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RESEARCH

Ulla-Maija Nakari

Identification and Epidemiological Typing of *Campylobacter* Strains Isolated from Patients in Finland

61

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Identification and Epidemiological Typing of *Campylobacter* Strains Isolated from Patients in Finland

ACADEMIC DISSERTATION

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Bacteriology Unit, Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Helsinki, Finland

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To my family

Abstract

Ulla-Maija Nakari. Identification and Epidemiological Typing of *Campylobacter* Strains Isolated from Patients in Finland. National Institute for Health and Welfare (THL). Research 61. 126 pages. Helsinki, Finland 2011.
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C. jejuni constitutes the majority of *Campylobacter* strains isolated from patients in Finland, and *C. coli* strains are also reported. To improve the species identification, a combination of phenotype- and genotype-based methods was applied. Standardising the cell suspension turbidity in the hippurate hydrolysis test enabled the reliable identification of hippurate-positive *Campylobacter* strains as *C. jejuni*. The detection of species-specific genes by PCR showed that about 30% of the hippurate-negative strains were *C. jejuni*.

Three typing methods, serotyping, PCR-RFLP analysis of LOS biosynthesis genes and pulsed-field gel electrophoresis (PFGE) were evaluated as epidemiological typing tools for *C. jejuni*. The high number of non-typeable strains lowered the discriminatory ability of serotyping. PCR-RFLP typing offered high discrimination for both serotypeable and non-typeable strains, but the correlation between serotypes and RFLP-types was not high enough to enable its use for molecular serotyping of non-typeable strains. PFGE was a highly discriminative typing method. Although the use of two restriction enzymes generally increases the discriminatory ability, KpnI alone offered almost as high discrimination as the use of SmaI and KpnI.

The characteristic seasonal distribution of *Campylobacter* infections with a peak in summer and low incidence in winter was mainly due to domestically acquired infections. Of the *C. jejuni* strains, 41% were of domestic origin compared to only 17% of the *C. coli* strains. Serotypes Pen 12, Pen 6,7 and Pen 27 were significantly associated with domestic *C. jejuni* infections, Pen 1,44, Pen 3 and Pen 37 with travel-related infections. Pen 2 and Pen 4-complex were common both in domestic and travel-related infections. Serotype Pen 2 was less common among patients 60 years or older than in younger patients, more prevalent in Western Finland than in other parts of the country and more prevalent than other serotypes in winter. The source of Pen 2 infections may be related to cattle, since Pen 2 is the most common serotype in isolates from Finnish cattle.

PFGE subtypes among isolates from patients and chickens during the summer 2003 and from cattle during the whole year were compared. The analysis of indistinguishable SmaI/KpnI subtypes suggested that up to 31% of the human infections may have been mediated by chickens and 19% by cattle.

Human strains isolated during two one-year sampling periods were studied by PFGE. Of the domestic strains, 69% belonged to SmaI subtypes found during both sampling periods. Four SmaI subtypes accounted for 45% of the domestic strains, further typing of these subtypes by KpnI revealed six temporally persistent SmaI/KpnI subtypes. They were only occasionally identified in travel-related strains, and therefore, can be considered to be national subtypes. Each subtype was associated with a serotype: Pen 2, Pen 12, Pen 27, Pen 4-complex, Pen 41, and Pen 57. Five of these subtypes were identified in cattle (S5/K27, S7/K1, S7/K2, S7/K5 and S64/K19), and 2 in chickens (S7/K1 and S64/K19) with a temporal association with human infections in 2003. Cattle are more likely potential sources of these persistent subtypes, since long-term excretion of *Campylobacter* strains by cattle has been reported.

Keywords: *Campylobacter*, C. *jejuni*, C. *coli*, identification, typing, epidemiology

Tiivistelmä

Ulla-Maija Nakari. Identification and Epidemiological Typing of *Campylobacter* Strains Isolated from Patients in Finland [Potilaista eristettyjen kampylobakteerikantojen tunnistus ja epidemiologinen tyypitys]. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 61. 126 sivua. Helsinki, Finland 2011.
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Kampylobakteeri on yleisin suolistoinfektioita aiheuttava bakteeri. Suomessa valtakunnalliseen tartuntatautirekisteriin ilmoitetaan vuosittain 3 000–4 000 tartuntaa. Yleisin potilaista eristetty laji on *C. jejuni*. Myös *C. coli* -löydöksiä ilmoitetaan jonkin verran. Lajien tunnistusta parannettiin käyttämällä kampylobakteerin ilmiasuun (fenotyyppi) ja perimään (genotyyppi) kohdistuvien tyypitysmenetelmien yhdistelmää. Hippuraattitestissä käytetyn solususpension solumäärän standardointi mahdollisti hippuraattipositiivisten kantojen luotettavan tunnistuksen *C. jejuni* -lajiksi. Kun lajispesifisiä geenejä tunnistettiin PCR:n avulla, todettiin noin 30%:n hippuraattinegatiivisista kannoista olevan *C. jejuni* -lajia. Negatiivisen testituloksen perusteella ne oli kliinisen mikrobiologian laboratorioissa nimetty *C. coli* -lajiksi.

Työssä tutkittiin kolmen tyypitysmenetelmän, serotyypityksen, PCR-RFLP-analyysin ja pulssikenttäelektroforeesin (PFGE), soveltuvuutta *C. jejuni* -kantojen epidemiologiseen seurantaan. Serotyypitysmenetelmän erottelukykyä alensi tyypittymättömien kantojen suuri osuus. PCR-RFLP-tyypitys oli hyvin erotteleva sekä serotyypittyville että -tyypittymättömille kannoille. Serotyyppien ja RFLP-tyyppien vastaavuus ei kuitenkaan ollut riittävän suuri, jotta PCR-RFLP-menetelmää voisi käyttää serotyypittymättömien kantojen molekyylitasoiseen serotyypitykseen. PFGE oli hyvin erotteleva menetelmä. KpnI-entsyymillä saavutettu erottelukyky oli lähes yhtä hyvä kuin SmaI ja KpnI -entsyymien yhteensä.

Kampylobakteeri-infektioille tyypillinen vuodenaikaisjakauma, jossa tartuntamäärät ovat kesällä korkeita ja talvella matalia, johtui pääasiassa kotimaisten tartuntojen määrän vaihtelusta. *C. jejuni* -kannoista 41 % oli kotimaista alkuperää, *C. coli* -kannoista vain 17%. Serotyypit Pen 12, Pen 6,7 ja Pen 27 liittyivät tilastollisesti merkitsevästi kotimaisiin tartuntoihin, Pen 1,44, Pen 3 ja Pen 37 puolestaan ulkomaanmatkalla saatuihin tartuntoihin. Pen 2 ja Pen 4 -kompleksi olivat yleisiä serotyyppejä sekä kotimaisissa että ulkomaanmatkaan liittyvissä tartunnoissa. Serotyyppi Pen 2 oli harvinaisempi 60-vuotiailla ja sitä vanhemmilla potilailla kuin nuoremmilla ja se oli yleisempi Länsi-Suomessa kuin muualla maassa. Lisäksi se oli muita serotyyppejä yleisempi talvella. Pen 2 -tartuntojen lähde voi liittyä nautakarjaan, koska Pen 2 on yleisin serotyyppi suomalaisissa naudoissa.

Kesällä 2003 potilaista eristettyjen kantojen PFGE-genotyyppejä verrattiin samaan aikaan kanoista eristettyihin kantoihin ja koko vuonna naudoista eristettyihin kantoihin. Samanlaisten tyyppien esiintymisen perusteella 31 % väestön infektioista voisi liittyä kanoihin ja 19 % nautoihin.

Kolmen vuoden aikana potilaista eristetyistä kannoista ensimmäisen ja kolmannen keräysvuoden kantoja tyypitettiin PFGE-menetelmällä. Kotimaisista tartunnoista eristetyistä kannoista 69 % kuului sellaisiin SmaI-PFGE-tyyppeihin, joita esiintyi molempina vuosina. Neljä SmaI-tyyppiä kattoi 45% kotimaisista kannoista. Nämä tyypitettiin myös KpnI-entsyymillä, jolloin löydettiin kuusi molempina keräysvuosina yleistä SmaI/KpnI-tyyppiä. Nämä genotyypit esiintyivät vain satunnaisesti ulkomaisissa kannoissa, joten ne olivat pysyviä kotimaisia genotyyppejä ja liittyivät serotyyppeihin Pen 2, Pen 12, Pen 27, Pen 4 -complex, Pen 41 ja Pen 57. Samoja PFGE-tyyppejä esiintyi myös kanoissa, kahdella genotyypillä (S7/K1 ja S64/K19) oli ajallinen yhteys väestön infektioihin vuonna 2003. Viisi tyypeistä (S5/K27, S7/K1, S7/K2, S7/K5 ja S64/K19) esiintyi naudoissa. Naudat voivat erittää samaa kampylobakteerikantaa pitkäaikaisesti, joten nautakarja on todennäköisempi lähde pysyvien tyyppien aiheuttamille infektioille.

Avainsanat: kampylobakteeri, lajinmääritys, tyypitys, epidemiologia

Sammandrag

Ulla-Maija Nakari. Identification and Epidemiological Typing of *Campylobacter* Strains Isolated from Patients in Finland [Identifiering och epidemiologisk typning av campylobacterstammar isolerade från patienter]. Institutet för hälsa och välfärd (THL). Forskning 61. 126 sidor. Helsingfors, Finland 2011.
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Campylobacter är den vanligaste orsaken till bakteriell tarminfektion. Årligen rapporteras 3 000–4 000 fall till det riksomfattande registret över smittsamma sjukdomar i Finland. Den oftast isolerade arten är *C. jejuni*, men även *C. coli* rapporteras i viss utsträckning. Artidentifieringen förbättrades genom kombination av typningsmetoder gällande dels bakteriens egenskaper (fenotyp), dels dess arvsmassa (genotyp). Genom standardisering av mängden celler i suspensionen för hippurattest möjliggjordes pålitlig identifiering av de hippuratpositiva stammarna som *C. jejuni*. Vid PCR-bestämningen av artspecifika gener identifierades cirka 30 % av de hippuratnegativa stammarna som *C. jejuni*. På grund av det negativa hippuratresultatet hade dessa klassificerats som *C. coli* på laboratoriet för klinisk mikrobiologi.

I arbetet undersöktes lämpligheten hos tre typningsmetoder för epidemiologisk uppföljning av *C. jejuni*-stammar. Metoderna var serotypning, PCR-RFLP-analys och pulsfältselektrofores (PFGE). Serotypningens diskriminationsförmåga reducerades av den stora andelen otypningsbara stammar. PCR-RFLP-analysen hade hög diskriminationsförmåga ifråga om både de stammar som kunde och inte kunde identifieras genom serotypning. Korrelationen mellan serotyperna och RFLP-typerna var dock inte tillräckligt god för att möjliggöra användning av PCR-RFLP-metoden för serotypning på molekylär nivå av stammar som inte kan bestämmas genom serotypning. PFGE hade god diskriminationsförmåga. Med enzymet KpnI uppnåddes nästan samma diskrimination som med enzymerna SmaI och KpnI tillsammans.

Campylobacterinfektionernas typiska årstidsfördelning, det vill säga ett stort antal infektioner sommartid och ett litet antal under vintern, berodde huvudsakligen på variationer i antalet inhemska infektioner. Av *C. jejuni* -stammarna var 41 % av inhemskt ursprung medan motsvarande andel av *C. coli* -stammarna endast var 17 %. Serotyperna Pen 12, Pen 6,7 och Pen 27 var statistiskt signifikant bundna till inhemska infektioner, medan serotyperna Pen 1,44, Pen 3 och Pen 37 förekom vid infektioner som patienterna ådragit sig vid utlandsresor. Serotyperna Pen 2 och Pen 4-komplexet var vanliga vid infektioner av både inhemskt och utländskt ursprung. Serotypen Pen 2 var ovanligare hos personer över 60 år än hos yngre patienter och vanligare i västra Finland än i övriga delar av landet. Serotypen var

därtill vanligare än de övriga serotyperna under vintern. Eftersom Pen 2 är den vanligaste serotypen hos finländsk nötboskap kan nötdjuren utgöra en smittkälla.

PFGE-genotypen hos bakteriestammar som isolerats från patienter under sommaren 2003 jämfördes med genotypen hos stammar som isolerats från höns under samma period och hos stammar som isolerats från nötdjur under hela året. Förekomsten av samma genotyper kan innebära att 31 % av de humana infektionerna var hönsrelaterade och 19 % relaterade till nötdjur. 69 % av stammarna från patienter med inhemsk infektion utgjordes av sådana SmaI-PFGE-typer som uppträdde under både det första och det tredje insamlingsåret. 45 % av de inhemska stammarna utgjordes av fyra SmaI-typer. Vid typning av dessa stammar med enzymet KpnI identifierades sex SmaI/KpnI-typer som var vanliga under vartdera insamlingsåret. Dessa genotyper uppträdde endast sporadiskt i de utländska stammarna, vilket innebär att de är inhemska genotyper förbundna med serotyperna Pen 2, Pen 12, Pen 27, Pen 4-complex, Pen 41 och Pen 57. Samma PFGE-typer uppträdde även hos höns. Två av genotyperna (S7/K1 och S64/K19) korrelerade tidsmässigt med de humana infektionerna år 2003. Fem av genotyperna (S5/K27, S7/K1, S7/K2, S7/K5 och S64/K19) uppträdde hos nötdjur. Nötkreatur kan utsöndra samma campylobacterstam under lång tid, vilket innebär att nötdjuren är en mer sannolik källa för infektioner med varaktiga genotyper.

Nyckelord: campylobacter, artbestämning, typning, epidemiologi

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List of original papers

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I-V). In addition, some unpublished data are presented.

I Nakari U-M, Puhakka A, Siitonen A. 2008. Correct identification and discrimination between *Campylobacter jejuni* and *C. coli* by a standardized hippurate test and species-specific polymerase chain reaction. *Eur J Clin Microbiol Infect Dis* 27(7):513-8.

II Nakari U-M, Laaksonen K, Korkeila M, Siitonen A. 2005. Comparative typing of *Campylobacter jejuni* by heat-stable serotyping and PCR-based restriction fragment length polymorphism analysis. *J Clin Microbiol* 43(3):1166-70.

III Nakari U-M, Huovinen E, Kuusi M, Siitonen A. 2010. Population-based surveillance study of *Campylobacter* infections in Finland. *Epidemiol Infect* 138(12):1712-8.

IV Hakkinen M, Nakari U-M, Siitonen A. 2009. Chickens and cattle as sources of sporadic domestically acquired *Campylobacter jejuni* infections in Finland. *Appl Environ Microbiol* 75(16):5244-9.

V Nakari U-M, Hakkinen M, Siitonen A. Identification of persistent subtypes of *Campylobacter jejuni* by pulsed-field gel electrophoresis in Finland. *Foodborne Pathog Dis*. Accepted.

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The author's contribution

Study I

Ulla-Maija Nakari selected the PCR methods, and did or supervised the typing of the strains. She planned and carried out the standardisation of the hippurate test. She analysed the results and was mainly responsible for writing the paper.

Study II

Ulla-Maija Nakari coordinated the setting up of the PCR-RFLP method and did or supervised the typing of the strains. She was mainly responsible for interpreting the results and writing the paper.

Study III

Ulla-Maija Nakari analysed the serotype data and participated in analysing the temporal and demographical data from the infectious disease register. She was mainly responsible for writing the paper.

Study IV

Ulla-Maija Nakari participated in planning the experimental design, and was responsible for the typing and analysis of the strains isolated from humans. She participated in writing these parts of the paper.

Study V

Ulla-Maija Nakari planned the study and performed the majority of the genotyping. She was mainly responsible for the analysis and for writing the paper.

Abbreviations

AFLP	Amplified fragment length polymorphism
ATCC	American type culture collection
BSA	Bovine serum albumin
CCDA	Charcoal cefoperazone deoxycholate agar
DI	Discrimination index
ECDC	European centre for disease control
EFSA	European food safety authority
HS	Heat stable
KTL	Kansanterveyslaitos, National public health institute; name changed 1st Jan 2009 to National institute for health and welfare (Terveyden ja hyvinvoinnin laitos, THL)
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable-number tandem repeat
NCTC	National collection of type cultures
NIDR	National infectious disease register
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism

1 Introduction

Campylobacter species are the most common bacterial causes of gastroenteritis in most developed countries (ECDC 2009, Friedman *et al*. 2000). In Finland, 3000-4000 cases are reported annually. The symptoms of a *Campylobacter* infection include fever, abdominal cramping, and diarrhea, and last from several days to more than a week (Fitzgerald and Nachamkin 2007). Of the 17 validly named species in the genus *Campylobacter*, *C. jejuni* ssp. *jejuni*, *C. coli*, *C. fetus* ssp. *fetus*, *C. upsaliensis*, *C. lari*, and *C. hyointestinalis* ssp. *hyointestinalis* are recognised causes of intestinal infections in humans (Fitzgerald and Nachamkin 2007, Lastovica and Allos 2008). *C. jejuni* is the most frequently reported *Campylobacter* species (80-90%) followed by *C. coli* (5-10%) (Fitzgerald *et al*. 2008).

Most clinical microbiology laboratories use basic biochemical tests for differentiation of *Campylobacter* species. The discrimination between two species often relies on a single biochemical test. Hippurate hydrolysis is the only phenotypic test differentiating *C. jejuni* and *C. coli*. Both false-positive and false-negative results have been reported (Denis *et al*. 1999, Wainø *et al*. 2003). A number of PCR applications, based on the detection of different species-specific genes, have been developed for species identification. However, the high number of *Campylobacter* cases limits the routine use of genotype-based identification methods. One aim of this study was to improve the species identification of C*ampylobacter* isolates in clinical microbiology laboratories. Correct species identification is important for surveillance, epidemiological investigations and risk assessment studies.

Campylobacter species are primarily zoonotic pathogens that are frequently isolated from a variety of animal species: poultry, cattle, pigs, sheep, pets, wild birds and rodents (Broman *et al*. 2002, Jacobs-Reitsma 2000, Meerburg *et al*. 2006, Modolo and Giuffrida 2004, Sandberg *et al*. 2002, Waldenström *et al*. 2002). Consumption of poultry or red meat is the most frequently indicated risk factor in case-control studies (Doorduyn *et al*. 2010, Neimann *et al*. 2003). In most European countries, the majority of *Campylobacter* infections are domestically acquired. Higher proportions of travel-associated cases (51-70%) are reported in Finland, Sweden and Norway (EFSA 2009, EFSA 2010a). *Campylobacter* infections show a characteristic seasonal distribution with a peak in summer (ECDC 2009, Nylen *et al*. 2002). The highest incidence is detected in children younger than 5 years of age, followed by a second peak in young adults 20-40 years of age (ECDC 2009, Nylen *et al*. 2002, Samuel *et al*. 2004).

Epidemiological typing methods enable the characterisation and discrimination of bacterial strains. The data is used for public health surveillance, disease cluster identification and outbreak investigations. Due to the large number of *Campylobacter* cases, routine subtyping of all strains isolated from patients is not feasible. However, even typing results from a limited collection of strains can be used as a baseline against which possible infection clusters can be compared. In addition, the contribution of different sources to the human infections can be studied by comparing subtypes of isolates from humans and possible infection sources. In this study, *Campylobacter* strains isolated from patients were typed by different epidemiological typing methods to see if the seasonal and demographical characteristics of *Campylobacter* infections would be associated with specific subtypes. Isolates from chickens and cattle were compared with human isolates to investigate the contribution of domestic animals to human illness.

2 Review of the Literature

2.1 Taxonomy and general characteristics of *Campylobacter* species

Campylobacter species belong to the epsilon subdivision of proteobacteria (Debruyne *et al*. 2008). Three closely related genera, *Campylobacter*, *Arcobacter* and *Sulfospirillum*, are included in the family *Campylobacteraceae* (On 2001, Vandamme 2000). *Campylobacter* species are curved, S-shaped or spiral rods that are 0.2-0.9µm wide and 0.5-5µm long. In old cultures or when exposed to air for prolonged time periods, *Campylobacter* transform from spiral form to coccoid morphology (Griffiths 1993). *Campylobacter* species are gram-negative, non-spore-forming rods, usually motile by means of a single polar unsheathed flagellum at one or both ends, but may also lack flagella. The species are oxidase positive, except for *C. gracilis* (Debruyne *et al*. 2008, Vandamme and De Ley 1991). They have a respiratory type of metabolism and are generally microaerophilic, requiring oxygen (3-10%) for growth but are unable to grow at normal atmospheric oxygen tensions. *C. concisus*, *C. rectus*, *C. curvus*, *C. showae*, *C. gracilis* and *C. mucosalis* require an increased hydrogen concentration (6%) for microaerobic growth. Also, some strains of *C. jejuni* ssp. *doyley*, *C. upsaliensis*, *C. lari* and *C. hyointestinalis* grow better in hydrogen-enhanced microaerobic conditions (Lastovica and Allos 2008).

Currently there are 17 validly named species in the genus *Campylobacter* (Fitzgerald and Nachamkin 2007, Lastovica and Allos 2008). *C. jejuni* ssp. *jejuni*, *C. coli*, *C. fetus* ssp. *fetus*, *C. upsaliensis*, *C. lari* and *C. hyointestinalis* ssp. *hyointestinalis* are recognised causes of intestinal infections in humans. Furthermore, *C. jejuni* ssp. *doyley* (Fernandez *et al*. 1997), *C. sputorum* biovar paraureolyticus (On *et al*. 1998a), *C. curvus* (Abbott *et al*. 2005), *C. concisus* (Engberg *et al*. 2000) and *C. insulaenigrae* (Chua *et al*. 2007) have been associated with intestinal infections, but their pathogenic role is not clearly determined. *C. rectus*, *C. concisus*, *C. curvus*, *C. showae* and *C. gracilis* are mainly considered to be causes of oral or dental infections in humans (Etoh *et al*. 1993, Han *et al*. 2005, Macuch and Tanner 2000). *C. helveticus*, *C. mucosalis*, *C. hominis* and *C. lanienae* have not been associated with human illness (Chaban *et al*. 2010, Inglis *et al*. 2005, Lawson *et al*. 2001, Stanley *et al*. 1992). In addition, several new species have been recently proposed: *Campylobacter canadensis* sp. nov. (Inglis *et al*. 2007), *Campylobacter volucris* sp. nov. (Debruyne *et al*. 2010a) and *Campylobacter subantarcticus* sp. nov. (Debruyne *et al*. 2010b) isolated from birds, *Campylobacter peloridis* sp. nov. isolated from humans and molluscs (Debruyne *et al*. 2009), *Campylobacter*

cuniculorum sp. nov. isolated from rabbits (Zanoni *et al*. 2009), *Campylobacter avium* sp. nov. isolated from poultry (Rossi *et al*. 2009), *Campylobacter troglodytis* (Kaur *et al*. 2011) isolated from chimpanzees, and the reclassification of *Bacteroides ureolyticus* as *Campylobacter ureolyticus* comb. nov. (Vandamme *et al*. 2010).

Campylobacter species are primarily zoonotic, with a variety of animals implicated as reservoirs for human infection. Humans appear to be the only recognised reservoirs for the periodontal pathogens *C. concisus*, *C. rectus*, *C. curvus* and *C. showae* (Lastovica and Allos 2008).

2.2 Isolation of *Campylobacter* species from faecal samples

In intestinal infections potentially caused by *Campylobacter*, a definitive diagnosis can only be made by detecting the bacteria in the faeces of the patient. However, there is no single gold standard for the routine isolation of all *Campylobacter* species. The isolation techniques currently used in many diagnostic laboratories, including incubation at 42ºC and cephalothin-containing media, may not support the growth of species other than *C. jejuni* and *C. coli* (Lastovica and Skirrow 2000). Only some strains of *C. fetus* ssp. *fetus* and *C. hyointestinalis* grow at 42ºC (Table 1). *C. upsaliensis* is susceptible to many antimicrobial agents but the commonly used blood free selective agar (the modified charcoal cefoperazone deoxycholate agar (CCDA) with 32ng cefoperazone per liter) and incubation at 42°C has been successfully used for the isolation of *C. upsaliensis* (Hald and Madsen 1997). A wide variety of *Campylobacter* species were isolated from human stool specimens without the use of selective media by filtration of stools through 0.65µm filters onto the surface of antibiotic-free blood-agar plates and incubation in hydrogen-enhanced microaerobic atmosphere at 37ºC (Lastovica 2006). In Finland, clinical microbiology laboratories search for *Campylobacter* in faecal samples of patients with acute diarrhea that is suspected to be caused by a bacterial pathogen. The faecal samples are cultivated on several selective media to identify *Salmonella*, *Shigella*, *Campylobacter* and *Yersinia*.

2.3 Species identification of *Campylobacter* strains

Correct identification provides important information on the prevalence of different species, which can be utilised in epidemiological investigations and risk assessment studies. Both phenotypic and genotypic methods are used.

2.3.1 Phenotypic methods

Most clinical microbiology laboratories use basic biochemical tests for differentiating *Campylobacter* species. The species identification is complicated because *Campylobacter* species are biochemically inert and the discrimination between

two species may rely on a single biochemical test (Table 1). Moreover, biochemical variants of well-known species have been identified, including the urease-positive nalidixic-acid susceptible subgroup of *C. lari* (Vandamme 2000).

Hippurate hydrolysis is the only phenotypic test differentiating *C. jejuni* and *C. coli*. The rapid tube hippurate test was first described by Hwang and Ederer (Hwang and Ederer 1975) and modified for use with *Campylobacter* by Harvey (Harvey 1980). Most *C. jejuni* strains hydrolyse hippurate to glycine and benzoic acid while other *Campylobacter* species lack this trait (Totten *et al*. 1987). Nevertheless, hippurate-negative *C. jejuni* strains have been reported in several studies (Cacho *et al*. 1989, Denis *et al*. 1999, Kos *et al*. 2006, Steinhauserova *et al*. 2001, Totten *et al*. 1987, Wainø *et al*. 2003). False-positive and variable hippurate test results have also been reported (Cacho *et al*. 1989, Denis *et al*. 1999, Wainø *et al*. 2003).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry has been shown to offer 100% correct identification for most *Campylobacter* species, and multiple species in mixed cultures can be identified more easily than by conventional methods (Mandrell *et al*. 2005, Martiny *et al*. 2010).

Api Campy (bioMérieux, Marcy l'Etoile, France) is a miniaturized identification system that uses 11 enzymatic and conventional tests, and 9 assimilation and inhibition tests. Api Campy offered no advantages when compared with conventional typing methods (Huysmans *et al*. 1995). In another study (Martiny *et al*. 2010), it correctly identified 94% of *C. jejuni* ssp. *jejuni* strains, and 100% of *C. upsaliensis* and *C. jejuni* ssp. *doyley* strains (Martiny *et al*. 2010). Of *C. coli* strains, 74% were correctly identified and 5% were misidentified as other species. The level of identification was lower for the other *Campylobacter* species: 60% for *C. lari*, 33% for *C. hyointestinalis* and 29% for *C. fetus*, but no strains were misidentified (Martiny *et al*. 2010).

VITEK 2 (bioMérieux, Marcy l'Etoile, France) is an automated identification system for diverse bacterial and fungal species. The Neisseria-Haemophilus card for the identification of *Neisseria*, *Haemophilus*, and other fastidious gram-negative or gram-variable microorganisms has been used for identification of *Campylobacter* species. This system correctly identified 90-94% of *C. jejuni* ssp. *jejuni* strains, while 6-10% were misidentified (Rennie *et al*. 2008, Martiny *et al*. 2010). Of *C. coli* strains, 75-88% were correctly identified and 8-25% misidentified (Rennie *et al*. 2008, Martiny *et al*. 2010). *C. usaliensis*, *C. fetus*, *C. hyointestinalis* and *C. jejuni* ssp. *doyley* were either misidentified or not identified in one study (Martiny *et al*. 2010), while another study reported 100% correct identification for *C. fetus*

(Rennie *et al*. 2008). Hippurate hydrolysis test is often required as an additional test for discriminating *C. jejuni* and *C. coli* (Valenza *et al*. 2007).

Of the other commercial identification systems, the Phoenix Automated Microbiology system (Becton Dickinson, Franklin Lakes, New Jersey, USA) seem to have lower identification rate for *Campylobacter* (Saffert *et al*. 2011). Thermophilic *Campylobacter* species are not included in the identification range of biochemical identification systems RapID Remel (Remel, Lexena, Kansas, USA) or BBL Crystal (Becton Dickinson). Several latex agglutination tests are available for the detection of *Campylobacter* species. They use polyclonal antibodies to detect antigenic outer membrane proteins or antigenic epitopes from flagella. The sensitivities and specificities of the tests vary (Miller et al. 2008), but they do not differentiate between *Campylobacter* species.

Table 1. Biochemical characteristics and test results used for identification of *Campylobacter* isolates from patients. Data from references Fitzgerald and Nachamkin 2007, Lastovica and Allos 2008.

Species	hippurate	catalase	indoxyl acetate	H2 required	urease	aryl sulfatase	selenite	nitrate	growth at 25ºC	growth at 42ºC
C. jejuni subsp. *jejuni*	+	+	+	-	-	V	V	+	-	+
C. jejuni subsp. *doyley*	V	V	+	-	-	-	-	-	-	-
C. coli	-	+	+	-	-	-	+	+	-	+
C. fetus subsp. *fetus*	-	+	-	-	-	-	V	+	+	V
C. upsaliensis	-	-	+	-	-	-	+	+	-	V
C. lari	-	+	-	-	V	-	V	+	V	+
C. hyointestinalis subsp. *hyointestinalis*	-	+	-	V	+	-	+	+	V	+
C. sputorum bv. sputorum	-	-	-	+	-	+	V	+	-	V
C. rectus	-	V	+	+	-	+	+	+	-	V

2.3.2 Genotypic methods

Genotype-based species identification methods have been developed to avoid the problems caused by the biochemical inertness of *Campylobacter* species. A number of PCR strategies, both conventional and quantitative, based on a variety of species-specific genes have been used in species identification. They include *cadF* (Cunningham *et al*. 2010), *hipO* (Persson and Olsen 2005, Wang *et al*. 2002), *mapA* (Best *et al*. 2003), *ceuE* (Houng *et al*. 2001), 23S rDNA (Eyers *et al*. 1993), *ipxA* (Klena *et al*. 2004) and *cdtA* (Asakura *et al*. 2008) for *C. jejuni*; *cadF* (Cunningham *et al*. 2010), *aspA* (Linton *et al*. 1997), *ceuE* (Gonzalez *et al*. 1997, Houng *et al*. 2001), *glyA* (Wang *et al*. 2002), 23S rDNA (Eyers *et al*. 1993), *ipxA* (Klena *et al*. 2004) and *cdtB* (Asakura *et al*. 2008) for *C. coli*; *glyA* (Wang *et al*. 2002) and *ipxA* (Klena *et al*. 2004) for C. lari; *glyA* (Wang *et al*. 2002) and *ipxA*

(Klena *et al*. 2004) for *C. upsaliensis*, and 16S rDNA (Oyarzabal *et al*. 1997), *sapB2* (Wang *et al*. 2002) and *cdtC* (Asakura *et al*. 2008) for *C. fetus*. Moreover, subspecies can be differentiated by PCR: *C. jejuni* ssp. *jejuni* from ssp. *doyley* (Miller *et al*. 2007), and *C. fetus* ssp. *fetus* from ssp. *venerealis* (Cunningham *et al*. 2010). In addition to species identification of *Campylobacter* strains isolated from humans or animals, many of the PCR methods have been used in direct detection and sometimes quantification of *Campylobacter* species in human, animal, food or environmental samples.

Although any of the PCR assays can be sensitive and specific when used on a limited number of strains, they may not work as well with a different set of strains. Assays for *C. jejuni* have shown variation in sensitivity and specificity probably due to the high heterogeneity of the species (On and Jordan 2003), implicating that a polyphasic strategy should be used to identify *C. jejuni* and *C. coli*.

Partial 16S rDNA sequencing is widely used for species identification of many enteric bacteria. For *Campylobacter*, the method is less useful because of the lack of discrimination between *Campylobacter jejuni*, *C. coli* and some *C. lari* strains, although it enables specific identification of most *Campylobacter* species (Gorkiewicz *et al*. 2003). Sequencing a housekeeping gene *groEL*/*cpn60* has been used successfully in identifying *Campylobacter* species (Hill *et al*. 2006, Kärenlampi *et al*. 2004).

2.4 Clinical features of *Campylobacter* infections in humans

The infective dose of *C. jejuni* has not been clearly defined but can be as low as 500-1000 cells (Black *et al*. 1988). *Campylobacter* enteritis is an acute diarrheal disease with clinical manifestations like those of other acute bacterial intestinal infections. The mean incubation period is 3.2 days but can vary from 18 hours to 8 days. Symptoms may include fever, abdominal cramping, and diarrhea (with or without blood or faecal white blood cells) that last from several days to more than a week (Fitzgerald and Nachamkin 2007). The symptoms can be severe, mild or even nonexistent. A symptomatic infection is usually self-limited but relapses may occur in 5-10% of untreated patients. The clinical symptoms of intestinal infections caused by *C. coli*, *C. upsaliensis*, *C. hyointestinalis*, *C. lari* and *C. fetus* ssp. *fetus* are similar to *C. jejuni* (Edmonds *et al*. 1987, Lastovica 2006, Patton *et al*. 1989, Rennie *et al*. 1994). In general, bacteremia is seldom reported (0.2-0.5 per 100 000 inhabitants/year) (Fernandez-Cruz *et al*. 2010, Schonheyder *et al*. 1995) but is much more common in infections caused by *C. fetus*, especially in elderly and compromised patients (Monno *et al*. 2004, Tremblay *et al*. 2003). Antibodies appear in the serum from about the fifth day of illness, peak within 2-4 weeks, and decrease over several months (Black *et al*. 1988, Strid *et al*. 2001). Late onset

complications include reactive arthritis and Guillain-Barré syndrome. The incidence of reactive arthritis after *Campylobacter* infection has been reported to be 1-5% (Pope *et al*. 2007). In Finland, 2.6% of patients developed reactive arthritis following an outbreak of *C. jejuni* (Hannu *et al*. 2004). *C. jejuni* infection precedes Guillain-Barré syndrome in 20-50% in Europe, North and South America, Japan and Australia (Jacobs *et al*. 2008). Cases of postinfectious irritable bowel syndrome have also been reported (Spiller 2007).

Generally, *Campylobacter* infections are self-limiting and antimicrobial treatment is not required. However, severe, prolonged or systemic infections do require treatment. Erythromycin and fluoroquinolones are the first- and second-choice drugs. The rate of resistance to fluoroquinolones varies substantially between countries, from 2% among domestically acquired infections in Australia (Unicomb *et al*. 2006) to 80% among strains isolated in Thailand (Boonmar *et al*. 2005). In Finland, the rate of fluoroquinolone resistance increased between 1995 and 2000 from 40% to 60% among travel-related infections (Hakanen *et al*. 2003). The resistance to macrolides has been low (1-2%) in Finland but the macrolide-resistant *Campylobacter* strains identified have been multidrug resistant (Hakanen *et al*. 2003, Lehtopolku *et al*. 2010).

2.5 Virulence factors and pathogenesis

The mechanism of pathogenesis of *Campylobacter* includes adhesion to intestinal cells, colonisation of the digestive tract, and invasion (Hu and Kopecko 2008). The ability to enter and survive within nonphagocytic cells is thought to be very important for pathogenesis. Chemotaxis and motility enabled by flagella probably have important roles in both the commensal and pathogenic lifestyles of *C. jejuni* (Hendrixson and DiRita 2003, Hugdahl *et al*. 1988). Flagella may also have a role in adhesion (Hu and Kopecko 2000). Several proteins have been implicated to have a role in the various steps of the pathogenesis process, including outer membrane protein CadF (Krause-Gruszczynska *et al*. 2007b), surface-exposed lipoprotein JlpA (Jin *et al*. 2001), secreted protein CiaB (Konkel *et al*. 1999), cytolethal distending toxin (Hu and Kopecko 2000), Rho-family GTPases (Krause-Gruszczynska *et al*. 2007a), and regulatory proteins (Kamal *et al*. 2007).

C. jejuni cells produce a polysaccharide capsule (Karlyshev *et al*. 2001, Parkhill *et al*. 2000) that is important for the adhesion and invasion of epithelial cells, and for serum resistance (Bachtiar *et al.* 2007, Bacon *et al*. 2001). Unlike most other gram-negative enteric pathogens, *C. jejuni* does not express lipopolysaccharide (LPS) but produces lighter-weight lipo-oligosaccharide (LOS). LOS differs from LPS by lacking an O-polysaccharide chain and has greater structural diversity in the outer core. Mutations in LOS biosynthesis genes affect serum resistance, ad-

herence and invasion (Fry *et al*. 2000). The *wla* gene cluster contains genes coding for the biosynthesis of LOS molecules and genes for regulating protein glycosylation (Fry *et al*. 1998, Fry *et al*. 2000, Szymanski *et al*. 1999). The LOS gene loci from multiple *C. jejuni* strains have been sequenced and grouped into 19 different LOS classes based on the gene content (Gilbert *et al*. 2008).

2.6 Occurrence and epidemiology

Campylobacter species are the most common bacterial causes of gastroenteritis in many developed countries. The overall incidence in Europe was 45.2/100 000 in 2007 and 40.7/100 000 in 2008 (EFSA 2009, EFSA 2010a). The reported incidences of culture-confirmed infections vary considerably between countries. Direct comparisons are difficult to make, however, since the culturing practices and reporting requirements vary. In Finland, the incidence of *Campylobacter* infections has been over 60/100 000 since 1999, and around 3000-4000 campylobacteriosis cases have been reported annually to the National Infectious Disease Register (NIDR) (Figure 1). In Europe, the majority of *Campylobacter* infections are domestically acquired. In Spain, Malta, Slovakia, Czech Republic and Hungary, 99-100% of reported cases are of domestic origin. In Finland, Sweden and Norway higher proportions (51-70%) of imported cases are reported (EFSA 2009, EFSA 2010a).

Campylobacter infections show a characteristic seasonality with the highest numbers of cases reported in the summer, from June to September (ECDC 2009, Nylen *et al*. 2002, THL 2010). Majority of these cases are sporadic whereas outbreaks usually occur in the spring and fall months. The difference between seasonality of sporadic cases and outbreaks imply that there might be different ecological events driving them (Olson *et al*. 2008). In Finland, the number of cases peak in July and August (Figure 1). Since 1998 there have been six large *C. jejuni* -related outbreaks in Finland, with more than one hundred to a thousand affected patients (KTL 2001, KTL 2005, KTL 2007). Each of the outbreaks was suspected or proved to be related to a municipal water supply. Three of them occurred in June-August, and three in October-November. In Finland, only minor food-related outbreaks with 3 to 15 patients have been detected. The vehicles or suspected vehicles were turkey, milk bought directly from a farm, chicken prepared with sour cream, fresh strawberries and chicken fillets (THL 2010).

The incidence of *Campylobacter* infections follows a bimodal age distribution. The highest incidence is detected in children younger than 5 years of age, followed by a second peak in young adults 20-40 years of age (ECDC 2009, Nylen *et al*. 2002, Samuel *et al*. 2004). In developing countries the incidence is much

higher than in industrialised countries, most symptomatic infections occur in early childhood and the incidence decreases with age (Oberhelman and Taylor 2000).

Overall, 80-90% of *Campylobacter* infections in industrialised countries are thought to be due to *C. jejuni* and 5-10% to *C. coli* when the diagnosis is performed solely on selective media (Fitzgerald *et al*. 2008). In Europe in recent years, *C. jejuni* has been the most frequently reported *Campylobacter* species accounting for 40-44% of the isolates, while *C. coli* accounted for 2-3 %, and other *Campylobacter* species for 7-9% (EFSA 2009, EFSA 2010a). Of the isolates, 46-49% were not identified to the species level (EFSA 2009, EFSA 2010a).

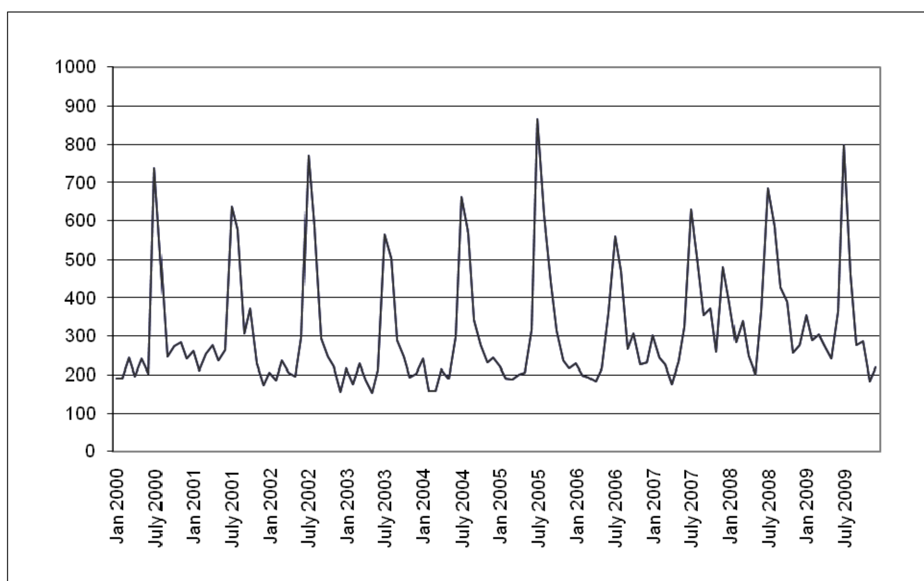

Figure 1. Number of campylobacteriosis cases reported to the NIDR by month during 2000-2009. Data from www3-ktl.fi/stat.

2.7 Sources and risk factors

Campylobacter species are widespread in nature. They colonise the intestinal tracts of a variety of wild and domesticated birds and mammals. They are prevalent in food animals such as poultry, cattle, pigs and sheep; in pets, and in wild birds and rodents (Broman *et al*. 2002, Jacobs-Reitsma 2000, Meerburg *et al*. 2006, Modolo and Giuffrida 2004, Sandberg *et al*. 2002, Waldenström *et al*. 2002).

In a recent study in the EU, the prevalence of *Campylobacter*-colonised broiler batches was 71% and the prevalence of *Campylobacter*-contaminated broiler carcasses 76% (EFSA 2010b). About two-thirds of the *Campylobacter* isolates from

the broiler batches and broiler carcasses were identified as *C. jejuni*, and one third as *C. coli*. In Europe, the occurrence of *Campylobacter* in fresh broiler meat was 25-30% in 2006-2008 (EFSA 2010a). In Finland, *C. jejuni* was found in 2.9% of broiler batches and 5.5% of broiler carcasses, but *C. coli* was not found. (EFSA 2010b). In Western Finland, 13% of Finnish poultry meat were positive for *Campylobacter* (Lyhs *et al*. 2010). Because of the high prevalence in broilers, poultry is regarded as the main source of *Campylobacter* infections in many countries. In Finland, the prevalence of *Campylobacter* in broiler flocks and retail broiler meat is low compared to other European countries. Thus, other infection sources may be more important. In Finnish cattle, the prevalence of *C. jejuni* was 19.5% in faecal samples and in 3.1% of carcass surface samples in 2003 (Hakkinen *et al*. 2007).

Most campylobacteriosis cases in humans are sporadic, which complicates the identification of infection sources. Case-control studies have identified a number of risk factors (Table 2), and consumption of poultry or red meat are indicated in many studies. The comparison of genotypes between isolates from humans and possible infection sources has been combined with mathematical models to estimate the attribution of different sources to human illness. Chicken and cattle seem to have the strongest association with the human cases (Table 2).

Table 2. Risk factors for *Campylobacter* infections identified in case-control studies (A); and attribution of different infection sources to human illness, studied by MLST typing of the isolates combined with different mathematical models (B).

Country	**A) Identified risk factors**	**Reference**
Denmark	consumption of undercooked poultry, consumption of red meat at barbecue, drinking unpasteurised milk, consumption of grapes, foreign travel	Neimann *et al*. 2003
Australia	consumption of undercooked chicken and offal, ownership of domestic chickens and dogs (aged <6 months)	Stafford *et al*. 2007
Finland	eating undercooked meat, swimming in natural sources of water, drinking water from a dug-well	Schönberg-Norio *et al*. 2004
The Netherlands	consumption of undercooked meat and barbecued meat, ownership of cats, use of proton pump inhibitors (*C. jejuni* and *C. coli*), consumption of chicken (*C. jejuni*), consumption of game and tripe, swimming (*C. coli*)	Doorduyn *et al*. 2010
Country	**B) Attribution of different sources to human cases**	**Reference**
Scotland	*C. jejuni*: chicken (58-78%), ruminants (18-23%), wild birds and environment (4%) *C. coli*: chicken (40-56%), sheep (40%), cattle (2-14%), pigs (<1-6%), turkey <1%	Sheppard *et al*. 2009
New Zealand	poultry (58-76%), bovine (11-18%), environment (1-12%), ovine (3-11%)	Mullner *et al*. 2009
England	chicken 57%, cattle 35%, sheep 4%, pigs 1%, wild animals 2%, environment 1%	Wilson *et al*. 2008

2.8 Epidemiological typing

Multiple methods are available for epidemiological typing of *Campylobacter* strains. They can be utilised in source tracking, and in determining the distribution of subtypes isolated from patients. The typing systems vary in complexities and abilities to discriminate between strains.

2.8.1 Phenotypic typing methods

The most widely accepted and well-evaluated phenotypic method to type *C. jejuni* strains is the Penner serotyping technique. It is based on passive haemagglutina-

tion, and differentiates the strains on the basis of soluble heat-stable (HS) antigens (Penner and Hennessy 1980). The antigenic material is extracted by heat or by chemical treatment and absorbed onto sheep or chicken erythrocytes. The sensitised erythrocytes are tested with antisera in microtitration plates with U-shaped wells. The plates are read by examining the wells for agglutination of erythrocytes. This method can detect 48 serotypes for *C. jejuni* and 17 serotypes for *C. coli* (Newell *et al*. 2000). Initially the method was thought to detect LPS antigenic determinants (Mandatori and Penner 1989, Mills *et al*. 1985, Moran and Kosunen 1989, Moran and Penner 1999, Perez and Blaser 1985, Perez *et al*. 1985, Preston and Penner 1987), but later, capsular polysaccharides have been shown to be the major serodeterminants (Karlyshev *et al*. 2000, Karlyshev *et al*. 2005, Wren *et al*. 2001).

High levels of non-typeability of human isolates with the Penner serotyping technique have been reported from some countries, but in general, the non-typeability has been less than 20% (Newell *et al*. 2000). A modification of the Penner serotyping, introducing absorbed antisera and using direct whole-cell agglutination of heated bacterial suspensions has been developed (Frost *et al*. 1998).

The Lior serotyping scheme is based on a slide agglutination procedure using live bacteria together with unabsorbed and absorbed antisera. This procedure is used for the detection of heat-labile antigens and can detect over 100 serotypes of *C. jejuni*, *C. coli* and *C. lari* (Lior *et al*. 1982). Uncharacterised bacterial surface antigens and flagellar antigens in some serotypes are the serodeterminants for this typing system (Alm *et al*. 1991).

In addition, biotyping (Lior 1984), phage typing (Frost *et al*. 1999, Khakhria and Lior 1992) and antimicrobial resistotyping (Ribeiro *et al*. 1996) have been used for epidemiological typing, but the discriminatory power of these methods is low (O'Reilly *et al*. 2006).

2.8.2 Genotypic typing methods

A number of genotypic methods have been developed for subtyping *Campylobacter* isolates. Pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and ribotyping are based on restriction analysis of the bacterial DNA. Multi-locus-sequence-typing (MLST), single-nucleotide-polymorphism (SNP) detection and sequencing of the flaA-gene are based on the identification of DNA sequence polymorphisms. Amplified fragment length polymorhism (AFLP) and randomly amplified polymorphic DNA (RAPD) methods are based on PCR amplification of particular genetic targets.

2.8.2.1 Pulsed-field gel electrophoresis (PFGE)

PFGE is a RFLP-based typing method in which the whole bacterial genome is digested with a rare cutting enzyme and the large DNA molecules are separated by the use of an alternating electrical field. The time needed for unravelling and re-orientation of large DNA fragments is size-dependent. Even up to 10 Mb fragments can be separated (Herschleb *et al*. 2007). Differences in the banding patterns are used for genetic comparisons between isolates. Commercially available software packages are used in the analysis.

The differences in the PFGE profiles were originally thought to be mainly caused by point mutations, but later, insertions and deletions were shown to play an important role (Kudva *et al*. 2002, Tenover *et al*. 1995). A point mutation in a restriction recognition site creates a 3-band difference, an insertion or a deletion results in a 2-band difference. According to the most widely cited Tenover criteria (Tenover *et al*. 1995), profiles differing from each other in the positions of up to three bands should be considered closely related since a single genetic event would result in up to three-band differences. However, these criteria were originally developed for the investigation of nosocomial outbreaks. According to more recent guidelines for food-borne outbreaks (Barrett *et al*. 2006), only isolates displaying indistinguishable PFGE patterns should be included when detecting infection clusters, and patterns with 2–3 band differences could be included if the outbreak persists for a longer time or if person-to-person spread is a prominent feature. In addition, the reproducibility of the PFGE method for a specific bacterium, the quality of the PFGE gel, the diversity of the organism and the prevalence of a specific PFGE pattern should be considered in interpreting the results (Barrett *et al*. 2006). In all situations, the subtyping results should be interpreted together with the epidemiological information.

PFGE is one of the most discriminatory genotypic typing methods for subtyping *Campylobacter* species (Hänninen *et al*. 2000, Michaud *et al*. 2004, On *et al*. 1998b, O'Reilly *et al*. 2006, Sails *et al*. 2003a), and PFGE is considered the gold standard of molecular typing methods for bacterial foodborne pathogens. A number of *Campylobacter* PFGE protocols have been described in the literature (Gibson *et al*. 1995, Owen *et al*. 1995, Yan *et al*. 1991). Interlaboratory comparisons of *Campylobacter* PFGE profiles are enabled by using standardised protocols and computer-assisted pattern normalisation techniques (Swaminathan *et al*. 2001). The most widely used standardised PFGE protocol is the PulseNet protocol developed for *Campylobacter* by Ribot *et al*. (Ribot *et al*. 2001). PFGE typing has a reasonable high intra- and interlaboratory reproducibility (Swaminathan *et al*. 2001).

Sometimes the banding patterns are not reproduced on repeated testing. The most common reason is incomplete restriction, resulting in additional bands in PFGE gels usually at the top of the gel. If a restriction site is methylated (McClelland *et al*. 1994), a single or a few extra bands that are typically weaker "ghost bands" may appear in the profiles. Variation in the PFGE profiles can be caused by plasmids, which could be present in some strains and absent in others. If plasmids are digested, the fragments will migrate according to their size. If plasmids are not in linear conformation, their migration is unpredictable (Barton *et al*. 1995). Cultures that are not pure result in variation in the PFGE patterns. Furthermore, genomic instability of *Campylobacter* strains causing changes in the PFGE profiles has been reported (Hänninen *et al*. 1999, Wassenaar *et al*. 2000).

The sensitivity of PFGE is dependent on the restriction enzyme used. KpnI has been shown to be more discriminative for *C. jejuni* strains than SmaI (Michaud *et al*. 2001, Saito *et al*. 2005), based on a higher number of fragments produced. Digestion with one enzyme is considered sufficient to show differences between isolates but not similarity. Identical profiles with SmaI should be further typed by KpnI for increased discrimination and confidence (Lindmark *et al*. 2004, On *et al*. 1998b).

2.8.2.2 Other genotypic typing methods

In MLST, the sequences of multiple genes are compared for nucleotide base changes (Maiden *et al*. 1998). The strain is assigned a specific sequence type based on the alleles from each of the genes sequenced. Additionally, isolates can be clustered into clonal complexes based on their sequence types (Feil *et al*. 2004). MLST is a highly reproducible method and Internet-based MLST databases facilitate a standardised nomenclature and rapid exchange of MLST results between laboratories. MLST typing of *C. jejuni* strains is based on the genetic variation present in seven housekeeping loci (Dingle *et al*. 2001) and has been expanded for typing of *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus* (Miller WG *et al*. 2005). MLST is more suitable for population genetic analyses and for understanding the overall genetic structure of *Campylobacter* populations than for outbreak investigations (Clark *et al*. 2005, Levesque *et al*. 2008, Sails *et al*. 2003b) The relatively high cost limits the use of MLST typing in the routine monitoring of *C. jejuni*.

The flagellin encoding flaA gene in *Campylobacter* has significant sequence heterogeneity (Fischer and Nachamkin 1991, Thornton *et al*. 1990). It has been used in the epidemiological typing of *Campylobacter* isolates as the target for PCR-RFLP typing (Nachamkin *et al*. 1993, Owen *et al*. 1993), and sequencing a short variable region (Heuvelink *et al*. 2009, Meinersmann *et al*. 1997, Wassenaar *et al*. 2009). Both methods offer low cost and high throughput, but interlaboratory stan-

dardisation of the PCR-PFLP-method can be difficult. On the other hand, flaA-RFLP has been shown to have a strong association with the clonal complex of MLST typing and may offer an alternative to MLST for use in routine surveillance (Djordjevic *et al*. 2007).

Ribotyping classifies bacteria based on differences in ribosomal RNA (Bouchet *et al*. 2008). Cellular DNA is cut with a frequently cutting restriction enzyme and the DNA fragments are separated by gel electrophoresis, transferred to a membrane, denatured and visualised with a labelled ribosomal RNA probe. Distinct band profiles are produced by differences in the number of rRNA genes and genetic variability in the flanking regions. There is an automated system (RiboPrinterTM, Qualicon, Wilmington, DE, USA) available for ribotyping. Although ribotyping is a highly reproducible method, it is not widely used for *Campylobacter* due to the low level of diversity (only 3 ribosomal operons) and relatively high cost (Bouchet *et al*. 2008, Ge *et al*. 2006, O'Reilly *et al*. 2006).

In RAPD-typing, random segments of genomic DNA are amplified by generic PCR primers under non-stringent conditions, the amplification products are separated by agarose gel electrophoresis and the subsequent banding patterns are used to compare the relatedness of bacterial strains (Franklin *et al*. 1999, Williams *et al*. 1990). RAPD shows a good discrimination power for several *Campylobacter* species (Hilton *et al*. 1997, Mazurier *et al*. 1992, Møller Nielsen *et al*. 2000, Ono *et al*. 2003) but variation in the assay is difficult to control. Even if the reproducibility is high in one laboratory, interlaboratory harmonisation has proven difficult.

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al*. 1995). The bacterial genome is digested with a frequently cutting enzyme to generate a large number of fragments. The multiple restriction sites throughout the genome result in high discrimination of the isolates. The differences in restriction fragment lengths are caused by mutations that create or abolish restriction endonuclease recognition sites. AFLP was adapted for *Campylobacter* by Duim *et al* (Duim *et al*. 1999). Fluorescently labelled primers help automate the analysis and facilitate higher throughput. AFLP has been used in epidemiological investigations of multiple *Campylobacter* species (Duim *et al*. 2000, Islam *et al*. 2009). Careful standardisation is needed for interlaboatory comparisons.

SNP is a nucleotide mutation at specific loci in the bacterial genome. By examining multiple SNPs, the relatedness of strains can be elucidated (Cebula *et al*. 2005). Microarrays based on single nucleotide polymorphisms are promising for future high-throughput typing of bacteria (Berget *et al*. 2007). For *Campylobacter* iso-

lates, the detection of SNPs at specific loci on each of the standard MLST genes has been used to genotype isolates (Best *et al*. 2004, Robertson *et al*. 2004). When this SNP analysis is combined with sequencing of the short variable regions of the flaA gene, the discrimination index is similar to that of the MLST analysis of the isolates (Price *et al*. 2006).

3 Aims of the Study

The aims of this study were:

To improve the species identification of *C. jejuni* and *C. coli*, which constitute the majority of *Campylobacter* strains isolated from patients in Finland.

To evaluate the usefulness of different typing methods for epidemiological typing of *C. jejuni*.

To utilise the data produced by these typing methods
- to describe the demographical, temporal and geographical trends of *Campylobacter* infections in Finland
- to find temporally persistent national subtypes of *C. jejuni*
- to investigate the contribution of poultry and cattle as sources of human *C. jejuni* infections

4 Material and Methods

4.1 *Campylobacter* strains (I-V)

Nine Finnish clinical microbiology laboratories of 9 hospital districts across the country (Figure 2) submitted all the *Campylobacter* strains (n=2364) isolated from patients between 1 July 2002 and 30 June 2005 to the Enteric Bacteria Laboratory of KTL (presently the Bacteriology Unit of THL). Some of these laboratories also submitted strains isolated in June 2002 and July 2005. Two additional laboratories collected strains for only part of the study period (Figure 2), and some of these strains were included in studies I and II. Altogether, 2566 *Campylobacter* strains were collected from 2532 patients, 2364 from the whole study period. The clinical microbiology laboratories isolated the strains from the samples of the patients using modified-CCDA plates. Most of the strains were isolated from faecal specimens, and 8 were from blood. The identification of isolates in these laboratories was based on standard biochemical tests (oxidase, growth in microaerobic atmosphere). The majority of the strains were tested for hippurate hydrolysis using diagnostic tablets (DiatabsTM, Rosco Diagnostica A/S, Denmark) according to the manufacturer's instructions. A special form, including information on the patient's travel history and the date when the specimen was taken, was submitted with the patient's strain. The strain was regarded as associated with foreign travel if the patient had travelled abroad and the onset of symptoms was within 10 days or the specimen was taken within 17 days after the patient's return. All *Campylobacter* cultures were preserved frozen at -70ºC in skim milk. The isolates were revived on blood agar plates and incubated in a microaerobic atmosphere at 37 ºC or 42 ºC for 18-48 h depending on the typing method.

The poultry isolates (n=43) and bovine isolates (n=201) were received and typed by the National Veterinary and Food Research Institute (currently: Finnish Food Safety Authority Evira). The poultry isolates were obtained from 2 of 3 Finnish broiler slaughterhouses between May and August 2003. One isolate from each *Campylobacter*-positive slaughter batch was submitted. The bovine isolates included isolates from carcass samples (n=15), and faecal isolates from dairy cattle (n=71) and beef cattle (n=115). They were obtained from 12 of 15 Finnish slaughterhouses in 2003.

Study I. 240 strains isolated from humans were selected based on the results of the hippurate hydrolysis tests carried out in the clinical microbiology laboratories: hippurate positive isolates (n=81), hippurate-negative isolates (n=152) and strains with a weak positive result or not tested for hippurate hydrolysis (n=7). In addition,

7 *Campylobacter* reference strains were studied: *C. jejuni* ssp. *jejuni* (ATCC 29428 and ATCC 33292), *C. jejuni* ssp. *doyley* (NCTC 11951), *C. coli* (ATCC 33559), *C. fetus* ssp. *fetus* (ATCC 27374), *C. upsaliensis* (ATCC 43954) and *C. lari* (ATCC 35221).

Study II. 139 *C. jejuni* strains isolated from humans between 1 June 2002 and 29 August 2003 were selected for PCR-RFLP analysis based on the serotypes of the strains. Of them, 79 were serotypeable and 60 strains were non-serotypeable by heat-stable serotyping. Both domestic and travel-related strains were included. In addition, 10 NCTC reference strains belonging to known Penner serotypes were studied: Pen 1 NCTC12500, Pen 6 NCTC 12505, Pen 7 NCTC 12506, Pen 12 NCTC 12511, Pen19 NCTC 12517, Pen 27 NCTC 12521, Pen 41 NCTC 12542, Pen 44 NCTC 12549, Pen 57 NCTC 12552 and Pen 58 NCTC 12553.

Study III. Of the 2364 strains received from the 9 clinical microbiology laboratories over the three-year period, 1407 *C. jejuni* strains were studied by serotyping. These included all *C. jejuni* strains isolated during two one-year periods (July 2002 to June 2003 and July 2004 to June 2005) from domestic (n=622) and foreign travel-related (n=785) infections.

Study IV. 419 *Campylobacter* isolates (175 human, 43 poultry and 201 bovine) were studied by PFGE. The human *C. jejuni* isolates included all domestic *C. jejuni* strains isolated between June and August 2003 by the 9 clinical microbiology laboratories.

Study V. From the strains isolated during two one-year periods (July 2002 to June 2003 and July 2004 to June 2005) that were serotyped in study III, 50% (n=311) of the domestic strains and 25% (n=197) of the travel-related strains were systematically selected to obtain a representative sample of the strains isolated during those time periods. The selection was based on serotype, hospital district, sampling date and travel destination of the patients with a foreign travel-related infection.

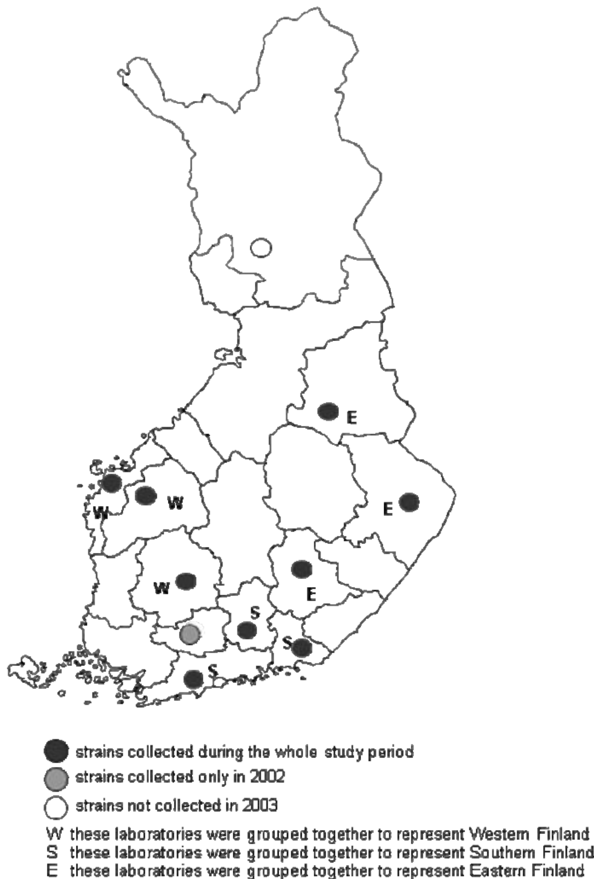

Figure 2. Location of the clinical microbiology laboratories and the hospital districts from which the strains were collected between 1 June 2002 and 8 July 2005.

4.2 Data from the National Infectious Disease Register (III)

Between 1 January 2002 and 31 December 2005, 14361 *Campylobacter* infections were reported to the NIDR. The NIDR provided data on the overall incidence, seasonal changes in the number of *Campylobacter* infections and demographical distribution of the cases diagnosed in Finland.

4.3 Standardisation of the hippurate hydrolysis test (I)

The hippurate hydrolysis test was carried out using Rosco diagnostic tablets (DiatabsTM, Rosco Diagnostica A/S, Denmark). The bacteria were grown on blood agar for 40 to 48 h in a microaerobic atmosphere and suspended in 0.9% NaCl. The diagnostic tablets were added and the tubes were incubated for 4 h at +37°C. Five drops of 3.5% ninhydrin solution was added and the results were read

immediately after 10 minutes re-incubation. Suspensions of 2 reference strains (*C. jejuni* ATCC 29428 and *C. coli* ATCC 33559) with OD450 values from 0.2 to 2.8 were used to determine the optimal concentration of bacteria used in the test. The optical absorbance of the cell suspensions was measured at 450nm with a photometer (GENE-TRAK®, GENE-TRAK Systems, Hopkinton, USA) and compared with McFarland turbidity standards by eye and with a photometer. Based on the results from the reference strains, the turbidity of suspensions from patient strains was adjusted between 0.8 (about McFarland 6) and 1.4 (about McFarland 10). The patient strains were tested 3 times. Blue or purple coloured reactions were read as positive and colourless or yellow reactions as negative.

4.4 Species identification by PCR (I)

Two multiplex PCR methods were used for species identification. In PCR method 1 (Houng *et al*. 2001), species-specific primers detected the distinctive *ceuE*-genes of *C. jejuni* and *C. coli* (Table 3). PCR method 2 (Wang *et al*. 2002) was based on species-specific genes from 5 major clinically relevant *Campylobacter* species and a 23S rRNA fragment from *C. jejuni* as an internal validation control (Table 3). First, both methods were validated with 7 reference strains, and 153 patient strains were typed. Second, method 2 was selected to identify 87 additional patient strains. Templates were prepared by suspending bacterial cells in sterile water and boiling for 5 to 10 minutes, 2µl of the suspension was used as a template. If no amplification product was obtained from a strain, PCR was repeated using isolated DNA (Thisted Lambertz and Danielsson-Tham 2005) as a template.

In method 1, the reaction mixtures (50µl) consisted of 2.5U of AmpliTaq Gold polymerase and 1x GeneAmp PCR buffer (Applied Biosystems, USA), 0.5µM of each primer, 0.2 mM concentrations of deoxynucleoside triphosphates and 1.5mM $MgCl_2$. The amplification cycle consisted of initial denaturation (2 min, 95°C), 30 cycles of denaturation (1 min, 95°C), primer annealing (1 min, 53°C) and chain extension (1 min, 72°C), and a final elongation step (7 min, 72°C).

In method 2, the reaction mixtures (25µl) consisted of 2.5U of AmpliTaq Gold polymerase and 1x GeneAmp PCR buffer (Applied Biosystems, USA), 0.5µM of primers CJF, CJR, CLF, CLR, 1.0 µM of primers CCF, CCR, CFF, CFR, 2.0µM of primers CUF, CUR, 0.2mM concentrations of deoxynucleoside triphosphates, 2.0mM $MgCl_2$ and 0.4M betaine.. The amplification cycle consisted of initial denaturation (2 min, 95°C) and 30 cycles of denaturation (1 min, 95°C), primer annealing (1 min, 59°C) and chain extension (1 min, 72°C), and a final elongation step (7 min, 72°C). The PCR amplification was performed on a PTC-200 thermal cycler (Bio-Rad, USA). The PCR products were analysed by electrophoresis on a

1.5% agarose gel. GeneRulerTM 100bp DNA Ladder (Fermentas, Canada) was used as the molecular size marker.

In case only the 23S rRNA internal control fragment was amplified with PCR method 2, partial sequencing of the 16S rRNA gene of the strain was carried out to support species identification. The fragment was amplified with primers FD1mod and 533reverse and sequenced with the 533reverse primer (Table 3). The templates for amplification were prepared as described by Thisted Lambertz *et al.* (2005).

Table 3. Primers used in Studies I and II

Primer	Sequence	Target gene	Product size	Reference	Used in study
CJ1	CTGCTACGGTGAAAGTTTTGC	*C. jejuni*	783bp	Houng *et al*. 2001	I
CJ2	GATCTTTTTGTTTTGTGC	*ceuE*		Houng *et al*. 2001	I
CC2	GATTTTATTATTTGTAGCAGCG	*C. coli*	645bp	Houng *et al*. 2001	I
CC3	TCCATGCCCTAAGACTTAACG	*ceuE*		Houng *et al*. 2001	I
CJF	ACTTCTTTATTGCTTGCTGC	*C. jejuni*	323bp	Wang *et al*. 2002	I
CJR	GCCACAACAAGTAAAGAAGC	*hipO*		Wang *et al*. 2002	I
CCF	GTAAAACCAAAGCTTATCGTG	*C. coli*	126bp	Wang *et al*. 2002	I
CCR	TCCAGCAATGTGTGCAATG	*glyA*		Wang *et al*. 2002	I
CLF	TAGAGAGATAGCAAAAGAGA	*C. lari*	251 bp	Wang *et al*. 2002	I
CLR	TACACATAATAATCCCACCC	*glyA*		Wang *et al*. 2002	I
CUF	AATTGAAACTCTTGCTATCC	*C. upsaliensis*	214bp	Wang *et al*. 2002	I
CUR	TCATACATTTTACCCGAGCT	*glyA*		Wang *et al*. 2002	I
CFF	GCAAATATAAATGTAAGCGGAGAG	*C. fetus*	435bp	Wang *et al*. 2002	I
CFR	TGCAGCGGCCCCACCTAT	*sapB2*		Wang *et al*. 2002	I
23SF	TATACCGGTAAGGAGTGCTGGAG	*23S-rRNA*	650bp	Wang *et al*. 2002	I
23SR	ATCAATTAACCTTCGAGCACCG			Wang *et al*. 2002	I
galE1	GCGGTGGTGCAGGTTATATAGG	LOS bio-	9600bp	Shi *et al*. 2002	II
wlaH3	TCAGTTCTTGCCATTAAATTTCTC	synthesis genes		Shi *et al*. 2002	II
FD1mod	AGAGTTTGATCYTGGYTYAG	16S-rRNA	525bp	Kotilainen *et al*. 1998	I
533reverse	TTACCGCGGCTGCTGGCAC			Lane *et al*. 1985	I

4.5 Heat-stable serotyping (II, III)

For serotyping, the strains were subcultured twice on blood agar and grown for 48 h at 42°C in a microaerobic atmosphere. Serotyping was performed according to the Penner serotyping scheme (Penner and Hennessy 1980) using a commercially available set of antisera (Denka-Seiken Co., Ltd., Tokyo, Japan), which contains 25 absorbed antisera against the following heat-stable serotypes (group antisera in brackets): (1,44), 2, 3, (4,13,16,43,50), 5, (6,7), 8, 10, 11, 12, 15, 18, 19, 21, (23,36,52), 27, 31, 32, 37, 38, 41, 45, 52, 55 and 57. The heat stable antigens were extracted by nitrite and absorbed onto fixed chicken red blood cells. The sensitised

red blood cells were tested with antisera for agglutination. The serotypes were called HS 1,44 etc in study II, and Pen 1,44 etc in Study III and hereafter.

4.6 PCR-RFLP typing of LOS biosynthesis genes (II)

The genomic DNA was isolated with Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and 200 ng of the DNA was used as a template in PCR. A 9.6 Kb DNA fragment of the LOS gene cluster was amplified with primers galE1 and wlaH3 (Table 2). The reaction mixtures (50 µl) consisted of 1x reaction buffer with 2.0 mM $MgCl_2$ (Finnzymes, Espoo, Finland), 1.0 U of DynaZyme Ext DNA polymerase (Finnzymes), 200 ng of primers, 0.2 mM concentrations of deoxynucleoside triphosphates, and 0.8 M betaine (N,N,N-trimethylglycine, Sigma, St Louis, Missouri, USA). The amplification cycle consisted of an initial denaturation step at 94°C for 2 min after which the enzyme was added and then 10 cycles of denaturation (25 s, 94°C), primer annealing (30 s, 57°C) and chain extension (7 min, 69°C). In cycles 11-22 the chain extension time was increased by 20 s per cycle. A final elongation step was performed for 10 min at 69°C. 10 µl of the PCR product was digested with 10 U of restriction enzymes HhaI and DdeI (Promega, Madison, USA) in separate reactions in a total volume of 20 µl with 2 µg of bovine serum albumin (BSA) for 3-4 h at 37°C. The digest was analysed by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. GeneRulerTM DNA Ladder Mix (MBI Fermentas) was used as a reference size marker. The types were called Hh and Dd types and were numbered separately according to their order of appearance (Hh1-30 and Dd1-32) and the two types were combined to give RFLP types (HhDd).

4.7 PFGE typing (IV, V)

The agarose plugs for PFGE were prepared according to the PulseNet protocol (http://www.cdc.gov/pulsenet/protocols/campy_protocol.pdf) and stored in Tris-EDTA buffer at 4°C. SmaI digestion was performed with 20U of SmaI restriction endonuclease (New England Biolabs Inc., Ipswich, MA) overnight at 25°C in a final volume of 200µl containing 2µl of BSA (New England Biolabs Inc., Ipswich, MA). KpnI digestion of patient strains was performed with 20 U of KpnI restriction endonuclease (Fermentas) in a final volume of 200µl at 37°C overnight and digestion of poultry and bovine strains with 20U of KpnI (New England Biolabs Inc., Ipswich, MA, USA) in a final volume of 200µl containing 2µl of BSA (Fermentas, Lithuania) at 37°C for a minimum of 4 h. Salmonella serotype Braenderup strain H9812 (ATCC BAA-664) was used as the fragment size marker. It was digested with 15U of XbaI restriction endonuclease (Roche, Germany) at 37°C for a minimum of 4 h.

PFGE was performed using pulsed-field electrophoresis systems CHEF-DR®III, CHEF Mapper® XA and GenePath® (Bio-Rad, CA, USA). An 1% agarose gel (SeaKem® Gold Cambrex Bio Science Rockland, Inc., USA) was prepared in 0.5× Tris-buffered EDTA. The SmaI-digested fragments were separated by electrophoresis for 19 h at 6 V and 14°C with ramped pulse times from 6.8 to 35.4 s. The KpnI-digested fragments were separated by electrophoresis for 18 h at 6 V and 14°C with ramped pulse times from 5.2 to 42.3s. PFGE profiles were analysed with Bionumerics v5.10 software (Applied Maths, Kortrijk, Belgium). Patterns differing by at least a single band were considered different subtypes. Subtypes obtained by SmaI and KpnI restriction were called S and K subtypes and were numbered separately according to their order of appearance. Subtypes S7 and S74 identified in Study IV were combined in Study V.

4.8 Statistical and mathematical methods (II, III, IV, unpublished data)

Yates corrected chi-square and Fisher's exact 1-tailed tests were used to compare the proportions of serotypes in domestic infections with those in travel-related infections, the distribution of PFGE types in the first and the second sampling period among domestic strains, and the overlap of *C. jejuni* subtypes between humans, chickens and cattle. A p-value of <0.05 indicated statistical significance.

A discrimination index (DI) (Hunter and Gaston 1988) was used to compare the discriminatory abilities of serotyping, PCR-PFLP and PFGE. The index describes the discriminatory power of a typing method by its ability to distinguish between unrelated strains. It is based on the number of types defined by the test method and the relative frequencies of these types.

5 Results

5.1 Species identified (I)

With PCR method 1, species-specific *ceuE* fragments were amplified from *C. coli*, *C. jejuni* ssp. *jejuni* ATCC 33292 and *C. jejuni* ssp. *doylei* reference strains as expected. DNA isolation was required for the *C. jejuni ceuE* to be amplified from *C. jejuni* ssp. *jejuni* strain ATCC 29428. With PCR method 2, the species-specific fragments and 23S rRNA internal control fragments were amplified from the *C. jejuni* ssp. *jejuni*, *C. jejuni* ssp. *doyley*, *C. coli*, *C. fetus* ssp. *fetus* and *C. lari* reference strains. Only the 23S rRNA fragment was amplified from the *C. upsaliensis* reference strain. Of the patient strains, 153 were studied with PCR method 1 and 240 with PCR method 2. When the original hippurate hydrolysis test results of the clinical microbiology laboratories were compared with species-specific fragments amplified by PCR, *C. jejuni*-specific genes were found in 60 (39%) of the 152 hippurate-negative strains and *C. coli* –specific genes in 2 (2.5%) of the 81 hippurate-positive strains.

The turbidity of cell suspension used was found to affect the colour reaction in the hippurate test (Figure 3). At optical absorbance 1.6 and above at 450nm, blue/purple colour started to appear in the reaction with the negative reference strain *C. coli* ATCC 33559. At optical absorbance 0.6 and below the colour reaction was weak or absent with the positive reference strain *C. jejuni* ATCC 29428. Thus, optimal turbidity was set between 0.8 (about McFarland 6 turbidity) and 1.4 (about McFarland 10) at 450nm. Of the 240 patient strains, 95 were hippurate-positive and 145 hippurate-negative when tested using these turbidity limits. Compared with the hippurate test results from the clinical microbiology laboratories, 16 (11%) of the 152 originally hippurate-negative strains were positive and 4 (5%) of the 81 originally positive strains were negative.

The results of the standardised hippurate test were combined with PCR results. With PCR method 1, *C. jejuni* ceuE was detected in 40 (95%) of the 42 hippurate-positive and 29 (26%) of the 111 hippurate-negative strains and *C. coli* ceuE in 65 (59%) of the hippurate-negative strains. No amplification products were obtained from 2 hippurate-positive and 17 hippurate-negative strains, even when isolated DNA was used as template. With PCR method 2, *C. jejuni* hipO was detected in all of the 95 hippurate-positive strains. A species-specific fragment was amplified from 142 (98%) of the 145 hippurate-negative strains: *C. coli* glyA in 93 (64%), *C. jejuni* hipO in 46 (32%), *C. upsaliensis* glyA in 2 (1%) and *C. fetus* ssp. *fetus* sapB2 in 1 (0.7%). Only the 23S rRNA internal control fragment was amplified

from 3 hippurate-negative strains. Partial 16S rRNA sequences suggested that 2 of them were *C. upsaliensis* (EMBL Nucleotide Sequence Database accession numbers AM497935 and AM497936) and 1 was *C. lari* (AM497937).

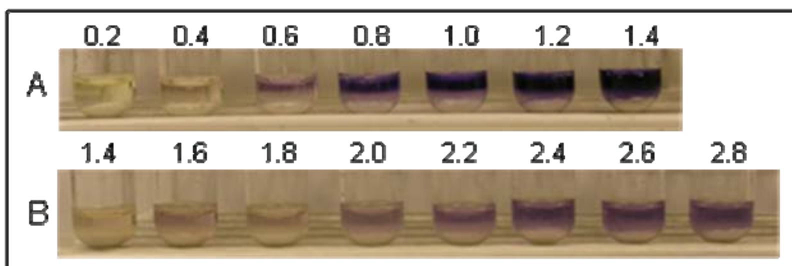

Figure 3. Hippurate test results of reference strains *C. jejuni* ATCC 29428 (A) and *C. coli* ATCC 33559 (B) with different OD450 values of the bacterial suspensions. Photo Anna Liimatainen

5.2 General epidemiology of *Campylobacter* infections in Finland (III)

Data from the NIDR was analysed together with the travel history of the patients obtained for 2186 (92%) of the 2364 strains that were collected over the three-year study period. The annual incidence during 2002-2005 varied from 61 to 76/100 000. Over the 4-year period, the mean incidence was lowest in patients aged 75 or older (24/100 000, 77% of infections domestic) and children aged 5 to 14 (22/100 000, 43% domestic) (Table 4). A seasonal peak in the number of cases in July and August was observed each year. In children younger than 5 years, the incidence and the proportion of domestic infections were higher (33/100 000, 58% domestic) than in older children. The highest incidence (148/100 000) was observed in age group 25-29 together with the highest proportion of travel-related cases (68%). Of the *C. jejuni* strains, 41% were of domestic origin and 51% were associated with foreign travel. Of the *C. coli* strains, 81% were associated with foreign travel and 17% were of domestic origin.

Table 4. The mean annual incidence of *Campylobacter* infections reported to the NIDR in different age groups of patients during 2002-2005, and the proportions of domestic and travel-related infections in the strains received (n=2364).

age group	incidence/ 100 000	No. of strains received	domestic	travel-related
0-4	33	83	48 (58%)	20 (24%)
5-9	21	63	30 (48%)	24 (38%)
10-14	23	58	22 (37%)	35 (60%)
15-19	49	121	36 (30%)	79 (65%)
20-24	112	224	67 (30%)	138 (62%)
25-29	148	236	61 (26%)	160 (68%)
30-34	124	204	72 (35%)	114 (56%)
35-39	97	209	79 (38%)	119 (57%)
40-44	90	206	68 (33%)	119 (58%)
45-49	86	220	71 (32%)	134 (61%)
50-54	77	211	74 (35%)	116 (55%)
55-59	58	181	72 (40%)	98 (54%)
60-64	49	110	56 (51%)	49 (45%)
65-69	41	75	47 (63%)	20 (27%)
70-74	37	65	37 (57%)	21 (32%)
75+	24	98	75 (77%)	13 (13%)

5.3 Distribution of serotypes among patient strains (III)

Of the 1407 *C. jejuni* strains, 814 (58%) were typeable by serotyping. Of the most common serotypes, Pen 12, Pen 6,7 and Pen 27 were statistically significantly associated with domestic strains, and Pen 1,44, Pen 3 and Pen 37 with travel-related strains (Table 5). Among the domestic strains, Pen 2, Pen 12, Pen 4-complex, Pen 6,7, Pen 1,44 and Pen 27 were the most common serotypes. A higher proportion of the Pen 2 strains (18%) were isolated in winter compared to the other serotypes (0-10%) (Figure 4). The distributions of the other specific serotypes followed the typical seasonal pattern showing a peak in summer and low incidences in other seasons (Figure 4). The patients were divided into four age groups to study the demographical distribution of the serotypes. Pen 2 was the most common serotype (13-18%) in 0-59 year old patients but accounted for only 7% of the isolates from older patients. Pen 1,44 was slightly more common in age group 0-19 years (7%) than in older patients (3-4%). The clinical microbiology laboratories were grouped together to represent three geographical areas. Pen 2 was more prevalent in Western Finland (18%) than in Southern (9%) or Eastern Finland (6%). The lower frequency of Pen 2 in patients aged 60 years or older was observed in Southern and Western Finland. The geographical distributions of Pen 12 and Pen 4-c were equal (10-15% and 6-12%), and there were only minor dif-

ferences in the distribution of the other serotypes: Pen 6,7 3-8%, Pen 1,44 1-7%, and Pen 27 2-6%.

Table 5. Serotypes identified among the *C. jejuni* patient strains (n=1407) isolated from domestic and travel-related infections. NS=not significant, ND=not determined

Serotype	Domestic	Travel-related	p-value
total number of strains	622 (44%)	785 (56%)	
Pen NT (n=593, 42%)	244 (41%)	349 (59%)	NS
Pen 2 (n=192, 14%)	80 (42%)	112 (58%)	NS
Pen 4-complex (n=118, 8%)	56 (47%)	62 (53%)	NS
Pen 12 (n=88, 6%)	72 (82%)	16 (18%)	<0.001
Pen 1,44 (n=79, 6%)	25 (32%)	54 (68%)	<0.05
Pen 6,7 (n=50, 4%)	37 (74%)	13 (26%)	<0.001
Pen 3 (n=46, 3%)	2 (4%)	44 (96%)	<0.001
Pen 37 (n=28, 2%)	1 (4%)	27 (96%)	<0.001
Pen 27 (n=25, 2%)	21 (84%)	4 (16%)	<0.001
other serotypes (n=188, less than 2% each)	84 (45%)	104 (55%)	ND

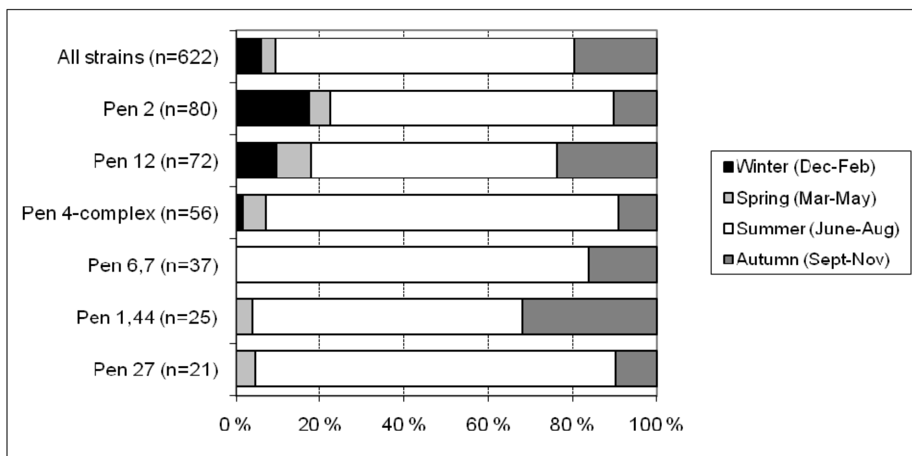

Figure 4. Seasonal distribution of the serotypes among the domestic *C. jejuni* strains (n=622).

Of the travel-related strains, Pen 1,44 and Pen 6,7 showed a peak in summer (44% and 62%, respectively), and the majority of the Pen 12 strains were isolated in summer (44%) and autumn (50%). The other serotypes among travel-related strains were evenly distributed seasonally. Among the travel-related strains, the

proportions of Pen 2 and Pen 1,44 were lower (6% and 3%, respectively) in patients 60 years or older than in younger patients (13-16% and 6-7%), whereas the proportion of Pen 4-complex was higher (16%) than in younger age groups (6-9%). There were no significant differences in the geographical distribution of the serotypes among the travel-related infections.

5.4 PCR-RFLP types of serotypeable and non-typeable *C. jejuni* strains (II)

All tested strains (n=149) were typeable by PCR-RFLP. Among the 139 patient strains, 36 different RFLP types were identified. The most common type, Hh1Dd1 accounted for 35% (n=49) of the strains, whereas 20 RFLP-types occurred only once (Table 6). The DI of RFLP typing was 0.85 for both serotypeable strains (23 RFLP types, n=79) and NT strains (22 RFLP types, n=60). The DI of Penner serotyping was 0.96 for the serotypeable strains. The 10 reference strains were divided into 7 RFLP types. Profile Hh1Dd1 was found in reference strains of serotypes Pen 6, Pen 12, Pen 27 and Pen 57, Hh3Dd3 in Pen 44, Hh7Dd7 in Pen 1 and Hh5Dd5 in Pen19. These profiles were also identified among the patient strains. Profiles Hh6Dd6 found in reference strain Pen 41, Hh24Dd26 in Pen 58 and Hh25Dd27 in Pen 7 were not found among patient strains.

Only a single RFLP type was found among patient strains within serotypes Pen 6,7 (Hh1Dd1, n=7), Pen 38 (Hh8Dd8, n=5), Pen 27 (Hh1Dd1, n=4), Pen 55 (Hh1Dd1, n=4), Pen 21 (Hh1Dd1, n=3), Pen 37 (Hh15Dd18, n=3), Pen 41 (Hh9Dd9, n=3) and Pen 1,44 (Hh7Dd7, n=2). Of these, RFLP types Hh8Dd8, Hh9Dd9 and Hh15Dd18 can be considered serotype specific since they were not found among other serotypes (Table 6). Strains of other serotypes with 2-7 strains (12 serotypes) were divided into 2 RFLP types, whereas the 3 Pen 15 strains analysed resulted in 3 RFLP types.

Table 6. PCR-RFLP types identified among the *C. jejuni* strains isolated from patients (n=139), and the serotypes of the strains found in each PCR-RFLP type.

RFLP-type	serotypes	number of strains
Hh1Dd1	10 serotypes and NT	49
Hh8Dd012	Pen 3, Pen 4-complex and NT	17
Hh7Dd7	Pen 1,44, Pen 2 and Pen 8 and NT	8
Hh8Dd8	Pen 38 and NT	8
Hh16Dd12	Pen 57, mixed serotypes and NT	5
Hh3Dd3	4 serotypes and NT	5
Hh23Dd24	NT	4
Hh9Dd9	Pen 41 and NT	4
Hh15Dd18	Pen 37	3
Hh2Dd2	Pen 2 and NT	3
Hh3Dd16	NT	3
Hh10Dd16	Pen 18 and NT	2
Hh14Dd14	Pen 11	2
Hh15Dd15	Pen 23,36,53	2
Hh22Dd10	NT	2
Hh5Dd5	Pen 19	2
Other RFLP-types (20 types)		20 (1 strain of each type)

5.5 PFGE subtypes among human *C. jejuni* isolates (V, unpublished data)

The 311 domestic strains were divided into 86 and the 197 travel-related strains into 172 SmaI subtypes. 37 of the domestic strains and 1 of the travel-related strains were not digested by SmaI. Four SmaI subtypes, S5, S7, S54 and S64 accounted for 45% of the domestic strains (Table 7), whereas subtype S25 was found in 4 of the travel-related strains and the other subtypes in 1-3 strains. SmaI subtypes S7 and S74, which differed in the size of one fragment in Study IV, were combined in Study V. In Study IV, the size of the fragment was 147-149 bp in S7 and 152-153 bp in S74. In Study V, the two types could no longer be separated. Of the most common SmaI subtypes among isolates from humans, profiles S7, S54 and S64 consisted of only 4 bands (without counting the compression zone), whereas S5 contained 7 bands (Figure 5). The dominating SmaI subtypes and strains that were not digested with SmaI were typed by KpnI. The KpnI profiles contained 9 or more bands (Figure 5). Some profiles belonging to subtypes K1 and K5 contained ghost bands that were not eliminated by repeated typing but the

intensity of the bands varied between runs (Figure 5). SmaI subtypes S7, S54 and S64 were divided into several KpnI subtypes whereas all 28 S5 strains had an identical KpnI subtype K27 (Table 7).

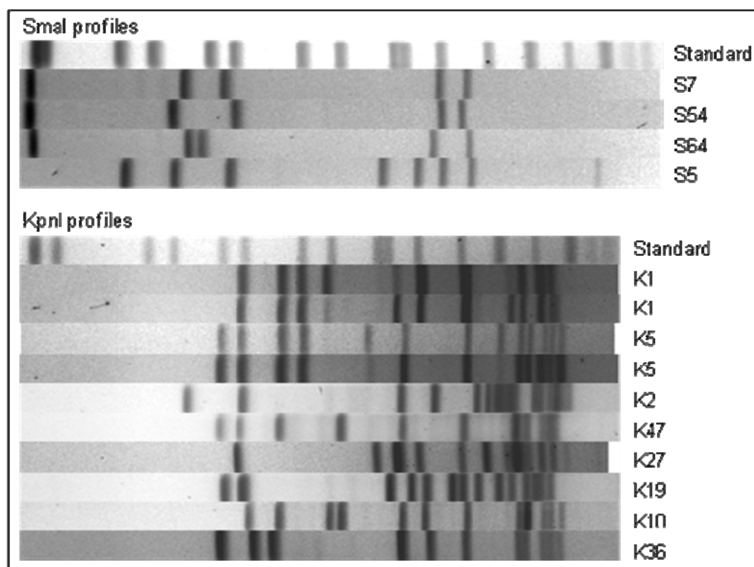

Figure 5. The most common SmaI and KpnI restriction profiles found among the human strains. Two examples of subtypes of K1 and K5 are included to show the variation in these profiles.

Seven SmaI/KpnI combination types with more than 5 strains were found (Table 7). All S7/K2, S7/K47, S54/K10 and S64/K19 strains were of domestic origin, while S5/K27, S7/K1 and S7/K5 and were found in 1 or 2 travel-related strains. Types S7/K1, S7/K2, S7/K5, S54/K10 and S64/K19 were evenly distributed among strains of the two sampling periods and were considered to be persistent subtypes. S5/K27 was also considered to be a persistent subtype. It was found during both sampling periods, but was not equally distributed. Of the 26 domestic strains, 4 (15%) were from the first sampling period, and 22 (85%) from the second ($p<0.01$). There was a cluster of 11 strains in Western Finland in July 2004. Of the 7 S7/K47 strains, all were from the first sampling period and none from the second ($p<0.05$). Thus, it was considered to be a transient subtype. Six of the S7/K47 strains were isolated in Western Finland between July and September 2002.

Subtype S7/K47 contained strains of 4 serotypes (14-43% each) whereas a dominating serotype accounting for 50-100% of the strains was identified for the other common combination types (Table 7). Pen 6,7 accounted for 29% of the NT/K36

strains. The DI of SmaI typing was 0.91 for domestic strains and over 0.99 for travel-related strains. The discriminatory ability of SmaI typing varied between serotypes among the domestic strains. The DI was 0 for Pen 27 (11 strains, 1 SmaI subtype), 0.50 for Pen 6,7 (18 strains, 2 SmaI subtypes, 14 strains NT), 0.54 for Pen 2 (40 strains, 2 SmaI subtypes, 1 strain NT), 0.59 for Pen 12 (36 strains, 7 SmaI subtypes), 0.83 for Pen 4-complex (28 strains, 12 SmaI subtypes) and 0.95 for Pen 1,44 (12 strains, 9 SmaI subtypes, 1 strain NT). A dominating SmaI/KpnI subtype accounting for 32-73% was identified for each serotype: S7/K1 for Pen 12 (36%), S5/K27 for Pen 2 (65%), S7/K5 for Pen 27 (73%), and S64/K19 for Pen 4-complex (32%). The Pen 1,44 strains (n=12) were divided into 9 SmaI subtypes and were not typed by KpnI. Of the Pen 6,7 strains, subtype K36 (non-typeable by SmaI) accounted for 33%. The other subtypes accounted for 17% or less of each serotype.

Table 7. SmaI and KpnI subtypes identified among the domestic strains (n=311), and the most common serotype among each subtype.

SmaI subtype	KpnI subtype	Most common serotype (% of SmaI-KpnI subtype)
S7 (21.9%, n=68)	K1 (n=17)	Pen 12 (76%, n=13)
	K5 (n=15)	Pen 27 (53%, n=8)
	K2 (n=8)	Pen 41 (50%, n=4)
	K47 (n=7)	Pen 55 (43%, n=3)
	K4 (n=5)	
	K7 (n=4)	
	Other KpnI-types (n=12; 7 types, 1-3 strains each)	
S5 (8.4%, n=26)	K27 (n=26)	Pen 2 (100%, n=26)
S64 (7.4%, n=23)	K19 (n=16)	Pen 4-complex (56%, n=9)
	Other KpnI-types (n=7; 5 types, 1-3 strain each)	
S54 (7.1%, n=22)	K10 (n=10)	Pen 57 (60%, n=6)
	Other KpnI-types (n=12; 8 types, 1-3 strains each)	
NT (11.9%, n=37)	K36 (n=21)	NT (57%, n=12), Pen 6,7 (29%, n=6)
	Other KpnI-types (n=16; 12 types, 1-3 strains each)	
Other SmaI-types 43.4%, n=135 (82 types, 1-10 strains of each type)		

5.6 Comparison of PFGE subtypes in humans, chickens and cattle (IV)

Isolates from human infections (n=175) were divided into 43, chicken isolates (n=43) into 15 and cattle isolates (n=201) into 61 SmaI subtypes. One isolate from chickens and 18 from humans were not digested by SmaI. Indistinguishable SmaI subtypes were found among human, chicken and cattle isolates (Table 8). These accounted for 114 (65%) of 175 human isolates. Isolates with the 14 overlapping SmaI subtypes were further typed using KpnI. S5, S6, S66 and S78 isolates were not further divided by KpnI, whereas 2-8 KpnI types were found among the other SmaI subtypes. Overlapping KpnI subtypes were found among the shared SmaI subtypes, except S1 and S38. Of the 45 combined SmaI/KpnI subtypes, 17 were shared between human and animal isolates (Table 8). 12 KpnI subtypes were found only in humans, 4 only in chickens, 11 only in cattle and 1 was shared between chickens and cattle (Table 8). Of the 175 human isolates, 97 (55%) belonged to the overlapping SmaI/KpnI subtypes. These subtypes accounted for 70% (30/43) of chicken isolates and 16% (32/201) of cattle isolates. Subtype S6/K12 predominated among the isolates from patients (12%) and was found in both chickens and cattle. The occurrence of identical SmaI/KpnI subtypes in human *C. jejuni* isolates was significantly associated with animal host species ($P < 0.001$), but not with herd type of cattle ($P = 0.056$). A temporal association of SmaI/KpnI subtypes among isolates from chickens and patients was possible in 55 (31%) of the 175 human infections. Of these 12 (7%) represented SmaI/KpnI subtypes not found in cattle. The SmaI/KpnI subtypes shared between humans, chicken and cattle but not temporally related to chickens accounted for 27 (15%) of the human infections, and 3 subtypes were shared only between humans and cattle (3% of human infections).

Table 8. Proportions of the shared SmaI/KpnI subtypes in humans, chickens and cattle

SmaI/KpnI subtype	human (n=175)	chicken (n=43)	cattle (n=201)
S4/K29	1 (0.6%)	1 (2.3%)	1 (0.5%)
S5/K27	1 (0.6%)	0	10 (4.9%)
S6/K12	21 (12%)	2 (4.7%)	7 (3.4%)
S7/K1	12 (6.9%)	2 (4.7%)	7 (3.4%)
S7/K2	4 (2.3%)	2 (4.7%)	2 (1.0%)
S7/K3	17 (9.7%)	2 (4.7%)	1 (0.5%)
S22/K16	1 (0.6%)	0	1 (0.5%)
S54/K10	6 (3.4%)	2 (4.7%)	0
S54/K11	3 (1.7%)	1 (2.3%)	0
S64/K19	7 (4.0%)	1 (2.3%)	1 (0.5%)
S66/K18	4 (2.3%)	0	1 (0.5%)
S74/K4	5 (2.9%)	8 (19%)	0
S74/K5	8 (4.6%)	4 (9.3%)	1 (0.5%)
S74/K7	2 (1.1%)	2 (4.7%)	0
S76/K20	3 (1.7%)	1 (2.3%)	0
S77/K30	1 (0.6%)	1 (2.3%)	0
S78/K6	1 (0.6%)	1 (2.3%)	0
Overlapping SmaI-KpnI subtypes	97 (55%)	30 (70%)	32 (16%)
Overlapping SmaI subtypes	114 (65%)	36 (84%)	62 (31%)
Other KpnI subtypes of shared SmaI types	12 KpnI subtypes found only in humans (1-3 strains each, n=17)	4 KpnI subtypes found only in chickens (1-2 strains each, n=5)	11 KpnI subtypes found only in cattle (1-6 strains each, n=27)
		1 KpnI subtype found in chickens (n=1) and cattle (n=3)	

6 Discussion

6.1 Quality of species identification (I)

Both false-positive (2.5%) and false-negative (39%) hippurate test results were found when the original hippurate hydrolysis test results reported by Finnish clinical microbiology laboratories were compared with the species-specific fragments amplified by PCR. It has been suggested in several studies that a combination of phenotypic tests and molecular methods should be used for the reliable identification of *C. jejuni* and *C. coli* (Denis *et al*. 1999, On and Jordan 2003, Totten *et al*. 1987). The positive results of strains identified as *C. coli* by PCR resulted from using a very high concentration of bacteria in the hippurate test. The manufacturer's instructions for the diagnostic tablets suggest that the turbidity of the bacterial suspension should be at least McFarland 4, and the use of a high concentration is also emphasised in literature (Fitzgerald and Nachamkin 2007). Introducing an upper limit for the cell suspension turbidity eliminated all false-positive test results, *C. jejuni* –specific genes were detected in all hippurate-positive strains with the two PCR methods. This means that all hippurate-positive strains could be reliably identified as *C. jejuni* without further testing when using the standardised hippurate test. Standardising the inoculum size has been shown to diminish or eliminate false-positive results (Nicholson and Patton 1995, Wainø *et al*. 2003). The lower turbidity limit was set in order to eliminate false-negative results caused by a very low inoculum size. However, strains with variable test results and hippurate-negative *C. jejuni* strains have been reported (Nicholson and Patton 1995, Wainø *et al*. 2003) and accounted for 32% of the hippurate-negative strains in this study. Thus, hippurate-negative *C. jejuni* strains cannot be reliably identified with phenotypic methods.

Correct species identification can be considered to be the first step in epidemiological surveillance. A cluster of 9 strains isolated in Eastern Finland in July 2005 was studied by PCR together with the other hippurate-negative strains. These strains were in fact *C. jejuni*. The reservoirs and thus the potential infection sources vary between species. *C. coli* accounts for 90% of the *Campylobacter* isolates from pigs, whereas *C. jejuni* predominates in cattle and poultry (Boes *et al*. 2005, EFSA 2010a). *C. upsaliensis* is the most frequently isolated species in cats and dogs (Parsons *et al*. 2010, Sandberg *et al*. 2002), and *C. lari* in wild birds (Waldenström *et al*. 2007). *C. fetus* is a veterinary pathogen that is seldom isolated from healthy animals, but infections in humans are often systemic (Monno *et al*. 2004, Tremblay *et al*. 2003). In most clinical microbiology laboratories in Finland, hippurate test is the only method used in to differentiate between *C. jejuni* and *C.*

coli. The laboratories were given instructions for standardising the hippurate test, which would improve the identification of hippurate-positive *C. jejuni* and make the epidemiological data reported to the NIDR more reliable. Identifying hippurate-negative species is more complicated. While some species can be identified by phenotypic tests or commercial phenotypic methods, molecular methods are required to differentiate between *C. coli* and hippurate-negative *C. jejuni* strains.

6.2 Demographical and temporal trends of *Campylobacter* infections in Finland 2002-2005 (III)

The highest incidence was observed in age group 25-29 years. A peak in the incidence in young adults has been observed in many countries (Friedman *et al*. 2000), and increased foreign travel in this age group has been suggested as an explanation (Kapperud and Aasen 1992). Although the proportion of travel-related cases was the highest in this age group (68%) it explains only part of the increased incidence. A higher rate of domestic infections in young adults in Finland has been reported previously (Vierikko *et al*. 2004). The finding of a higher incidence in children younger than 5 years compared to older children was in agreement with other reports from different countries (Friedman *et al*. 2000, Koehler *et al*. 2006, Vierikko *et al*. 2004). Oversampling or specific risk factors in young children have been suggested as explanations (Doorduyn *et al*. 2010, Fullerton *et al*. 2007, Kapperud and Aasen 1992, Koehler *et al*. 2006, Schönberg-Norio *et al*. 2006, Tenkate and Stafford 2001). More than 40% of the *C. jejuni* infections in Finland seem to be domestically acquired. The domestic cases were defined as not travelling abroad within 10 days prior to the onset of symptoms or 17 days prior to providing the specimen. Since the mean incubation period of a *Campylobacter* infection is usually 2 to 5 days, with a range of 1 to 10 days (Skirrow and Blaser 2000), it is certain that there were no travel-related infections among the ones classified as domestic. However, some of the infections classified as travel-related could have been acquired in Finland after returning from abroad. The seasonal variation observed in the number of *Campylobacter* infections was mostly due to low occurrence of domestic infections in winter and a peak in July and August, whereas the number of travel-related infections was more stable throughout the year. Possible risk factors, such as barbecue-prepared meals and attending outdoor parties, coincide with the seasonal peak of *Campylobacter* infections (Eberhart-Phillips *et al*. 1997, Sopwith *et al*. 2006) as does the prevalence of *C. jejuni* in broiler flocks and cattle (Bang *et al*. 2003, Hakkinen *et al*. 2007, Hansson *et al*. 2004, Hofshagen and Kruse 2005, Nylen *et al*. 2002, Perko-Mäkelä *et al*. 2002) Foreign travel could be considered to be the major risk factor for *C. coli* infections for Finnish patients, since 81% of the cases were travel-related.

6.3 Subtyping methods and their applicability to epidemiological investigations (II, III, IV, V, unpublished data)

6.3.1 Evaluation of serotyping and PCR-RFLP analysis

PCR amplification of the LOS biosynthesis gene cluster and RFLP-analysis of the fragment was tested as a supplementary method for typing non-serotypeable strains. Serotypeable strains were included in the study to see the correlation between Penner serotypes and RFLP-types. A technical finding was that addition of 0.8M betaine in the reaction mixture was required to decrease smearing produced by the DyNazyme EXT DNA-polymerase when long extension times are used, as was reported by Diakou and Dovas (2001). Betaine improves the amplification of by reducing the formation of a secondary structure caused by GC-rich regions (Henke *et al*. 1997). Later, betaine has been used in other technically demanding PCR applications in the Bacteriology Unit of THL. It improves the amplification of fragments in multiplex-PCR.

The main limitation of serotyping is the high proportion of non-typeable strains, especially when commercially available antisera are used (Boonmar *et al*. 2005, Newell *et al*. 2000). 42% of the strains serotyped in this study were non-typeable. 100% typeability of non-serotypeable strains was achieved by PCR-RFLP, but the discriminatory index for the PCR-RFLP typing was lower (0.85) for both serotypeable and NT strains than that of the Penner serotyping for serotypeable strains (0.96). Some serotype-specific RFLP types (Hh8Dd8 for Pen 38, Hh9Dd9 for Pen 41 and Hh15Dd18 for Pen 37) were found. Most of the serotypes were divided into 2-3 RFLP-types, and the most prevalent RFLP type Hh1Dd1 contained strains of 8 serotypes. This is in agreement with previous findings of variation in the LOS biosynthesis genes within a single Penner serotype (Dorrell *et al*. 2001). The *C. jejuni* heat stable antigens forming the basis of the Penner serotyping were originally reported to be LOS-molecules (Moran and Penner 1999, Preston and Penner 1987). Later, the role of capsular polysaccharides became evident (Karlyshev *et al*. 2000, Oza *et al*. 2002). Loci for both LOS and capsule are present in the *Campylobacter* genome (Parkhill *et al*. 2000). Although the non-serotypeable strains were all typed by the PCR-RFLP method, the correlation between serotypes and RFLP types was not high enough to enable grouping all NT isolates with serotypeable isolates. Therefore, the PCR-RFLP types of non-serotypeable strains cannot be directly compared with serotype data. Instead, the method serves as an epidemiological typing method and gives information about the diversity of strains within a serotype or a serogroup.

6.3.2 Advantages and limitations of the PFGE method

PFGE has been shown to be a highly discriminatory genotypic typing method for subtyping *Campylobacter* isolates (Møller Nielsen *et al*. 2000, On *et al*. 1998b, O'Reilly *et al*. 2006, Sails *et al*. 2003b). Identical SmaI and KpnI profiles have been shown to reflect true genetic relatedness, based on the comparison of PFGE types and comparative genomics hybridisation data (Rodin *et al*. 2008). On the other hand, the usefulness of genetic subtyping of *C. jejuni* has been questioned due to the weakly clonal population structure, alterations in the PFGE patterns caused by temperate bacteriophages, and the easy exchange of DNA between strains (Barton *et al*. 2007, de Boer *et al*. 2002, Dingle *et al*. 2001, Dingle *et al*. 2005). Long-term persistence of PFGE-subtypes has been reported, however. Indistinguishable SmaI subtypes were found in chicken and human isolates over a 12-year period in Japan (Yabe *et al*. 2010), and five of the six most common SmaI/KpnI combination types in chickens and humans in Sweden in 2000 were still predominant in 2003 (Lindmark *et al*. 2004, Lindmark *et al*. 2009). In Finland, recurrent genotypes in a defined area have been previously reported (Hänninen *et al*. 2000).

SmaI and KpnI are the first and second enzymes suggested by the Pulse-Net network (http://www.cdc.gov/pulsenet/protocols/campy_protocol.pdf) and were used for PFGE typing in this study. The discriminatory ability of SmaI-typing varied between serotypes among the domestic strains. Pen 1,44 and Pen 4-complex strains were divided into several SmaI subtypes, whereas all Pen 27 strains were of type S7. The low number of bands limited the discriminatory ability of SmaI-typing. Three of the four most common SmaI profiles contained only four bands. In Sweden, SmaI profiles with only a few bands have also been identified (Lindmark *et al*. 2004). On the other hand, all SmaI profiles contained 6 or more bands in a study in Japan (Yabe *et al*. 2010). In agreement with with previous reports (Michaud *et al*. 2001, Saito *et al*. 2005), KpnI was more discriminative than SmaI, due to the higher number of fragments produced. SmaI subtypes S7, S54 and S64 were further divided by KpnI, whereas all S5 strains were indistinguishable by KpnI. In addition, KpnI typing was necessary for the isolates that were not digested by SmaI. These accounted for 12% of all domestic strains and 78% of the domestic Pen 6,7 strains in this study and have also been reported previously (Hänninen *et al*. 2003, Sails *et al*. 2003b). The variable bands or ghost bands occurring in some profiles complicated the analysis of KpnI profiles. Generally, digestion with one enzyme is considered sufficient to show differences between isolates but not similarity. The use of a second enzyme can significantly increase the discriminatory power and the reliability of the results. Therefore, we typed the strains with identical SmaI subtypes shared between humans, chickens and cattle with KpnI to confirm which isolates are truly identical. Strains of the most com-

mon SmaI subtypes isolated from humans were also further analysed by KpnI to find clonal groups of strains. Subtypes, which differed in at least one band position, were considered different. The relatedness of the subtypes was not studied. There are criteria for the degree of genetic relatedness between PFGE profiles (Barrett *et al*. 2006, Tenover *et al*. 1995), but they are generally only applicable for outbreak investigations.

6.3.3 Other potential subtyping methods

Multi-locus Variable-Number Tandem Repeat (MLVA) is a PCR- based genotyping technique that utilizes the differences in the number of repeated copies at specific loci. MLVA protocols have been developed for other foodborne bacteria. It has demonstrated better discriminatory ability than PFGE for *Salmonella* serovar Typhimurium and *E. coli* O157:H7 (Keys *et al*. 2005, Torpdahl *et al*. 2007). MLVA provides typing results in less time than PFGE. The results are easier to interpret, highly reproducible and can be readily compared between laboratories. MLVA seems like an ideal genotyping method, but so far, no protocols for *Campylobacter* have been published.

DNA microarrays have been used in comparative genomic hybridization studies to analyze the genomic variability of *C. jejuni* strains (Champion *et al*. 2005, On *et al*. 2006, Taboada *et al*. 2004). Also, this technique offers high discriminatory power for molecular epidemiology investigations, and clustering of strains have been similar to PFGE (Hannon *et al*. 2009, Leonard *et al*. 2003, Rodin *et al*. 2008). The results are easy to interpret and compare between laboratories, but the relatively high cost and low throughput have limited the use.

6.4 Epidemiology of *C. jejuni* in Finland (III, IV, V, unpublished data)

6.4.1 Distribution of serotypes in *C. jejuni* isolates from patients

Of the most common serotypes among domestic *C. jejuni* strains, Pen 12, Pen 6,7 and Pen 27 were significantly associated with domestic infections, whereas Pen 2, and Pen 4-complex were common both among domestic and travel-related strains, and Pen 1,44 was associated with travel-related strains. Serotypes Pen 2, Pen 4-complex and Pen 1,44 have been reported as the most prevalent serotypes in Denmark, Canada and New Zealand, whereas Pen 12, Pen 6,7 and Pen 27 were found more rarely (Devane *et al*. 2005, Fussing *et al*. 2007, McMyne *et al*. 1982). Pen 2 was more prevalent in Western Finland than in other parts of the country and more prevalent than other serotypes in winter. The other serotypes followed the overall seasonal pattern of domestic infections with a peak in summer and low

occurrence in winter, and were evenly distributed geographically. Pen 1,44 was more common among patients younger than 20 years. Pen 2 was less common among patients 60 years or older than in younger patients. A lower mean age of Finnish patients infected with a strain of serotype Pen 2 has been previously observed (Schönberg-Norio *et al*. 2006). A reduced frequency of common serotypes in older age groups has been reported, possibly due to people developing immunity to the most common serotypes (Linneberg *et al*. 2003, Miller G *et al*. 2005). In this study, however, the other common serotypes were evenly distributed across age groups of the patients in domestically acquired infections.

6.4.2 Distribution of PFGE types among isolates from patients

In other studies, the systematic collection and typing have revealed clusters of infection (Fussing *et al*. 2007, Gilpin *et al*. 2006, McTavish *et al*. 2008). Two previously unidentified clusters were found when the distribution of the most common SmaI/KpnI subtypes in the two sampling periods was studied. All seven S7/K47 strains were isolated between July and September 2002, and this subtype was not found later. Subtype S5/K27 formed a cluster of 11 strains in Western Finland in July 2004 but was also identified in other years. The higher prevalence of domestic Pen 2 strains in Western Finland is not fully explained by the S5/K27 cluster. In addition to S5/K27, other SmaI subtypes were more prevalent in Western Finland than other parts of the country. Furthetmore, the higher prevalence of Pen 2 strains in the winter was not due to any specific subtype. The six common persistent national subtypes identified were also found among the domestic strains in the summer 2003. The proportions of S7/K1, S7/K2, S7/K5, S54/K10 and S64/K19 were about the same, but S5/K27 was found in only one human strain (0.6%) in 2003 compared to 8% in the two one-year periods. The difference is explained by the cluster in 2004. Subtypes S6/K12 and S7/K3 were the most common in the summer 2003, but both were found in only one or two isolates in the other years. Both subtypes were geographically dispersed in 2003. Thus, it seems that certain subtypes are temporally persistent, and are found in equal proportions over several years. In addition, the temporally persistent subtypes can occasionally cause clusters. On the other hand, different transient subtypes appear each year, and they can be either geographically clustered or dispersed.

The diversity of SmaI subtypes was much higher among the travel-related strains than among the domestic strains. A high proportion of the domestic strains (69%) belonged to SmaI subtypes that were isolated during both sampling periods, indicating that persistent sources of certain subtypes may exist. Serotype Pen 2 was frequent in Finland both in domestically acquired and travel-related infections, but the PFGE subtypes in domestic and travel-related Pen 2 strains were different. PFGE pattern S5/K27 accounted for 65% of the 40 domestic Pen 2 strains but was

found in only 7% of the 28 travel-related strains, suggesting that the domestic infections could be associated with a specific source.

6.4.3 Sources of domestic *C. jejuni* infections in humans

Of the most common serotypes identified among the domestic human strains, Pen 6,7 has been reported as the predominant serotype in Finnish poultry (Perko-Mäkelä *et al*. 2002). Pen 12, Pen 4-complex and Pen 27 strains have also been found in poultry (Perko-Mäkelä *et al*. 2002). Subtypes S74/K4 (19%) and S74/K5 (9%) were predominant among the chicken strains. They accounted for 3% and 5%, respectively, of the human strains during the summer 2003, and 1.6% and 7.1% of the strains isolated during July 2002-June 2003 and July 2004-June 2005 (where they were named S7/K4 and S7/K5). Altogether, the SmaI/KpnI subtypes isolated from chickens were detected in 52% of human cases during the summer 2003. A similar percentage of overlap was reported in Denmark (Møller Nielsen *et al*. 2006). The analysis of the temporal distribution of indistinguishable SmaI/KpnI subtypes in isolates from patients and their appearance in chicken slaughter batches indicated that up to 31% of the human infections could have been caused by *C. jejuni* strains from chickens in summer 2003, which is in agreement with a previous estimate (Kärenlampi *et al*. 2003). This implies that chickens are the most important single source of *C. jejuni* in sporadic, domestically acquired infections in Finland, although the prevalence of *Campylobacter* in Finnish chicken slaughter batches is lower than in most other countries (EFSA 2010b, Nadeau *et al*. 2002, Stern *et al*. 2003). Five (S7/K1, S7/K2, S7/K5, S54/K10 and S64/K19) of the temporally persistent genotypes identified were found among chickens in during summer 2003. A temporal association with the positive broiler flock and the human infections was found in 92% of the infections caused by S7/K1 and in 14% of the infections caused by S64/K19. In addition, strains isolated from chickens seem to vary from year to year, as indicated by MLST data (de Haan *et al*. 2010). Thus, chickens do not seem to be the main source of the infections caused by these persistent subtypes.

In Finnish cattle, Pen 2, Pen 4-complex and Pen 12 are frequently isolated, and Pen 1,44 and Pen 6,7 have also been found (Hakkinen *et al*. 2007). Pen 2 strains originating from cattle could explain the higher prevalence of domestic Pen 2 strains in Western Finland, since many cattle farms are located in that area. Moreover, the higher prevalence of Pen 2 strains in winter could be associated with cattle, since long-term excretion of *Campylobacter* by cattle has been reported (Hakkinen and Hänninen 2009, Kwan *et al*. 2008). SmaI subtypes S1 (10,9%), S2 (7,7%), S3 (7,7%), S5 (5,5%), S7 (5,5%) and S11 (5,5%) were the most frequently identified among cattle faecal samples (Hakkinen *et al*. 2007). Of these, S1, S5 and S7 were identified among the human isolates and typed by KpnI. S1 was di-

vided into seven KpnI subtypes. Thus, no predominant SmaI/KpnI subtypes were found in cattle. The SmaI/KpnI subtypes isolated from faecal samples of cattle were detected in 42% of human cases. Similar findings in New Zealand have been reported (Gilpin *et al*. 2008), but a much higher percentage of overlap (83%) was observed in Denmark (Møller Nielsen *et al*. 2006). *Campylobacter* colonization in cattle can results in occurrence of bacteria in unpasteurised milk (EFSA 2010a, Schildt *et al*. 2006). None of the *C. jejuni* strains isolated from bovine carcasses represented SmaI/KpnI subtypes similar to those of human isolates. The most prevalent of the shared *C. jejuni* subtypes in cattle, S5/K27 (4.9%), was detected in only one patient in 2003. However, it was the most common subtype found in human strains during the two one-year sampling periods. Cattle are common carriers of *Campylobacter* in Finland (Hakkinen *et al*. 2007) and have been reported to be an important source of *C. jejuni* infections in many countries (French *et al*. 2005, Garrett *et al*. 2007, Møller Nielsen *et al*. 2006, Nygard *et al*. 2004). Isolates from human infections with overlapping SmaI/KpnI subtypes with cattle and not temporally related to chickens accounted for 19% of the domestic human strains in summer 2003. These infections could have been caused by *C. jejuni* strains originating from cattle. They included all strains of the persistent genotypes S5/K27, S7/K2, S7/K5 and most of the S64/K19 strains. On the other hand, the same genotypes may exist in other animal or environmental sources and some of the human infections temporally associated with chicken isolates could also have been caused by cattle.

About half of the domestic human cases could have originated from chickens or cattle in summer 2003. However, many of the overlapping subtypes were found among both chicken and bovine strains. Thus, the human infections cannot be connected to a specific animal source without additional epidemiological investigation. The human infections could have originated from either animal source or from a common environmental source. In addition, the other half of human infections remained unexplained. For example, subtype S54/K10 was not found in cattle and the human infections were not temporally related to isolates from positive chicken flocks. The associations between *C. jejuni* isolates from humans, chicken and cattle suggest a common environmental source of infection (Møller Nielsen *et al*. 1997, Møller Nielsen *et al*. 2000, Schouls *et al*. 2003), as does the temporal overlap in serotypes and genotypes found in Finnish patients and chicken flocks at slaughter (Kärenlampi *et al*. 2003). K36, the KpnI subtype accounting for 57% of the domestic strains not digested by SmaI, was identified in a strain isolated from mink in 2009 (unpublished results from M. Hakkinen). Genotypically similar *C. jejuni* strains are able to colonise a range of hosts (Hopkins *et al*. 2004). Wild birds are carriers of *Campylobacter*, especially during autumn migration (Broman *et al*. 2002, Waldenström *et al*. 2002). Pet animals are possible infection

sources, contact with cats and dogs has been identified as a risk factor in case-control studies (Stafford *et al*. 2007, Doorduyn *et al*. 2010), and *Campylobacter* have been isolated from pet animals (Hald and Madsen 1997, Sandberg *et al*. 2002). *Campylobacter* species are frequently isolated from surface waters (Van Dyke *et al*. 2010, Hörman *et al*. 2004). The origin of the bacteria can be faecal contamination by domestic animals or wild birds, agricultural runoffs or sewage leaks (Abulreesh *et al*. 2006, Bopp *et al*. 2003). Low water temperature enhances the survival of the bacteria (Thomas *et al*. 1999). In addition to infecting humans, water can be the origin of *Campylobacter* colonization in farm animals (Jacobs-Reitsma *et al*. 2008) and seafood (Whyte *et al*. 2004). Vegetables can be contaminated by surface water or by wild birds, and consumption of raw vegetables and fruits has been identified as a risk factor, although the detection rates in vegetables are generally low (Verhoeff-Bakkenes *et al*.2010, Park and Sanders 1992, Abadias *et al*. 2008, Bohaychuk *et al*. 2009). Vegetables and other ready-to-eat foods serve as vehicles in cross-contamination situations in the kitchen. *Campylobacter* are easily transferred from raw chicken meat to cutting boards, knives, and hands, and further to other foods (Kusumaningrum *et al*. 2004). This may be a more important infection route than direct consumption of undercooked poultry meat (Luber *et al*. 2006, Luber 2009). The environmental sources of infections are difficult to find. The diversity of *C. jejuni* genotypes among isolates from environmental sources (water, wild birds and mammals) is high and the overlap with human isolates is usually low (Broman *et al*. 2002, Lindmark *et al*. 2004, Petersen *et al*. 2001).

7 Conclusions

Standardising the cell suspension turbidity of the hippurate test allows reliable identification of hippurate-positive *Campylobacter* strains as *C. jejuni*. Hippurate-negative *C. jejuni* strains cannot be reliably identified with phenotypic methods. Thus, molecular methods, such as the detection of species-specific genes by PCR, are needed.

There is no perfect typing method for the epidemiological surveillance of *C. jejuni*. The large proportion of non-typeable strains lowered the discriminatory ability of serotyping. PCR-RFLP typing of LOS biosynthesis genes offered a high discrimination of both serotypeable and non-typeable strains. However, the method cannot substitute for serotyping since the correlation between serotypes and RFLP-types was not high enough. PFGE was a highly discriminative but a rather labour-intensive typing method. Although the use of two restriction enzymes generally increases the discriminatory ability, KpnI alone offered almost as high discrimination as the use of two enzymes. PFGE typing with KpnI will be the first-choice method in epidemiological investigations in the Bacteriology unit of THL. In the future, other subtyping methods with more easily interpreted and portable results will be considered.

The mean incidence of *Campylobacter* infections was highest in the age group 25-29 and lowest in children aged 5 to 14 and in patients aged 75 and older. Serotype Pen 2 was less common among patients 60 years or older than in younger patients. Pen 2 was also more prevalent in Western Finland than in other parts of the country and more prevalent than other serotypes in winter. These characteristics were not specific to any particular PFGE subtype. Cattle can be considered to be a potential source of Pen 2 infections in humans.

The number of domestic cases was low in winter and peaked in summer, whereas the travel-related infections were more equally distributed. The serotype distribution was different in strains isolated from domestically acquired and travel-related infections. Serotypes Pen 12, Pen 6,7 and Pen 27 were significantly associated with domestic infections, Pen 1,44, Pen 3 and Pen 37 with travel-related infections. Pen 2 and Pen 4-complex were common in both groups but PFGE analysis showed that the genotypes were different. Six temporally persistent genotypes were identified among the domestic strains. They were only occasionally identified in travel-related strains, and therefore, can be considered to be national subtypes. In the future, the serotype and PFGE data will serve as baseline against which possible infection clusters can be compared.

The analysis of indistinguishable SmaI/KpnI subtypes in isolates from patients, chickens and cattle, and their temporal distribution in chickens at slaughter indicated that up to 31% of the human infections could have been mediated by chickens and 19% by cattle during summer 2003. The six persistent subtypes identified in humans were also found in chickens and/or cattle, but there are also other sources of these subtypes.

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