Palmer, 1990; Eberhart-Phillips *et al.*, 1997; Studahl & Andersson, 2000), eating barbecued food (Kapperud *et al.*, 1992), contact with diarrhoeic animals (Saeed, Harris & DiGiacomo, 1993), contact with dogs or puppies (Kapperud *et al.*, 1992) and contact with cats or kittens (Deming *et al.*, 1987). Recently, swimming in natural waters was found to be an independent risk factor for domestic campylobacteriosis in Finland (Schonberg-Norio *et al.*, 2004). There are also case reports on other infection routes, such as eating raw shellfish (Abeyta *et al.*, 1993). However, most sporadic cases remain unexplained (Neal & Slack, 1997; Vellinga & Van Loock, 2002).

Seasonal variation

A striking feature of campylobacteriosis in temperate countries is the seasonal variation, with one or two incidence peaks occurring in spring, summer or early autumn (Nylen *et al.*, 2002; Kovats *et al.*, 2005). The seasonal variations in nine European countries show a remarkably consistent pattern from year to year (Nylen *et al.*, 2002). The seasonality pattern is still largely unexplained, although it has been shown to be related to climatic factors (Patrick *et al.*, 2004; Louis *et al.*, 2005). Seasonal peaks in *Campylobacter* prevalence in broilers and other potential sources have been suggested to be related to the seasonal variations in humans. Others propose flies as important vectors for infection transmission during the summer (Ekdahl, Normann & Andersson, 2005; Nichols, 2005). In Wales,

consumption or handling of milk contaminated by birds (magpies and jackdaws) picking at milk bottles was associated with *Campylobacter* infection during a spring incidence rise (Southern *et al.*, 1990). Another suggested cause of the seasonal peaks is human behaviour that may be more common during the warmer season, such as barbecuing, camping, swimming in lakes and rivers, and drinking water from streams and lakes (Nylen *et al.*, 2002).

Campylobacters in broilers

The prevalence of *Campylobacter* spp. in commercial broiler flocks is high in most industrialised countries (Pokamunski *et al.*, 1984; Nielsen, Engberg & Madsen, 1997). In Sweden, a national surveillance programme was started in 1991, and the prevalence of *Campylobacter*-positive flocks within this programme was reduced from 16% to 10% 2000. In July 2001 a new programme was initiated which showed an apparently higher prevalence (14–20%), probably due to more extensive sampling (Hansson *et al.*, 2004; Swedish Zoonosis Center, 2004). The *Campylobacter* prevalence in Swedish broilers shows seasonal variation, with a seasonal peak occurring in late August.

It is rarely possible to isolate *Campylobacter* in broiler chickens less than two weeks old (Jacobs-Reitsma *et al.*, 1995; Berndtson, Danielsson-Tham & Engvall, 1996a), but the colonisation of the flock often occurs from then and during the weeks until slaughter. Maternal antibodies may at least partly protect very young chicks from *Campylobacter* infection (Sahin *et al.*, 2003b). Colonised birds excrete *Campylobacter* in large quantities; up to 10^8 CFU *Campylobacter* per gram caecal content (Altmeyer, Krabisch & Dorn, 1985).

Once introduced, the spread of campylobacters within the flock is rapid. In a few days 100% or close to 100% of the birds are colonised (Lindblom, Sjögren & Kaijser, 1986; Jacobs-Reitsma *et al.*, 1995). However, data from the Swedish surveillance programme show that in at least one fifth of the *Campylobacter*-positive flocks at slaughter the intra-flock prevalence is considerably lower than 100% (Hansson *et al.*, 2004). Also, in breeder flocks, i.e. older birds, the colonisation rate within the flock is often lower than 100% (Jacobs-Reitsma, 1995).

Horizontal transmission is the most important transmission route for campylobacters in broiler chickens (Jacobs-Reitsma *et al.*, 1995). Most studies indicate that vertical transmission does not occur (Jacobs-Reitsma, 1995; Jacobs-Reitsma *et al.*, 1995; Petersen, Nielsen & On, 2001b; Sahin, Kobalka & Zhang, 2003a), although there is contradicting evidence (Pearson *et al.*, 1996; Cox *et al.*, 2002a). The protective effect of hygiene barriers (Kazwala *et al.*, 1990; Humphrey, Henley & Lanning, 1993; Berndtson *et al.*, 1996b; van de Giessen *et al.*, 1996; Gibbens *et al.*, 2001) suggests that introduction into the flock occurs when campylobacters from outside are transported into the chicken house by the staff, on shoes and clothes. Introduction through the water system has been described in England (Pearson *et al.*, 1993). Feeding the chickens undisinfected water has been shown to be a risk factor for broiler flock colonisation in Norway (Kapperud *et al.*, 1993). However, water and feed are unlikely sources in Sweden

(Lindblom *et al.*, 1986; Berndtson *et al.*, 1996b). Aerosols may also play a role in transmission (Shreeve *et al.*, 2000; Gibbens *et al.*, 2001; Refregier-Petton *et al.*, 2001).

Contamination of previously *Campylobacter*-free broiler flocks with campylobacters during transport (Mead, Hudson & Hinton, 1994; Newell *et al.*, 2001; Slader *et al.*, 2002) and slaughter (Rivoal *et al.*, 1999; Miwa *et al.*, 2003) has been shown in several studies. Slader *et al.* (2002) showed that transport crates were often contaminated with *Campylobacter* spp. when reused, despite washing and disinfection. The probable cause of the ineffectivity of disinfection was that organic material remained on the crates.

Subtyping of *C. jejuni* and *C. coli*

Methods of differentiating between bacterial strains below the species or subspecies level are generally known as bacterial typing or subtyping. The main purposes for bacterial subtyping are to evaluate taxonomy, evolutionary mechanisms and phylogenetic relationships, population genetics and bacterial epidemiology (van Belkum *et al.*, 2001). Here, focus will be primarily on epidemiological typing. A basic assumption in epidemiological typing is that isolates from the same transmission chain, for example causing a disease outbreak, are clonally related, i.e. originate from a common ancestor (Struelens, 1998). Some criteria that may be worth considering in choosing a typing method, depending on the aims and settings, are the typeability, reproducibility, discriminatory power, ease of performance and interpretation, and availability, rapidity and cost of a method (van Belkum *et al.*, 2001).

Phenotyping

For many years, methods based on phenotypic traits formed the foundation for bacterial typing, and so also for *Campylobacter* typing. Several biotyping systems, i.e. typing based on biochemical tests, intended for *C. jejuni* and *C. coli*, have been described (Bolton, Holt & Hutchinson, 1984; Lior, 1984; Roop, Smibert & Krieg, 1984; Huysmans, Turnidge & Williams, 1995). Some of the individual tests included in these biotyping schemes are also utilised for species differentiation.

Resistotyping is typing based on an organism's sensitivity to selected antibiotics, and has been used for characterisation of *Campylobacter* isolates, mostly in combination with other methods (Bopp *et al.*, 1985; Ribeiro *et al.*, 1996; Owen, Lorenz & Gibson, 1997a; Rönner *et al.*, 2004). With regard to the increasing prevalence of antibiotic-resistant *Campylobacter* strains in humans and food-producing animals, its greatest value may be as a monitoring tool, and as an aid in therapy choice. Other phenotypic methods used for *Campylobacter* subtyping are phage typing (Grajewski, Kusek & Gelfand, 1985; Owen, Hernandez & Bolton, 1990; Gibson, Fitzgerald & Owen, 1995), protein profiling (Diker, Esendal & Akan, 2000; Scarcelli *et al.*, 2001) and fatty acid methyl ester (FAME) analysis (Steele *et al.*, 1998).

The most widely used phenotypic method for *C. jejuni* and *C. coli* is serotyping. Two serotyping systems have been extensively used: Lior and Penner serotyping. Lior *et al.* (1982) performed slide agglutination of heat-labile antigens present in the bacterial cell. The antigen types are labelled with the prefix HL. Penner & Hennessy (1980) based their method on passive agglutination of heat-stable antigens on the cell surface, and theses types are given the prefix HS. The identified heat-stable antigens were initially thought to be lipopolysaccharide (LPS) somatic O antigens (Penner & Hennessy, 1980; Preston & Penner, 1987; Moran & Penner, 1999), but have later been shown, at least in some cases, to be capsular antigens (Chart *et al.*, 1996). Penner serotyping is labour-intensive, and modified protocols have been developed to make it simpler and more economic for use in the routine laboratory (Fricker *et al.*, 1986; Fricker, Alemohammad & Park, 1987; Mills *et al.*, 1991). Another shortcoming with both Lior and Penner serotyping is that they leave a substantial proportion of the strains untypeable (Patton, Barrett & Morris, 1985; Mills *et al.*, 1991; Jacobs-Reitsma & Jansen, 1995; Frost *et al.*, 1998; Rautelin & Hänninen, 1999).

Genotyping

Phenotypic methods are based on the detection of phenotypic properties, which depend on the organism's production of certain proteins. Gene expression may vary in the same bacterial strain, depending, for example, on the nutrients available in the medium or other culture characteristics, and hence the phenotype may not be the same under different conditions. In contrast, genotyping is based on a more stable marker, DNA, and identifies the genotype regardless of gene expression.

Some genotypic methods employing different approaches are plasmid analysis (Bopp *et al.*, 1985; Patton *et al.*, 1991), DNA-DNA hybridisation (Hernández *et al.*, 1991) and flagellin gene sequencing (Meinersmann *et al.*, 1997; Clark *et al.*, 2005). Multilocus sequence typing, MLST, is based on sequencing of a set of so-called housekeeping genes, i.e. essential genes (mostly involved in the metabolism of the bacterium) that are present in all strains (Maiden *et al.*, 1998). An MLST system for *C. jejuni* has been developed (Dingle *et al.*, 2001) and is being increasingly used to study epidemiology (Duim *et al.*, 2003; Manning *et al.*, 2003; Sails, Swaminathan & Fields, 2003a, 2003b; Clark *et al.*, 2005) as well as the population structure of the bacterium (Dingle *et al.*, 2005). Microarrays based on the sequencing of the entire genome of *C. jejuni* have been constructed, and may enable identification of variable markers of potential value in developing new typing techniques (Leonard *et al.*, 2003. 2004; Taboada *et al.*, 2004).

Restriction enzyme analysis

In the late 1960s, the first cleavage-site-specific restriction endonuclease (type II restriction enzyme), from a *Haemophilus influenzae* strain, was discovered and shown to cleave DNA at specific sites by the 1978 Nobel Prize Laureates Hamilton O. Smith and Daniel Nathans (Smith & Wilcox, 1970; Danna & Nathans, 1971; Smith & Nathans, 1973). Since then, thousands of type II

restriction enzymes have been characterised, and more then 500 are commercially available (Roberts *et al.*, 2005). Many bacterial genotypic methods make use of restriction enzymes. Separation of DNA fragments in an electrophoretic gel produces a banding pattern (fingerprint) that is specific to the cleavage site positions in the restricted DNA. Analysis based on differences between such patterns is known as restriction fragment length polymorphism (RFLP) analysis.

A number of specific methods based on restriction enzyme analysis have been used for *Campylobacter* subtyping. In restriction endonuclease analysis, REA, the genomic DNA from the sample is restricted by an enzyme producing 30–100 distinct DNA fragments (Owen *et al.*, 1990; Korolik, Moorthy & Coloe, 1995; Jimenez *et al.*, 1997). Other restriction-enzyme-based methods used for typing of *C. jejuni* and *C. coli* are ribotyping (Owen *et al.*, 1990; Gibson *et al.*, 1995), random amplified polymorphic DNA (RAPD) analysis (Hernández *et al.*, 1995; Hilton *et al.*, 1997) and amplified fragment length polymorphism, AFLP, analysis (Kokotovic & On, 1999; Hänninen *et al.*, 2001). Some methods combine PCR and REA, i.e. after amplification of a specific locus, the PCR product is cleaved with restriction enzymes to produce RFLP patterns. The tandem-arranged flagellin genes in *C. jejuni* (*fla*A and *fla*B) constitute a locus containing both highly conserved and variable regions (Meinersmann *et al.*, 1997), and are the most frequently utilised target for such PCR-REA, the method being called PCR-RFLP analysis of the flagellin locus or *fla* typing (Nachamkin, Bohachick & Patton, 1993; Burnens *et al.*, 1995; Ayling *et al.*, 1996). Other gene loci used for PCR-REA of *C. jejuni* include the LPS gene cluster (Shi *et al.*, 2002), and the genes *gyr*A and *pfl*A (Ragimbeau *et al.*, 1998).

Macrorestriction and pulsed-field gel electrophoresis

Conventional REA of whole-genomic DNA gives complex banding patterns which may lead to difficulties in interpretation. Use of restriction enzymes with recognition sites of six to eight base pairs, so-called rare-cutting enzymes, produces banding patterns consisting of relatively few but large fragments, ranging from about 1 to 1,000 kbp (McClelland *et al.*, 1987). This is known as macrorestriction profiling, MRP.

In conventional electrophoresis, the electrophoretic mobility of DNA fragments above 20 kbp is independent of molecular size. Larger fragments could not be resolved by electrophoresis until Schwartz and Cantor (1984) introduced electrophoresis in an alternately perpendicularly oriented field, which enabled resolution of yeast chromosomes up to 2000 kbp. To prevent shear degradation of DNA, microbial cells were embedded in agarose before cell lysis and DNA. The contour-clamped homogeneous electric field, CHEF, the PFGE variant most widely used today, was introduced in 1986 (Chu, Vollrath & Davis, 1986). The same year, Bernards *et al.* (1986) showed that agarose-embedded DNA could be digested by restriction enzymes before loading into the gel. This forms the basis of bacterial macrorestriction profiling by PFGE, as used today.

PFGE has been extensively used in genetic and epidemiological studies of *C. jejuni* and *C. coli*. The many applications include genomic mapping and sizing

(Yan & Taylor, 1991; Kim *et al.*, 1992; Taylor *et al.*, 1992), outbreak investigations (Lehner *et al.*, 2000; Olsen *et al.*, 2001), exploring the diversity of campylobacters causing sporadic infections (Owen *et al.*, 1997b; Hänninen *et al.*, 1998), examining the persistence of genotypes in a population (Petersen & Wedderkopp, 2001) or the environment (Slader *et al.*, 2002), and comparison of genotypes between hosts (Hänninen *et al.*, 2000; Petersen *et al.*, 2001a) or humans and foodstuff (Lindmark *et al.*, 2004).

Aims

The main objective of the present work was to gain insight into *Campylobacter* epidemiology by genetic subtyping. The aims of the included studies were:

- to investigate the genotype diversity of *Campylobacter* isolates from humans infected in Sweden,
- to relate genotypic results to epidemiological information on human infections, and to detect possible clustering of *Campylobacter* genotypes in time, season, location, age or sex,
- to investigate the seasonal distribution of *Campylobacter* genotypes from humans infected in Sweden,
- to explore the genotype diversity and dynamics of *Campylobacter* during rearing and slaughter of a commercial broiler flock, and
- to evaluate the use of a sequenced strain of *C. jejuni* as a reference profile and molecular size marker for pulsed-field gel electrophoresis in epidemiological subtyping of *C. jejuni*.

Comments on materials and methods

The materials and methods are explained in detail in each paper.

Human populations (Papers III and IV)

Human campylobacteriosis is a notifiable disease that has been reported according to the Swedish Communicable Disease Act since 1989. Both clinicians and microbiological laboratories are required to report campylobacteriosis cases to the County Medical Officer (Smittskyddsläkare), CMO, and to the Swedish Institute for Infectious Disease Control, SMI (Anon., 2004). Sweden is divided into 21 counties, and the surveillance system is based on these administrative regional units. The CMO organises the surveillance of communicable diseases in the county, and the SMI compiles data on communicable diseases for the whole of Sweden (Swedish Institute for Infectious Disease Control, 2005b). Most campylobacteriosis case reports (90%) include information about whether the infection was acquired in Sweden or abroad.

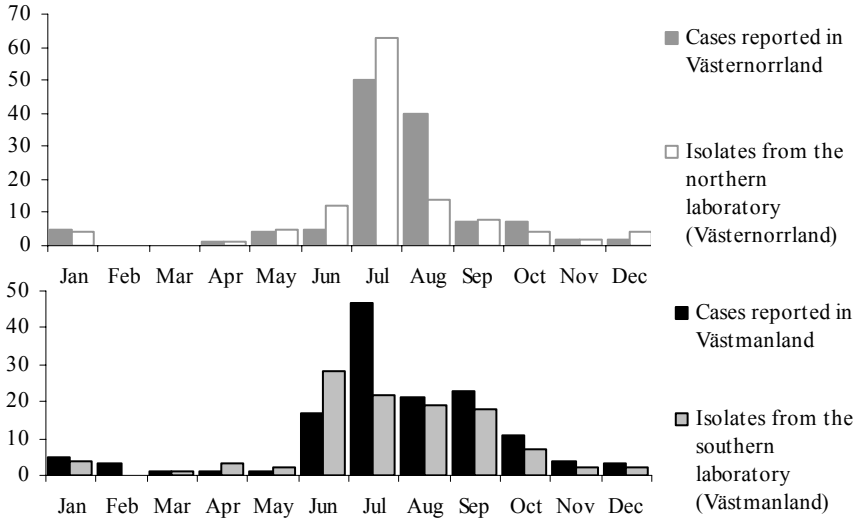

Figure 3. Reported domestic campylobacteriosis cases per month during the periods studied (data from the two years aggregated) in study IV compared with the numbers of isolates collected in each county. Dates for reported cases are the dates of notification, whereas dates for isolates are the stated or estimated illness onset dates. Note the inverse ratios between the bars for July and August in the diagram for Västernorrland, and the bars for June and July in the diagram for Västmanland. This incongruence is probably the result of the delay in reported cases, and gives the largest effect on the bars around the seasonal peak in the respective county. The numbers of reported cases are obtained from the official statistics of communicable diseases in Sweden (http://gis.smittskyddsinstitutet.se/mapapp/build/22-141000/Disease_eng.html).

Isolates for studies III and IV were collected in the microbiological laboratories of two Swedish county hospitals. Individual clinicians in a county may send specimens to other laboratories, and the county hospital laboratory may also receive specimens from other counties. However, the overlap of cases diagnosed in a county hospital laboratory and cases reported for that county can be expected to be high. The total number of reported cases in the two counties of Västernorrland and Västmanland during the study periods for study IV were 123 and 130, respectively, compared with the 117 (95%) and 108 (83%) isolates that were collected for the study. The official numbers of reported cases in Sweden are based on the notification date, which for salmonellosis has been shown to be delayed by 7 days (median) from sample collection (Jansson *et al.*, 2004). In the present studies, the illness onset date was used when this was available, and an estimated onset date calculated by median deviation from specimen or diagnosis date when it was unavailable. Therefore, the dates for cases in these studies are not the same as the dates for the same cases in the official statistics. This slight incongruence is illustrated in Figure 3.

The design of studies III and IV, in which all isolates of documented domestic origin from essentially one or two regions were collected during a defined period, was chosen rather than random selection of isolates from the whole of Sweden. The main reason for this was to enable detection of genotype distribution differences associated with other epidemiological factors, in spite of possible geographical differences in genotype distribution. For example, the degree of seasonality of campylobacteriosis is known to vary between different Swedish counties (Lindbäck & Svensson, 2001), which could possibly override and prevent recognition of more general seasonal trends in genotype distribution. Another aim of the region-based study design was to enable detection of clustering in space and time, indicating small but until then undetected outbreaks. However, the region-based design limits the ability to draw general conclusions from the studies. To elucidate whether the genotype seasonality trends seen in studies III and IV apply to the whole of Sweden, or other countries, more research is needed.

Macrorestriction and pulsed-field gel electrophoresis

Choice of restriction enzymes

The restriction enzyme or enzymes used for PFGE are of course of fundamental importance for the results. Many aspects must be considered in the choice of enzymes. First, which enzymes, among the hundreds available, will work with the organism in question? Many different enzymes, for example *Sma*I, *Sal*I, *Kpn*I, *Sac*II/*Ksp*I and *Bam*HI, have been shown to give interpretable banding patterns with most *C. jejuni* strains (On *et al.*, 1998a; Hänninen *et al.*, 2000; Petersen *et al.*, 2001a). Theoretical cleavage (Bikandi *et al.*, 2004) of the sequenced genome of *C. jejuni* NCTC 11168 shows that in addition to the above-mentioned enzymes, at least 20 recognition sequences of existing restriction enzymes (each recognition sequence considered once regardless of cleavage site) produce 4 to 20 restriction fragments in the size range 15 to 650 kbp (http://www.in-silico.com/s_digest/index.php?mo=Campylobacter, accessed 23 Sep 2004).

Technical aspects – restriction cleavage

A number of technical problems may be associated with restriction cleavage. These may be visible in gels as "empty" profiles, extra, usually weak, bands or a more or less uninterpretable "smear".

"Empty" profiles, i.e. profiles with no bands except one large, intense band in the region of unseparated fragments, may be the logical result of a genome containing no or very few cleavage sites for the restriction enzyme used. However, a more probable explanation of individual undigested genomes is the presence of methylated bases in the DNA sequences. Moreover, some *C. jejuni* strains have been shown to produce DNAse, which degrades their chromosomal DNA during the preparative steps of PFGE (Gibson, Sutherland & Owen, 1994). This may be prevented by formaldehyde fixation of the bacterial cells.

Weak and inconsistent bands in profiles may be due to either incomplete digestion or star activity, i.e. relaxation of specificity. Star activity means that in addition to sites with the defined recognition sequence the enzyme, to some extent, restricts sites with similar sequences. In practice, it may be difficult or impossible to distinguish between partial digestion and star activity only from examining gels. The solutions to incomplete digestion and relaxed specificity are the opposite of each other: complete digestion is achieved by increasing the amount of enzyme used (in relation to DNA) and/or prolongation of the incubation time, whereas relaxed specificity may be avoided by decreasing the quantity of the enzyme and/or reduction of the incubation time.

In the present studies, *Sma*I consistently gave reliable results without weak bands or smearing, with a minimum enzyme quantity and incubation time. However, restriction with *Sal*I (Papers III & IV) sometimes gave profiles that were almost impossible to interpret, with too many bands or smearing. This problem was overcome by decreasing the amount of enzyme and limiting the incubation time to five hours. It thus appears that relaxed or unspecific cleavage was responsible for this problem. Examining extra bands in profiles of CCUG 6824 (Paper IV) revealed that these were not the result of incomplete digestion, as the bands were not of sizes expected for any combinations of restriction fragments (assuming the same fragment order as in the sequenced genome NCTC 11168). Relaxed specificity has been reported for *Sal*I (Malyguine, Vannier & Yot, 1980), although manufacturers state that an extreme overload of enzyme (more than 100 units per gram of substrate DNA) would be necessary for star activity. In our experience, the amount of enzyme is a critical factor that requires fine adjustment to obtain complete digestion but not overdigestion.

Extra bands and/or smearing of *Sal*I profiles arose repeatedly when starting with a new enzyme batch, and therefore the amount of enzyme used had to be adjusted. This is the reason for the varying amount of *Sal*I (Papers II–IV). In study III, a similar problem (resulting in more or less smeared profiles) appeared with *Ksp*I. Also in this case it was corrected by decreasing the amount of enzyme and the incubation time, although the problem remained to some extent: distinct profiles could not be obtained for five isolates despite repeated runs.

Analysis and interpretation of macrorestriction profiles

Although PFGE is generally considered to have excellent intralaboratory reproducibility, its interlaboratory reproducibility has been questioned. Problems encountered in interlaboratory comparisons are related to differences in PFGE protocols, as the variation in plug preparation procedures and running parameters used for PFGE analysis of campylobacters is large (Klena, 2001). Moreover, analysis and interpretation of the produced banding patterns entail assessment which should be standardised to allow reliable comparisons between studies and laboratories (van Belkum *et al.*, 1998).

Computer software for the analysis of RFLP patterns produced by various DNA fingerprinting methods has been developed and is being used increasingly in epidemiological typing studies (de Boer *et al.*, 2000; On & Harrington, 2001). Such software may be a suitable aid in the analysis of PFGE gels, especially in the analysis of large datasets (Gerner-Smidt *et al.*, 1998; de Boer *et al.*, 2000). However, although computer-based analysis applies some degree of automation to the analysis, the analyst is required to make a number of critical decisions that may affect the final results (Gerner-Smidt *et al.*, 1998). Difficulties may be encountered already in the first step of the analysis: determination of the number and positions of visible bands in each pattern. This basic pattern recognition may be a simple task for human visual perception, but requires the application of complicated algorithms for computer programs to perform (Wang, Keller & Carson, 2001). The automated band search function in the computer programs may be misled by artefacts on gel images, and frequently produces errors (Gerner-Smidt *et al.*, 1998). Consequently, it is necessary to examine and sometimes correct the computer-generated results in this regard (Gerner-Smidt *et al.*, 1998).

Recognition of superimposed fragments on gels is a critical step which may affect the subsequent quantification of the similarity between patterns. The automated band search feature in gel analysis software seldom recognises such possible double bands, and hence this may need extra assessment during computer-based analysis (Broman, 2003). In the present studies, suspected double bands were reviewed by plotting densitometric curves of the profiles and summing of fragment sizes. Although we did not perform confirmatory PFGE runs in all cases, the double criterion (twice the amplitude in the densitometric curve *and* agreement with the approximate genome size) to identify a double band was deemed sufficiently reliable.

At least two approaches to enable comparisons of profiles on different gels exist (Gerner-Smidt *et al.*, 1998). The original approach includes comparison of band positions with a DNA size standard and transformation to fragment molecular sizes. Some commercial computer programs for the analysis of enzyme restriction patterns instead utilise a reference pattern, present in each gel, for normalising gel positions. A comparison of two computer programs utilising these different approaches showed that the program that utilised normalised reference patterns for the comparison performed slightly better in identifying visually identical conventional electrophoresis restriction patterns than the program that calculated fragment sizes from comparison with size standards (Gerner-Smidt *et al.*, 1998). However, this conclusion not necessarily applies to the analysis of PFGE data, as

DNA migration in PFGE gels follows a more complicated pattern. In the present studies, transformation of gel positions to molecular sizes was the primary method for obtaining intergel-comparable data, and performing a rough analysis of banding pattern similarities. However, the subsequent band matching was derived directly from gel pictures by comparing the gel position for each band in isolate patterns with approximately equal-sized bands in the DNA size standard (Paper III & IV) or the reference pattern (Paper IV).

Although dendrograms generated from DNA restriction data are a convenient way of quantifying and illustrating pattern similarities, they do not necessarily reflect the true phylogenetic relationships between strains (Clewley, 1998). To draw conclusions on the degree of genetic homology between two or more isolates, methods utilising direct comparison of the DNA sequences, for example MLST, are required.

Results and discussion

Pulsed-field gel electrophoresis for subtyping of *C. jejuni* and *C. coli* (Papers I–IV)

In the present studies, pulsed-field gel electrophoresis was demonstrated to be a useful method for subtyping of campylobacters from various hosts and for evaluating *Campylobacter* epidemiology and colonisation dynamics. Although the DNA from all isolates could not be digested by each of the enzymes used, all isolates gave interpretable restriction patterns with at least one enzyme. By using a combination of restriction enzymes, all isolates could be assigned a genotype.

Campylobacter in the broiler flock (Paper I)

In study I, the dynamics of *Campylobacter* colonisation of a broiler flock was followed. In total, 220 *Campylobacter jejuni* isolates collected on four sampling occasions during rearing and from routine sampling during slaughter were subtyped by *Sma*I restriction and PFGE. During rearing, a subsequent addition of genotypes was observed, with two *Sma*I types being found at two weeks of age and six types the day before slaughter. All types that were detected in more than one isolate were also found on all succeeding sampling occasions, including the slaughter sampling. Two new types were found in the slaughter samples.

Previous studies have indicated that colonisation with several *Campylobacter* subtypes rarely occurs in Swedish broiler flocks (Berndtson *et al.*, 1996b). In some other countries, broilers flocks colonised by multiple strains seem to be a relatively common finding (Pokamunski *et al.*, 1986; Jacobs-Reitsma *et al.*, 1995; Hiett *et al.*, 2002). The proportion of broiler flocks colonised by multiple subtypes could be expected to be correlated to the overall *Campylobacter* prevalence, as a more abundant occurrence of campylobacters in the farm environment probably means that more different subtypes may colonise the broiler flocks. The *Campylobacter* prevalence in Swedish broilers shows a marked variation, between both seasons and farms (Hansson *et al.*, 2004). Hence, colonisation of broiler flocks with multiple subtypes may be more common in the high-prevalence season and farms. Irrespective of the frequency of broiler flocks colonised by more than one strain, study I revealed interesting points in the dynamics of *Campylobacter* colonisation of chickens.

Subsequent addition of *Campylobacter* genotypes during rearing may be explained by either subsequent introductions or frequent mutations of the present clones. Indications that both processes took place in the studied flock were found. At least type 3a, which first appeared on day 38 and did not share any bands with patterns found earlier, probably entered the flock at a later point than the first *Campylobacter* introduction. Other types showed pattern similarities which may have been derived from genetic recombinations of pre-existing types. Assuming genomes of similar sizes, the four-band difference between types 2a and 2b is compatible with the inversion of a large fragment (180–420 kbp). For types 3a, 3b,

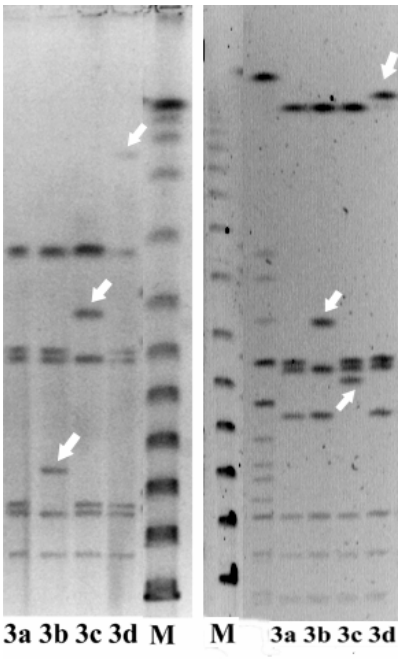

*Figure 4. Sma*I (left) and *Sal*I (right) profiles of type 3a to 3d in study I. M indicates lambda ladder (DNA size standard) profiles. Arrows indicate the differing fragment in each profile, compared with type 3a. The largest *Sma*I band in types 3a, 3b and 3b consists of two fragments. Observe that positions of bands cannot be compared between the two gels, as they were run with different electrophoretic parameters.

3c and 3d the indication of a genetic relationship was stronger. Each of the types 3b, 3c and 3d differed from type 3a by two bands, in that one band had shifted to a higher gel position (Figure 4). If we consider type 3a as the original variant, this is compatible with the insertion of a DNA fragment, at different positions, in these three variants. The size difference of the differing fragment was about 35 kbp in types 3b and 3c, and about 75 kbp in type 3d, compared with type 3a. Further analysis of one isolate of each type with a second restriction enzyme, *Sal*I, revealed two-band differences of the same approximate sizes (Figure 4). Although all isolates of the same *Sma*I type may not necessarily show the same *Sal*I pattern, this gives additional support to the hypothesized genetic changes as the reason behind the band differences in these types. In the two individual samples in which variant types 3b and 3c were found, type 3a was also isolated.

In contrast to the findings regarding types 2a and 3a, which were possibly the origin of genetically rearranged variants found at the same time or later in time, no types very similar to type 1 were found. Although type 1 was isolated during the whole study and in the greatest numbers of all types, the *Sma*I pattern remained stable throughout the study. This is in agreement with the findings of other studies, where some *Campylobacter* strains have been shown to remain genetically stable over long periods in both natural and laboratory environments (Manning *et al.*, 2001).

Although the distribution of genotypes varied between different sampling occasions, no indication was found that any subtype excluded another during the rearing of the broiler flock. Petersen *et al.* (2001b) found that different *Campylobacter* genotypes in broiler flocks, as well as parent flocks, sampled over a longer period, coexisted rather than excluded each other. Fluctuations in

distribution, but no extinction, were also found in a commercial broiler flock investigated by Thomas *et al.* (1997). In contrast, other authors showed experimentally that some *C. jejuni* strains were able to exclude or prevent colonisation by other strains in chickens (Korolik *et al.*, 1998; Barrow & Page, 2000).

In the present study, four rearing types were found in the slaughter samples, together with two types not found before: type 3d, found in one isolate, and type 4. All isolates from one of the pooled cloacal samples were of type 4, which was not detected in any other samples. Because of the different sampling methods between rearing and slaughter, the interpretation of the results from slaughter should be cautious. However, contamination during transport or slaughter is a possible explanation, and has been shown to occur in several studies (Rivoal *et al.*, 1999; Newell *et al.*, 2001; Slader *et al.*, 2002; Miwa *et al.*, 2003). The ten cloacal samples in each pooled sample were collected at short intervals on the slaughter line, which implies that the individuals contributing to one sample were probably located close to each other during transport and the wait before slaughter. Thus, the local spread of a new *Campylobacter* type, originating, for example, from transport crates and with the ability to exclude other campylobacters, may explain the total dominance of type 4 in this sample. If so, this demonstrates the importance of collecting samples throughout the flock during routine *Campylobacter* slaughter sampling. If all samples are collected from birds from a restricted part of the transport vehicle, a subtype originating from local spread during transport may be isolated and assumed to be the most prevalent type, or a partially colonised or contaminated flock may be regarded as *Campylobacter*-free.

Campylobacter epidemiology in humans (Papers III & IV)

Diversity of campylobacters from humans

A large number of distinct genotypes (combined *Sma*I/*Sal*I patterns) were found among *Campylobacter* isolates from humans reported as infected in Sweden. A high diversity among campylobacters of human origin has been reported by other authors, from results obtained by PFGE typing (Hänninen *et al.* 1998, 2000; Hedberg *et al.*, 2001) as well as other genotyping methods (Hernández *et al.*, 1995; Duim *et al.*, 2000; Dingle *et al.*, 2001; Fitzgerald *et al.*, 2001b). The genotype diversity of human strains may be a reflection of the epidemiology of *Campylobacter* enteritis, demonstrating that cases are usually sporadic and may be derived from numerous sources rather than a few predominant ones. Alternatively, the observed diversity in humans may reflect the *Campylobacter* population structure in reservoirs or sources. A large genotype diversity has also been demonstrated in animal hosts (Manning *et al.*, 2003).

The isolates from Västmanland in study III revealed a diversity (expressed as Simpson's index of diversity) of similar degree to isolates from Västmanland in study IV, for both combined *Sma*I/*Sal*I types (0.980 compared with 0.987) and clusters representing half of the total dendrogram (0.738 compared with 0.758). Hence, the higher resolution in study IV did not considerably influence the maximum discriminatory power of the method, i.e. the calculated genotypic

diversity of isolates. However, it did affect the degree of diversity in comparisons of clustering at a specific level of average Dice similarity, e.g. 63% which was the cluster level in the study III. Taking the diversity in the total dendrogram into account enabled cut-offs for clustering to be set at comparable levels, with respect to differences in methods. This is based on the assumption that the total diversity of isolates, collected during two (but not the same two) consecutive years in the same county, should be approximately the same. The finding that the difference between diversity indices for the two counties in study IV was larger than the difference between the diversity indices for Västmanland in studies III and IV supports this assumption.

Potential outbreaks

Outbreaks are a possible reason for concentration of cases in time. PFGE has been extensively used in outbreak investigations to confirm the epidemiological association between *Campylobacter* isolates from different sources (Fitzgerald *et al.*, 2001a; Olsen *et al.*, 2001; Kuusi *et al.*, 2005), and to relate supposed sporadic cases to already known outbreaks (Lehner *et al.*, 2000). In the present studies of *Campylobacter* isolates from humans, several sets of isolates with indistinguishable PFGE types were found to be derived from cases that occurred close in time. Some of these were known family outbreaks. Family outbreaks may be due to secondary infection from the first sporadic case or to primary infection from a common infection source, for example, when family members have all consumed contaminated food. Person-to-person transmission is considered responsible for only a small proportion of the total number of cases (Norkrans & Svedhem, 1982; Oosterom *et al.*, 1984), and small children more often infect their parents than the opposite (Butzler & Skirrow, 1979). In study III, the index cases in the three family outbreaks occurred in three-year-old or younger children, with a parent and/or sibling falling ill 4 to 9 days later. Transmission of infection from child to parent or sibling seems to be the most probable infection route in these outbreaks, as the time spans from the index case to the next case correspond well to the incubation period for campylobacteriosis. In contrast, the two family outbreaks in study IV each included two adults, which were diagnosed on the same day. A common infection source seems more likely in these cases.

In other sets of type- and time-related cases, no known connection between the cases indicated a common infection source. Since most of the PFGE types in question were found in other periods as well, some or all of the sets of clustered cases may just be coincidences. However, a point source may be responsible for cases during an extended period of time, for example, when food from the same batch is consumed at different times. Furthermore, continuous source outbreaks, which may continue for months or even years (Pearson *et al.*, 2000), could possibly be behind accumulation of cases with indistinguishable genotypes during a longer period. The majority of isolates (12/17) in cluster III in study IV were from Västernorrland in July 2001, some of them grouped in sets of type- and time-related isolates with common geographical origin. A continuous source outbreak not limited to a specific geographical location and caused by several different but

related *Campylobacter* PFGE types may be the cause of this accumulation of cases.

The aim with the limited analysis of type- and time-related isolates in study IV was to indicate potential but previously undetected point source outbreaks, which could possibly affect the overall seasonal distribution of PFGE types. Although the analysis implied nine small potential outbreaks, excluding of these cases (except the first case in each set) from the data did not alter the seasonal distribution of different clusters.

Seasonal variations

The results from Västmanland in 1998 and 1999 (Paper III) indicated that some groups of related *Campylobacter* genotypes were more concentrated to the high-incidence season from June to September than other types. In order to further evaluate the possible genotypic seasonality, a new study (Paper IV) was conducted in the same county for another two years, and also expanded to another Swedish county. The results from study III were confirmed in study IV: some clusters of similar PFGE types were significantly more seasonally concentrated than other types. Although differences in banding pattern analysis between studies did not allow a reliable common clustering, the overall patterns of seasonally more concentrated types in study IV agreed with patterns of such types in study III. Hence, the *Sma*I and *Sal*I profiles in the more summer-frequent cluster in study III correspond to profiles in cluster II in study IV. Types with *Sma*I patterns corresponding to the patterns in the seasonally concentrated subcluster Ib in study IV were found in only five isolates in study III. However, these were also concentrated to summer or early autumn in study III (group K, Figure 2, Paper III).

A number of possible explanations of the seasonal variation of human campylobacteriosis have been suggested, some of them evaluated in observational studies. However, few studies have investigated seasonality in different genotypes. The variations found in these studies may be explained by seasonal variations in *Campylobacter* genotype distribution in reservoirs and infection sources, or seasonal variations in their relative importance for human infection.

Studies from several countries show that humans and poultry often share similar or indistinguishable *Campylobacter* genotypes (Hänninen *et al.*, 2000; Kramer *et al.*, 2000; Dickins *et al.*, 2002; Nadeau, Messier & Quessy, 2002; Chu *et al.*, 2004; Lindmark *et al.*, 2004; Campy-SET, 2005). However, this finding does not in itself reveal the direction of the relationship. In Sweden (National Veterinary Institute, 2001) as well as other European countries (Meldrum *et al.*, 2005), the *Campylobacter* prevalence in slaughtered broilers shows a similar seasonality to that of human campylobacteriosis, but peaks somewhat later. A common environmental source for humans and chickens, rather than the infection route from chickens to humans, has been suggested as a more likely explanation of the seasonal increase in both species (Meldrum *et al.*, 2005). There is considerable evidence that poultry consumption is an important risk factor for campylobacteriosis in humans (Eberhart-Phillips *et al.*, 1997; Studahl &

Andersson, 2000; Neimann *et al.*, 2003). However, despite efforts to estimate its relative importance (Effler *et al.*, 2001; Vellinga & Van Loock, 2002), the extent to which poultry consumption contributes to the total incidence of campylobacteriosis is largely unknown, as is its possibly varying role as an infection source during different seasons. Studies that address the seasonal distribution of genotypes in poultry are needed to establish whether seasonal genotype variation in poultry is related to the differences seen in humans.

Comparisons of campylobacters from humans and other *Campylobacter* sources than poultry, such as other meats, other domesticated animals (Engvall *et al.*, 2003), wild birds (Broman *et al.*, 2004) and raw water (Lindmark *et al.*, 2004), generally reveal a lower degree of common types. Interestingly, seasonality in *C. jejuni* prevalence has been reported in black-headed gulls (*Larus ridibundus*) in southern Sweden, with the highest rates found in late autumn, several months after the seasonal peak in humans and chickens (Broman *et al.*, 2002). The differences in seasonal distribution in different species indicate that ecological factors, not yet understood, may be important for *Campylobacter* epidemiology in humans and other species.

Several studies have focused on the seasonality of human campylobacteriosis in relation to various climate factors, such as temperature, precipitation, relative humidity and sunlight. Higher maximum or average temperatures, especially in combination with many hours of sunlight, are associated with higher campylobacteriosis incidence (Patrick *et al.*, 2004; Louis *et al.*, 2005). Climate factors that are beneficial for campylobacters in the environment may be so to varying degrees for different genotypes of the bacterium.

Flies have been suggested as a possible transmission vector for *Campylobacter* infections in humans, with special reference to seasonal variations (Ekdahl *et al.*, 2005; Nichols, 2005). This hypothesis could be compatible with the present results, as flies acting as vectors could pick up any campylobacters in the environment (from faeces, food or waste) and contaminate food ready for consumption. However, one expected effect of fly-vector-driven seasonality would be a higher diversity of campylobacters infecting humans in the season when flies are most abundant, as infections would be assumed to origin from a larger variety of different reservoirs, some of them improbable as infection sources without the vector. In the present studies, however, the *Campylobacter* isolates from the high-incidence season (coinciding with the high-prevalence season for flies) showed a lower degree of diversity than isolates from the rest of the year.

Differences between counties (Paper IV)

The campylobacteriosis seasonality in the two counties differed, with a more pronounced seasonality in the more northern county Västernorrland. Although a similar seasonality of campylobacteriosis incidence is seen in most temperate countries, the peak time and the amplitude of the peak differ between countries (Nylen *et al.*, 2002). A north–south gradient with a later peak and more marked seasonality (greater peak amplitude, shorter high-incidence period) at higher latitudes has been observed in Norway (Kapperud & Aasen, 1992). The same tendency has been reported in Sweden, although seasonality differences between

neighbouring counties, not explained by a latitude gradient, also exist (Lindbäck & Svensson, 2001). Lindbäck and Svensson (2001) developed a statistical model for seasonal distribution of domestic campylobacteriosis cases for all counties in Sweden, based on surveillance data from seven years (1992–1998). According to their model, the ratio between high and low incidence is larger, the peak week occurs later and the two-times-base incidence period starts later, in Västernorrland than in Västmanland. The same parameters calculated from data in study IV (approximately estimated averages from both study years) roughly agree with averages for the two counties from the seven-year model.

In addition to the overall seasonal distribution, the seasonal concentration for some groups differed between counties. Although the order of more and less concentrated groups was the same in both counties, the differences between groups with different seasonal patterns were larger in Västernorrland. Hence, the geographic region may influence the concentration tendency for different genotypes. However, this tendency may also vary between years. As the collecting periods were different in the two counties, the effect of county seen in our study could be a combined effect of both spatial and temporal differences. In order to evaluate this, isolates from the second study year (2002) in Västernorrland were compared with isolates from the first study year (June 2002 to May 2003) in Västmanland. Although these periods were not perfectly matched, they mainly included the same high incidence period, i.e. June to October 2002. The numbers of observations were too low to test for significance, but the concentration of each group in each county, expressed as median and 75 percentile deviation from the peak week, was essentially the same as the total data (Table 2, Paper IV).

Age differences

Children under ten years contributed with 24% and 9% of the isolates in studies III and IV, respectively. In study III, the genotype diversity tended to be higher (lower degree of Dice similarity to other isolates, or types that were more infrequently found) in isolates from children than isolates from adults. Also in study IV, isolates from children, especially small children (0–4 years), tended to be of more infrequently found types. This could indicate that adults and children, at least partly, get infected from different sources. Small children, who crawl and may ingest things from the ground, may get infected more often than adults with *C. jejuni* from non-food origins, for example pet animals and faeces from wild birds.

Colonisation with more than one genotype (Papers I & III)

Co-colonisation in chickens (Paper I)

In the broiler study, two-thirds of the birds sampled the day before slaughter were colonised by more than one *Sma*I type, although there was a clear tendency for the dominance of one type in individual birds. Thomas *et al.* (1997) found five *fla* genotypes among 300 isolates from a commercial broiler flock, and more than one genotype in 37% of the individual faecal samples. However, they could not

exclude cross-contamination due to sample collecting from the floor of the poultry shed. Also several different serotypes have been found in the same individual (Pokamunski *et al.*, 1986). In contrast, in a study where different *Campylobacter* strains were given to chickens then allowed to mix to enable cross-infection, co-colonisation with different strains of *C. jejuni* in individual birds was a very rare finding, while co-colonisation with *C. jejuni* and *C. coli* occurred (Korolik *et al.*, 1998).

Co-infection in humans (Paper III)

In study III, PFGE runs with different plugs prepared from different cultures of the same original isolate resulted, in two cases, in entirely disparate profiles with all enzymes used, although only in one of these cases could the different genotypes be confirmed and subsequently isolated. Recombination of the genome, either in the patient or in the laboratory, is not a probable explanation, as one single genetic event is not sufficient to explain the substantial differences in these cases. Hence, these patients were probably infected with two different strains, which were not separated during primary isolation. Co-infection with more than one *Campylobacter* species (Zöllner & Wuthe, 1993; Linton *et al.*, 1997; Gorkiewicz *et al.*, 2002), serotype (Zöllner & Wuthe, 1993) or genotype (Yan, Chang & Taylor, 1991; Steinbrueckner, Ruberg & Kist, 2001) has been reported, but has been found in less than 10% of the cases in studies directly addressing this issue (Ruberg, Steinbrueckner & Kist, 1998; Richardson *et al.*, 2001). It should be noted that the detection of co-infection with different genotypes in the present study was an accidental finding, as the routine isolation procedure did not include picking of more than one isolate per patient. Therefore, no conclusions about the frequency of co-infection in this population can be drawn.

Campylobacter species (Papers III & IV)

Species identification showed that all isolates in study III were *C. jejuni*, whereas three isolates (1.4%) in study IV were identified as *C. coli*. In other studies on sporadic human campylobacteriosis, *C. coli* constituted from 3% to 14% of the total number of *C. jejuni/coli* isolates (Goossens *et al.*, 1990; Nielsen *et al.*, 1997; Hudson *et al.*, 1999).

There is evidence that children are more often infected with *C. upsaliensis* than adults (Lindblom *et al.*, 1995), a *Campylobacter* species commonly isolated from healthy and diarrhoeic dogs and cats (Olson & Sandstedt, 1987; Sandberg *et al.*, 2002). Other *Campylobacter* species than *C. jejuni* and *C. coli* may be more common causes of gastroenteritis than hitherto recognised, especially in children (Lindblom *et al.*, 1995). The routine methods used for isolation are designed specifically for the detection of *C. jejuni* and *C. coli* (Lindblom *et al.*, 1995), although other thermotolerant campylobacters or even species not regarded as thermotolerant are occasionally recovered (Goossens *et al.*, 1990).

Use of a sequenced *C. jejuni* strain as a reference and molecular size marker (Papers II & IV)

*Sma*I and *Sal*I restricted genomic DNA from the sequenced strain *C. jejuni* NCTC 11168 was found to be a useful reference for the analysis of *C. jejuni* PFGE patterns. The reference pattern enabled fragment size estimates of lower variability than those from comparison with a DNA size standard based on lambda ladder (Paper II). The better performance of NCTC 11168, in terms of variability, was seen despite the fact that the lambda ladder showed a lower variability in migration distances within each gel. This indicates that the advantage may be due to the more sample-like migration patterns for NCTC 11168.

Calculations of *Sma*I fragment sizes for NCTC 11168 from comparison with lambda ladder resulted in systematic overestimation in the size range of 5 to 15 kbp. Hence, in addition to the lower variability, fragment size estimates based on NCTC 11168 may agree better with the actual fragment sizes.

Another advantage of using the NCTC 11168 pattern as a reference is related to its resemblance to PFGE patterns from other *C. jejuni* strains derived from humans. This was observed during the analysis in study IV, in which the reference patterns of CCUG 6824 (equivalent to NCTC 11168) were a convenient aid in deciding which bands were equal and unequal. The resulting resolution of the band matching procedure was considerably higher in study IV than in study III, in which only lambda ladder was used for intergel comparisons. In study IV, the *Sma*I band designation resulted in 58 distinct bands (with 54 distinct positions, considering double bands) with fragment sizes below 550 kbp (the largest fragments considered in study III), while 28 distinct *Sma*I bands were designated in study III. This difference is partly attributable to the larger number of isolates in study IV, and consequently the occurrence of more patterns containing infrequently found bands. However, the ability to discriminate between two unequal fragments close in size was definitely greater in study IV.

Restriction failure (Papers III & IV)

Although all isolates gave interpretable banding patterns with at least one enzyme, *Sma*I and *Sal*I repeatedly failed to cleave the DNA from a small fraction (1–9%) of the isolates. Cleavage failure of this degree (2–10%) has been reported in several PFGE studies, with different enzymes (Harrington, Thomson-Carter & Carter, 1999; Lindmark *et al.*, 2004). In most cases, isolates with indigestible genomes are classified as untypeable and are excluded from further analyses (Broman *et al.*, 2002). In study IV, isolates with genomes that were not restricted by *Sma*I apparently had a distinctive seasonal distribution, as a vast majority (16/19) of these isolates were concentrated to July, and no isolates at all were found from October to May. Even isolates that were refractory to *Sal*I restriction (although only six isolates in total) seemed mainly concentrated to a short season. With regard to the uneven seasonal distribution of isolates with restriction failure, it was considered implausible to exclude them from the analysis, and "empty" profiles were included and regarded as single-band-patterns. However, this may

have led to either over- or underestimation of their genetic similarities, with the Dice coefficient computed from relatively few bands (especially in "empty" *Sma*I profiles, whose corresponding *Sal*I profiles had only four or five bands in addition to a large band seen in most *Sal*I profiles). Similarly, the differences between "empty" profile isolates and isolates with banding patterns for both enzymes could possibly be overestimated. However, study IV types that clustered together in cluster III and cluster IV (with "empty" *Sma*I and *Sal*I profiles, respectively) also clustered together in total dendrograms based solely on the other enzyme (data not shown). In study III, isolates with "empty" *Sma*I or *Sal*I profiles were too few to reveal any possible disparity in distribution. However, the three isolates with zero-band *Sma*I patterns showed indistinguishable or closely similar (with a Dice coefficient above 90%) *Sal*I and *Ksp*I patterns, the latter enzyme pattern consisting of considerably more bands.

Concluding remarks

The research presented in this thesis shows that PFGE is a useful tool for acquiring insight in *Campylobacter* epidemiology and colonisation dynamics in humans and chickens.

The results show that multiple genotypes of *C. jejuni* may be present in a commercial broiler flock during rearing and even in the gastrointestinal tracts of individual birds. Subsequent addition of genotypes during rearing occurred, and indications of both recurring environmental exposure and genetic changes within the population were found. Although the distribution of genotypes varied between different sampling occasions, no evidence of competitive exclusion was found. The diversity and colonisation dynamics of campylobacters in broilers could be important in investigations of *Campylobacter* infection sources and routes in broilers as well as humans.

Considerable diversity, with many distinct PFGE types, was found in *Campylobacter* isolates from humans infected in Sweden. However, a large proportion of the isolates could be sorted into a few clusters, based on PFGE pattern similarities. Analysis of epidemiological information in relation to typing data revealed sets of type- and time-related isolates, possibly representing small outbreaks. The genotype diversity tended to be higher in isolates from children than in those from adults, which may indicate that adults and children, at least partly, become infected from different sources. Some clusters of similar genotypes were significantly more seasonally concentrated than other types. These genotypes may account for a substantial proportion of campylobacteriosis cases during the high-incidence season.

The sequenced strain *C. jejuni* NCTC 11168 was used as a reference and molecular size marker on PFGE gels. Fragment size estimates obtained from comparison with NCTC 11168 showed lower variability and were closer to the actual sizes than estimates from comparisons with a standardised size marker for PFGE. This facilitates the interpretation and analysis of PFGE patterns, and enables higher resolution of the method.

Future perspectives

PFGE has been shown to be a reproducible and convenient typing method with many applications. It is especially suitable for investigations of disease outbreaks in linking isolates from cases to suspected infection sources. In addition, as has been exemplified in the present studies, PFGE typing may help in further evaluating *Campylobacter* epidemiology in humans and animals, beyond the strict outbreak setting. However, to benefit as much as possible from PFGE typing results in more complex epidemiological issues, there is a need for standardisation of the method (both laboratory techniques and analysis and interpretation of the data). For example, a standardised nomenclature for describing PFGE types is needed. The use of a sequenced strain as a reference and/or molecular size marker may be one step in this direction. More reliable fragment size estimates may form the basis for a standardised definition of restriction patterns, avoiding dependence on internal standards (i.e. normalised gel positions) in specific software.

Studies of the seasonal distribution of genotypes in reservoirs and suspected sources of human infection may help to elucidate the hitherto insufficiently explained seasonality of campylobacteriosis in humans. PFGE, as well as other genotyping methods, such as MLST, may be suitable tools for such investigations. Association of certain summer-frequent genotypes with a specific reservoir or infection source may enable measures to be taken to reduce the number of summer infections, which constitute a large part of the total number of campylobacteriosis cases.

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