EESTI MAAÜLIKOOL
ESTONIAN UNIVERSITY OF LIFE SCIENCES

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CAMPYLOBACTER SPP. IN POULTRY AND RAW POULTRY MEAT PRODUCTS IN ESTONIA WITH SPECIAL REFERENCE TO SUBTYPING AND ANTIMICROBIAL SUSCEPTIBILITY

KAMPÜLOBAKTERITE ESINEMINE EESTIS KODULINDUDEL JA TOORETES LINNULIHATOODETES, TÜVEDE TÜPISEERIMINE JA ANTIBIOOTIKUMIDELE TUNDLIKKUSE MÄÄRAMINE

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To my mother Tiiu Roasto

CONTENTS

ABSTRACT

Nowadays, *Campylobacter jejuni* and *Campylobacter coli* are the most common registered bacterial causes of human intestinal infections in many developed countries. Several epidemiological studies have shown that handling or eating poultry is an important risk factor for acquisition of campylobacteriosis. Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, contains microbiological criteria for specific food/microorganism combinations and the implementing rules to be complied with by food business operators at all stages of the food chain. To date no criteria have been established for *Campylobacter* spp. in foodstuffs.

The objectives of the present study were: 1) To determine *Campylobacter* spp. in raw retail poultry meat in Estonia in order to provide data for understanding the significance of poultry as a potential source of human *Campylobacter* infection in Estonia, 2) To serotype and PFGE genotype *Campylobacter* isolates originating from raw retail poultry meat to understand the distribution and diversity of serotypes and PFGE genotypes in Estonia, 3) To determine the antimicrobial susceptibility of the isolated *Campylobacter* strains in order to compare it to respective levels in other EU countries and to understand the problem severity in Estonia.

In present study it was found: 1) High proportion of *Campylobacter* spp. positive samples on fresh chicken products of the small-scale company (35.6%) which was significantly more prevalent ($P < 0.001$) than on those originated from the large-scale company (6.3%). Proportion of *Campylobacter* positive samples on fresh chicken products of Estonian origin was 9.1% compared to 15.9% obtained from imported frozen raw poultry products in 2002 and 2003. Compared to raw poultry products collected in Tallinn retail outlets, more commonly *Campylobacter* spp. positive samples were obtained from products collected from Tartu markets. Analysis of seasonality of *Campylobacter* positive samples indicated that the seasonal peak of *Campylobacter* on chicken meat was from June to October, 2) High serotype and genotype diversity among *Campylobacter* isolates from raw retail poultry meat in Estonia. The serotype distribution did not show association with the origin of the sample. The genotyping of the 70 *Campylobacter* isolates showed *Kpn*I to be more discriminatory, yielding 34 PFGE types compared to 29 obtained by *Sma*I. PFGE with the enzymes *Kpn*I and *Sma*I for digestion proved to be discriminatory, repeatable and

reproducible, 3) High resistance patterns of isolated *Campylobacter* spp. strains for several antimicrobials. Multidrug resistance in Estonian broiler chicken isolates was one of the highest reported in latest studies of broiler chicken *Campylobacter* isolates all over the world. Our findings in 2005 and 2006 suggest that the use of fluoroquinolones may select multiresistant strains since resistance to erythromycin, gentamicin or oxytetracycline was exceptional without simultaneous resistance to fluoroquinolones.

Finally, this study which was the first of its kind performed in Estonia, revealed that there are several areas where further studies are required. More studies to monitor the potential *Campylobacter* levels and the reasons for changes in contamination levels with time are needed in Estonia. Antimicrobial susceptibility studies need to be continued to find the trends in levels of *Campylobacter* resistance as well as the mechanisms for resistance and potential to decrease the *Campylobacter* resistance in Estonia. Research based risk assessment, risk management and risk communication has to be performed in Estonia in relation with *Campylobacter* spp. in entire food production chain, and similar *Campylobacter* spp. control programs used in the Nordic countries could be applied in Estonia.

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original articles referred to in the text by the Roman numerals I to III:

I. **Roasto, M**., Praakle, K., Korkeala, H., Elias, P., Hänninen, M.-L. 2005. Prevalence of *Campylobacter* in raw chicken meat of Estonian origin. *Archiv für Lebensmittelhygiene*. **56(3)**, 61-62;

II. Praakle-Amin, K., **Roasto, M**., Korkeala, H., Hänninen, M-L. 2006. PFGE genotyping and antimicrobial susceptibility of *Campylobacter* in retail poultry meat in Estonia. *International Journal of Food Microbiology*. **114**, 105-112;

III. **Roasto, M**., Juhkam, K., Tamme, T., Hörman, A., Häkkinen, L., Reinik, M., Karus, A., Hänninen, M.-L. 2007. High level of antimicrobial resistance in *Campylobacter jejuni* isolated from broiler chickens in Estonia in 2005-2006. *Journal of Food Protection*. **70(8)**, 1940-1944.

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Mati Roasto´s contribution to the papers

I planned the studies, sampled the material, analyzed the samples, evaluated the results, and wrote the manuscript.

II planned the studies, sampled the material, analyzed the samples, and in co-operation with the first author performed sero- and genotyping, and antimicrobial susceptibility tests of the isolated *Campylobacter* strains, had a significant role in writing of the manuscript.

III planned the studies, sampled the material, analyzed the samples, performed antimicrobial susceptibility tests, evaluated the results, and wrote the manuscript.

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
CAMHB	Cation-adjusted Mueller-Hinton broth
CCDA	Charcoal cefoperazone deoxycholate agar
CCP	Critical Control Point
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute, USA
CRL	Community Reference Laboratory
DANMAP	Danish Programme for Surveillance of Antimicrobial Resistance
EELA	National Veterinary and Food Research Institute of Finland (*Eläinlääkintä- ja elintarviketutkimuslaitos*). Since 01.01.2006 Finnish Food Safety Authorithy (Elintarviketurvallisuusvirasto, Evira)
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylene-diamine-tetra-acetic acid
ESP	Buffer including proteinase K
FBP	Ferrous sulphate, sodium metabisulphite and sodium pyruvate
FSIS	Food Safety and Inspection Service, USA
GBS	Guillain-Barré syndrome
GHP	Good Hygiene Practice
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis of Critical Control Points
ISO	International Organization of Standardization
LMP	Low-melting-point agarose
MH	Mueller-Hinton
MIC	Minimal Inhibitory Concentration
MIK	minimaalne inhibeeriv kontsentratsioon
NARMS	National Antimicrobial Resistance Monitoring System

NMKL/NCFA	*Nordisk Metodik Kommite*/Nordic Committee of Food Analysis
OIE	World Organisation for Animal Health
OR	Odd´s ratio
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PHA	Passive hemaglutination
PFGE	Pulsed-field gel electrophoresis
PIV	Tris + NaCl buffer
RAPD	Random amplified polymorphic DNA
REA	Restriction endonuclease analysis
RBC	Red blood cell
SCVMPH	Scientific Committee on Veterinary Measures Relating to Public Health
TBE	Tris-Borate-EDTA solution

1. INTRODUCTION

The name *Campylobacter* is derived from the Greek word "kampylos", which means curved. Organisms resembling campylobacters were first described in 1880 by Theodore Escherich in stool samples of children with diarrhea (Friedman *et al*., 2000). In 1913, McFaydean and Stockman identified campylobacters (called related *Vibrio*) in fetal tissues of aborted sheep. In 1957, King described the isolation of related Vibrio from the cultivation media of blood samples of children with diarrhea, and in 1972, clinical microbiologists in Belgium first time isolated campylobacters from stool samples of patients with diarrhea (Dekeyser *et al*., 1972). Although there have been few ealier case reports, campylobacters have actually been known as important human pathogens only since the late 1970s (Skirrow, 1977). This limited understanding is due to the fact that the organism is very fastidious/fragile and requires specific conditions for growth in vitro in laboratory. Nowadays, *Campylobacter jejuni* and *C. coli* are the most common registered bacterial causes of human intestinal infections in many developed countries (Hänninen *et al*., 2003). In industrialized countries, including Western Europe, USA, Canada, Australia and New Zealand, the rate of human *Campylobacter* infections has been increasing steadily, and the number of reported culture-verified *Campylobacter* cases has exceeded that of salmonella (U.S. Food and Drug Administration, 1999; Friedman *et al*., 2000). The Scientific Committee on Veterinary Measures Relating to Public Health (SCVMPH) issued on 12 April 2000 an opinion on foodborne zoonoses (SCVMPH, 2000). In this opinion the Committee identified *Campylobacter* spp. as one of the public health priorities among the foodborne zoonotic pathogens. The Committee also addressed *Campylobacter* spp. in its opinion of 26-27 March 2003 on the human health risk caused by the use of fluoroquinolones in animals. Campylobacteriosis represent an important public health problem with considerable socio-economic impact in the European Union (EU). *Campylobacter* species, primarily *Campylobacter jejuni* and *Campylobacter coli*, are recognized as a major cause of human gastroenteritis worldwide, with a total of 200,122 cases of laboratory confirmed campylobacteriosis were reported from 22 EU member states and two non-member states in 2005 (EFSA, 2006). In the year 2005, 2631 confirmed campylobacteriosis cases were reported in Norway, 4002 cases in Finland, 3677 cases in Denmark, 5969 cases in Sweden and 124 cases in Estonia. The overall incidence of campylobacteriosis in the EU was 51.6 per 100,000 population, ranging from <0.1 to 302.7 cases per 100,000 population

(EFSA, 2006). However, the true number of positive cases is certainly higher, and in some countries it has estimated to be as much as 30-40 times more than is reported in official registers (Friedman *et al*., 2000). In 2000, 78 campylobacteriosis cases were registered in Denmark but the estimated incidence of *Campylobacter* infections may have been 600-8300 cases per 100,000 population (Rosenquist *et al*., 2003). Transmission to man usually results in sporadic infection and the majority of cases of clinical *Campylobacter* enteritis are sufficiently mild or self-limiting and do not require antimicrobial chemotherapy (Allos and Blaser, 1995). *C. jejuni* infection has been induced with doses as low as 500 bacteria in experimental human infection (Black *et al*., 1988). Infection occurs within 2 to 10 days after exposure to the organism. Symptoms include fever, headache, muscle pain, nausea and bloody diarrhea. Infections, in most cases, are not serious, and symptoms last only about for a week. In a few incidences, the infection can spread to other parts of the body like the vascular or nervous system. *Campylobacter* infections can also cause post-infection complications as reactive arthritis, Miller-Fisher syndrome and Guillain-Barré syndrome (GBS), a disease that affects the nervous system causing paralysis (Patterson, 1995).

Several epidemiological studies have shown that handling or eating poultry is an important risk factor for acquisition of campylobacteriosis (Friedman *et al*., 2000; Kapperud *et al*., 1993; Schönberg-Norio *et al*., 2004). The cross-contamination from raw poultry to food items like fruits and berries is thought to be an important source of infection (Kapperud *et al*., 2003). By proper cooking and handling, *Campylobacter* infection can be reduced (Center for Disease Control and Prevention, 2000). *Campylobacter* require amino acids and tricarbocylic acid cycle intermediates for metabolism which makes the intestinal tracts of most mammalian and avian species ideal for *Campylobacter* colonization. Poultry share a commensal relationship with *Campylobacter*. The type of relationship poultry has with *Campylobacter* makes it a major reservoir for this pathogen. Colonization by this organism may result in carcass contamination during processing and it may potentially spread and cause disease in humans. Studies carried out in slaughterhouses have shown that the main source of the *Campylobacter* contamination of poultry carcasses is their intestinal contents (Mead *et al*., 1995; Newell *et al*., 2001; Stern *et al*., 2003; EC, 2004). *Campylobacter* has been isolated at all phases of poultry production chain, from the live bird throughout the production cycle to the retail products (Doyle, 1984).

Food safety is of paramount importance to consumers and food industry in Europe. For many years, the community of food safety professionals has been trying to draw the attention of consumers and society to the importance of food safety, for health and economy. The importance of public health and its high standard are fundamental objectives of the European Union (EU) food laws as laid down in a European Commission (EC) Regulation No 178/2002. Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, contains microbiological criteria for specific food/microorganism combinations and the implementing rules to be complied with by food business operators at all stages of the food chain. To date no criteria have been established for *Campylobacter* spp. in foodstuffs.

Monitoring programmes are implemented to identify trends in *Campylobacter* infections and evaluate the feasibility of control programmes. A good example is the obligatory monitoring of *Campylobacter* in broilers in the European Union, as required by Directive 2003/99/EC. This directive implemented the monitoring of broiler flocks from 01.01.2005 with the European Food Safety Authority (EFSA) as the agency responsible for compiling and reporting data collected by the EU Member States (Wagenaar *et al*., 2006).

Declines in the incidence of foodborne disease have been reported in some European countries. However, as a total, the incidences are still high and cause considerable economic loss due to health care costs and lost production. In order to reduce the incidence of campylobacteriosis in humans, a number of preventive measures are needed throughout the way from farm to fork. The most efficient measures for preventing *Campylobacter* contamination of broilers are estimated to be biosecurity measures and farm practices aimed at preventing the introduction of *Campylobacter* into flocks (Rosenquist *et al*., 2003). It is necessary to reduce the prevalence of *Campylobacter* both in live birds and in poultry products.

2. REVIEW OF THE LITERATURE

2.1. General characteristics of *Campylobacter* spp.

The genus *Campylobacter* consists of 17 species and 6 subspecies (Euzeby, 2006). These bacteria are microaerophilic (85% N_2, 10% CO_2 and 5% O_2), but some can also grow aerobically or anaerobically. The most important species of *Campylobacter* are the thermophilic species: *C. jejuni* subsp. *jejuni*, *C. coli* and *C. lari*. The species *C. jejuni* comprises two subspecies (*C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*) that can be discriminated on the basis of nitrate reduction, subsp. *doylei* does not reduce nitrate. Subspecies *jejuni* is much more frequently isolated than subspecies *doylei* (OIE, 2004). Other species which are known as gastrointestinal pathogens include *C. sputorum*, C. *upsaliensis*, *C. hyointestinalis*, *C. mucosalis*, *C. fetus* ssp. *fetus* and *C. curvus* (EFSA Journal, 2004; Abbott *et al*., 2005; Euzeby, 2006). *Campylobacter jejuni* and *C. coli* are gram-negative, spirally shaped microaerophilic bacteria, formely classified as *Vibrio fetus*. *Campylobacter* cells are mostly slender, spirally curved rods, 0.2 to 0.5 µm wide and 0.5 to 5 µm long. The rods may have one or more helical turns and can be as long as 8 µm. They also appear S-shaped and gull-wing-shaped when two cells form short chains (Holt *et al*., 1994). Cells of some species are predominantly curved or straight rods. Cells in old cultures may form coccoid bodies which are considered degenerative forms rather than a dormant stage of the organism (Hazeleger *et al*., 1994). Cells of the most species are motile with a characteristic corkscrew-like motion by means of a single, unsheathed, polar flagellum at one or both ends of the cells. Cells of some species like *C. hominis* and *C. gracilis* are nonmotile or have multiple flagella as *C. showae*. The cellular morphology of certain *Helicobacter* spp. is very similar to that of *Campylobacter* spp.; however, the flagella of most *Helicobacter* spp. are sheathed (Rautelin *et al*., 1999). *Campylobacter* spp. are relatively inactive biochemically, obtaining their energy from amino acids or tricarboxylic acid cycles intermediates rather than carbohydrates. Carbohydrates are neither fermented nor oxidized. This makes them difficult to speciate by use of classical biochemical tests (On, 1996), so they are often identified to species level by use of PCR-based methods (Bolton *et al*., 2002; On and Jordan 2003). No acidic or neutral end products are produced. Gelatin is not hydrolyzed. Methyl red and Voges-Proskauer tests are negative and no lipase activity occurs. Pigments are not produced (Holt *et al*., 1994). Only a few biochemical tests

including catalase production, indoxyl acetate hydrolysis, H_2S production and hippurate hydrolysis are useful for differentiation between species (Euzeby, 2006). Problems in the identification to the species level can be related to the fact that many of biochemical tests give variable results for different strains that belong to the same species (On and Holmes, 1995). Campylobacters grow optimally in an atmosphere containing 5% oxygen and have an optimum growth temperature between 30 ºC and 45 ºC. They survive storage at refrigerated temperatures better than at room temperature. The cells are sensitive to freezing, drying and to salt concentrations above 1% sodium chloride. Campylobacters are sensitive to standard concentrations of common disinfectants (National Advisory Committee on Microbiological Criteria for Foods, 1994). *Campylobacter* spp. are relatively sensitive to heat and irradiation, and so can readily be inactivated during cooking (ICMSF, 1996). Their sensitivity to environmental stresses seems to be confirmed by their lack of genes analogous to those in other bacteria, enabling physicological adaptation to adverse environments – e.g., oxidative stress, osmoregulation, starvation/stationary phase, heat and cold shock (Park, 2002).

2.2. *Campylobacter* spp. as a human pathogen

Campylobacter jejuni subsp. *jejuni* and *C. coli* are the main cause of *Campylobacter* enteritis in human (Skirrow and Blaser, 2000). Doses, as low as 500 organisms, have been reported to cause illness (Friedman *et al*., 2000). Accidental ingestion of one drop of raw chicken juice can easily constitute an infectious dose (Newell and Wagenaar, 2000). Children less than one year of age and young adults are more susceptible to developing this disease, and immunocompromised individuals can develop prolonged or more severe disease (Friedman *et al*., 2000). *C. jejuni* is responsible for 80-90% of all campylobacteriosis cases. It causes more disease than *Shigella* spp. and *Salmonella* spp. Main symptom observed is diarrhea which can vary from limited to voluminous stools which may be watery or bloody (Moore *et al*., 2005). *Campylobacter* enteritis is an acute diarrheal disease with clinical manifestations like those of other acute bacterial intestinal infections such as salmonellosis or shigellosis. Clinically it cannot be distinguished from these infections, although the presence of a prodromal period of fever without diarrhea, intense abdominal pain, or prostration would favor a diagnosis of *Campylobacter* enteritis. Most *Campylobacter* gastroenteritis cases do not require other medication besides oral fluid

therapy, but quite a large number of patients are hospitalized and require more intensive care including antibiotic therapy.

A definitive diagnosis can be made only by detecting campylobacters in the fecal samples. Diagnosis of *Campylobacter* gastroenteritis is traditionally done by bacterial culture of fecal sample at selective media and isolation and detection of typical colonies (Skirrow and Blaser, 2000). *Campylobacter* infection may lead to severe but rare sequelae, reactive arthritis (Hannu *et al*., 2004), Guillain-Barré syndrome (Kuwabara, 2004) or even myocarditis (Cunningham and Lee, 2003). Risk for developing Guillain-Barré syndrome is very low, less than 1 per 1,000 infections (Kuwabara, 2004). Guillain-Barré syndrome is a debilitating inflammatory polyneuritis characterized by fever, pain and weakness that progress to paralysis. Other possible autoimmune diseases from *Campylobacter* infections include Miller Fisher syndrome (MFS) and Reiter´s syndrome (Kuroki *et al*., 1993). Deaths attributed to *Campylobacter* infection in the USA are estimated at 680 to 730 per year (Saleha *et al*., 1998).

2.3. Food- and waterborne outbreaks

Most *Campylobacter* infections are sporadic but outbreaks have been traced to raw milk, contaminated water, and contact with pets and farm animals (Hänninen *et al*., 2003; Kuusi *et al*., 2005; Schildt *et al*., 2006). Examples of food- and waterborne outbreaks caused by *Campylobacter* spp. are shown in Table 1. Chicken meat, either directly or via cross-contamination of other produce, was identified as the source of several outbreaks (EFSA, 2005; Eggertson 2005; Mazick *et al*., 2006). Outbreaks occur all over the year, but waterborne outbreaks are most common in the period from August to October (Hänninen *et al*., 2003; Kuusi *et al*., 2004). In contrast, sporadic *Campylobacter* infections are most common in July and August (Altekruse *et al*., 1999).

Table 1. Food- and waterborne outbreaks caused by *Campylobacter* spp.

Country	Product	No. of affected persons	Reference
Germany	chocolate drink made from raw milk	24	Farmer *et al.*, 1980
United Kingdom	contaminated milk	2500	Jones *et al.*, 1981
USA	raw milk	190	Taylor *et al.*, 1982
Norway	drinking water	680	Melby *et al.*, 1991
Canada	drinking water	241	Millson *et al.*, 1991
Hungary	raw milk	52	Erkmen, 1996
United Kingdom, Wales	stir-fried chicken meat pieces	12	Evans *et al.*, 1998
Hungary	raw milk	52	Kalman *et al.*, 2000
Switzerland	drinking water	1607	Maurer and Sturchler, 2000
USA, Kansas	gravy	129	Olsen *et al.*, 2001
Canada	drinking water	> 2000	Clark *et al.*, 2003
Finland	drinking water	~1500	Hänninen *et al.*, 2003
USA	undercooked barbecued chicken meat	11	Eggertson, 2005
Finland	drinking water from municipal water supply	2700	Kuusi *et al.*, 2005
Spain	custard	253	Jimenez *et al.*, 2005
Finland	unpasteurized milk	6	Schildt *et al.*, 2006
Denmark	salad with chicken meat	79	Mazick *et al.*, 2006

2.4. Reservoirs and transmission routes of *Campylobacter* spp.

Campylobacter spp. are fastidious organisms capable of surviving in a wide range of environments. They have been isolated from rivers, lakes, estuarine and coastal waters (Bolton *et al.*, 1987; Jones, 2001; Hörman *et al.*, 2004). The primary reservoir of thermophilic *Campylobacter*, the etiological agents of campylobacteriosis, is the alimentary tract of wild and domesticated birds and mammals. Consequently thermophilic *Campylobacter* spp., especially *C. jejuni* and *C. coli*, are commonly isolated from water sources,

food animals such as poultry, cattle, pigs and sheep, as well as from cats and dogs (Jones, 2001; FAO/WHO, 2002). Overlap is reported between serotypes of *C. jejuni* found in humans, poultry, and cattle, indicating that foods of animal origin may play a major role in transmitting *C. jejuni* to humans (Nielsen *et al*., 1997). Person-to-person transmission is uncommon (Deming *et al*., 1987). Transmission of campylobacters from pets to humans has been confirmed in previous case studies and identified as a potential risk factor in epidemiological investigations, particularly young children in contact with puppies (Sopwith *et al*., 2003). The data of Engvall *et al* (2003) showed that younger dogs shed thermophilic *Campylobacter* spp, which could be of impact of Public Health. Species that carry *Campylobacter* include migratory birds such as cranes, ducks, geese, shorebirds, thrushes and seagulls (Glunder *et al*., 1992; Broman *et al*., 2004; Waldenström *et al*., 2007). Horizontal transmission is belived to be mainly through contaminated water, litter, insects, wild birds, rodents, fecal contact, and by farm personnel via their boots (Line 2001). Feed has not been implicated in the spread of *Campylobacter* because it is often too dry to favour the survival of organism. Some studies have shown vertical transmission as a means of contamination of a breeder flock (Van de Giessen *et al*., 1992; Chuma *et al*., 1994; Pearson *et al*., 1996).
As a result of the widespread occurrence of thermophilic *Campylobacter* spp. in nature and in food animals, the bacteria can readily contaminate various foodstuffs and foods represent a significant risk in regard to human campylobacteriosis (EFSA, 2004).

2.4.1. Prevalence of *C. jejuni* and *C. coli* in poultry and other sources

The avian species are the most common hosts for *Campylobacter*, probably because of their higher body temperature (Skirrow, 1977). Monitoring studies indicate that *C. jejuni* and *C. coli*. colonization in commercial poultry flocks is widespread in many countries. Studies in Europe indicate flock prevalences ranging from 18% to over 90%, with northern European countries showing a lower proportion of positive flocks (Barrios *et al*., 2006). According to the data of the European Food Safety Authority (EFSA) in 2005 six EU member states and one non-member state reported data on prevalence of *Campylobacter* in broiler flocks over the past four years (EFSA, 2006). High flock prevalences (up to 91%) were reported by several countries. Austria, Germany, France and the Veneto

region of Italy have repeatedly reported high prevalences over the years. Denmark observed more moderate prevalences, whereas Sweden, Finland and Norway have consistently reported low flock prevalences (EFSA, 2006). *Campylobacter* contamination has been shown to increase during crating, transportation and holding before slaughter. Potential sources of *Campylobacter* contamination on poultry carcasses are fecal contamination of feathers and skin during transport to the slaughterhouse, leakage of fecal content from the cloaca, intestinal breakage, and contact with contaminated equipment, water, or other carcasses (Jacobs-Reitsma *et al*., 1994).

Studies carried out in slaughterhouses have shown that the main source of the spread of *C. jejuni* on poultry carcasses is the intestinal contents of birds (Stern *et al*., 2003). Intestinal colonisation usually leads to contamination of the final product, which cannot be prevented in the processing plant. Of the fresh broiler meat samples taken at the slaughter in Belgium, Estonia and Sweden 19.6%, 2.2% and 18.5% were *Campylobacter* positive, respectively (EFSA, 2006). Potential for cross-contamination of *Campylobacter* is very high inside the poultry processing plant since poultry entering the processing plant have *Campylobacter* counts ranging from 10^5-10^8 colony forming units (CFU)/g of fecal material (Byrd *et al*., 1998). Contamination may also occur from the environmental sources during the whole production chain. It is well established that poultry products are a vehicle for foodborne campylobacteriosis, and they are suspected to be an important source of infection (Kapperud *et al*., 1992; Hänninen *et al*., 2000; Neimann, 2001; Domingues *et al*., 2002). Disease control studies have demonstrated that in some countries 50% to 70% of human campylobacteriosis is attributed to consuming poultry and poultry products (Allos, 2001). Recent Danish study (Wingstrand *et al*., 2006) showed clearly fresh chicken meat as a main risk factor for campylobacteriosis in Denmark. However, an actual health risk exists only when meat consumed is raw or undercooked (Domingues *et al*., 2002). Examples of prevalence data of *Campylobacter* on fresh poultry products are shown in Table 2.

Table 2. *Campylobacter* spp. on fresh poultry meat

Product	Country of origin	No. of positive samples (%)	Reference
Chicken carcass[a]	Finland	28 (14)	Aho and Hirn, 1988
Goose carcass	Poland	76 (38)	Kwiatek *et al*., 1990
Chicken wings	Nothern Ireland	99 (65)	Flynn *et al*., 1994
Poultry meat[a]	Chile	117 (93)	Fernandez and Pison, 1996
Retail poultry meat	The Netherlands	431 (37)	de Boer *et al*., 1997
Turkey meat	Denmark	78 (25)	Hald *et al*., 1998
Chicken carcass	Japan	13 (59)	Ono and Yamamoto, 1999
Retail chicken meat	Spain	98 (50)	Dominguez *et al*., 2002
Retail poultry meat	South Africa	1 (7)	van Nierop, *et al*., 2005
Retail poultry meat	Estonia	32 (20)	EFSA, 2006
Retail poultry meat	Latvia	125 (10)	EFSA, 2006
Retail poultry meat	Denmark	2686 (20)	EFSA, 2006
Retail poultry meat	Sweden	32 (3)	EFSA, 2006

[a]frozen products

Other foods are also considered as potential sources of infection. *Campylobacter* have also been isolated from such food items as raw milk, pork, beef, lamb, and seafood (Hudson *et al*., 1999; Jakobs-Reitsma, 2000; Duffy *et al*., 2001). Untreated drinking water has been the source of *Campylobacter* infection in many reported cases and waterborne outbreaks associated with contamination of drinking water by *Campylobacter jejuni* are rather common in the Nordic countries Sweden, Norway or Finland, were groundwater in some districts is used without disinfection (Hänninen *et al*., 2003; Kuusi *et al*., 2005; Moore *et al*., 2005). A study done in Finland 2000-2001 reported 17.3% of randomly taken surface water samples to be postitive for *Campylobacter* spp. (Hörman *et al*., 2004).

2.5. Detection of *Campylobacter* spp.

The method of identification/detection of choice depends on whether we need to identify the isolate to the genus or to species level, the proportion of negative samples expected, the spectrum of species required to be detected, the cost in terms of staff-time, materials and equipment available. Furthermore, an important factor affecting the method of choice is whether pure cultures of strains are required for further examination, such as typing for epidemiological studies or examination for antimicrobial resistance. The most frequently used methods for detecting *Campylobacter* in animals at farm, slaughter and in foods are the cultivation methods NMKL Method, vol. 119 and ISO10272-1:2006 E (EFSA 2006). The current ISO method for detection of *Campylobacter* in foods (ISO, 10272-1, 2006) recommends using Bolton broth (1:10 ratio of food to broth), incubating in microaerobic atmosphere at 37 ºC for 4 to 6 hours and then at 41.5 ºC for 44 hours ± 4 hours. The mCCDA plating medium plus one other medium that is based on a principle different from mCCD agar (Skirrow agar, Karmali agar, Preston agar) and incubation at 41.5 ºC in a microaerobic atmosphere are recommended.

2.5.1. Cultivation methods

Campylobacter spp. are sensitive to toxic products of oxygen and therefore most media are supplemented with substances such as whole or lysed blood, FBP (a mixture of ferrous sulphate, sodium metabisulphite and sodium pyruvate), charcoal or haematin plus ferrous sulphate. They grow better on solid media if the surface is not too dry. Consequently, the appearance of colonies can vary considerably, and it is advisable to check colonies growing on selective media for positive oxidase reaction as well as characteristic morphology by Gram stain or phase contrast microscopy (NMKL Method, vol. 119; ISO 10272-1, 2006).

2.5.1.1. Isolation of *Campylobacter* spp.

Isolation of *Campylobacter* from fecal/cecal or intestinal samples is usually performed by direct plating on the selective medium or by using the filtration method on nonselective agar. Enrichment is recommended to enhance the culture sensitivity of potentially environmentally stressed organisms or in the case of low levels of organisms in feces, for example from cattle,

sheep or pigs (OIE, 2004). However, enrichment of the fecal samples is not carried out routinely. Food products generally need enrichment for the culture of usually environmentally stressed and low numbers of campylobacters. After selective enrichment, the samples are subcultured on to solid selective media (NMKL Method, vol. 119; ISO 10272-1, 2006).

2.5.1.2. Selective media for isolation

Many media are currently used for the bacteriological cultivation of *Campylobacter* spp. The selective media can be divided into two main groups: blood-containing media and charcoal-containing media. Blood components and charcoal serve to remove toxic derivatives of oxigen. Most media are commercially available. The selectivity of the media is determined by the antibiotics used. Cephalosporins, cefoperazone most commonly, are used in combination with other antibiotics (e.g. vancomycin, trimethoprim, polymyxin B). Cycloheximide (actidione) and recently more often amphotericin B are used to inhibit yeasts and moulds (Martin *et al*., 2002). The main difference between the various media is the degree of inhibition of contaminating flora. All the selective agents do not inhibit the growth of *C. jejuni* and *C.coli*. There is no medium available that allows growth of *C. jejuni* and inhibits *C. coli* or vice versa. To some extent, other *Campylobacter* species (e.g. *C. lari*, *C. upsaliensis*, *C. helveticus*, *C. fetus* and *C. hyointestinalis*) are also able to grow on most media, especially at the less selective temperature of 37 ºC. Where required, the species of the isolated *Campylobacter* should be determined.

2.5.1.3. Inoculation of media

For samples that do not need enrichment, a small quantity (a loopful) is spread directly, using a loop, on to a solid selective medium to facilitate isolation of single colonies. For food samples that need enrichment, usually 25 g of material is diluted 1/10 in the enrichment medium. Meat samples or complete chicken carcasses can also be washed with saline or phosphate-buffered saline (PBS), after which one volume proportion of this washing fluid is added to nine volume proportions of enrichment medium. Larger volumes of washing fluid can be added to an equal volume of double-strength enrichment broth. When smaller meat samples are used for analysis, they can be washed with enrichment fluid, which is subsequently incubated.
Fecal material/cecal swabs can be enriched. They are placed into 10 ml of enrichment broth, either individually or pooled, and incubated.

2.5.1.4. Incubation

Incubation at a microaerobic atmosphere of 5-10% oxygen, 5-10% carbon dioxide (and preferably 5-9% hydrogen) is required for optimal growth (Corry *et al.*, 1995). Appropriate microaerobic atmospheric conditions may be produced by a variety of methods. In some laboratories, gas jar evacuations followed by atmosphere replacement with specific commercial gas mixtures are used. Gas generator kits are also available from commercial sources. Variable atmosphere incubators are more suitable if large numbers of cultures are undertaken. For enrichment, no specific atmosphere is needed when a small head space (< 2 cm) in the enrichment bottle is used, provided the lid is tightly sealed.

Incubation temperature: Media may be incubated at 37 ºC or 42 ºC temperatures, but a common practice is to incubate at 42 ºC to minimise growth of contaminants and for optimal growth of *C. jejuni*/*C. coli*. For enrichment, specific protocols are sometimes used in which the temperature is increased over a time of incubation in order to recover sublethally injured cells. Enrichment broth is incubated for 24 to 48 hours and streaked after that on to a solid selective medium.
Campylobacter jejuni and *C. coli* usually show growth on solid media within 24-48 hours at 42 ºC. As the additional number of positive samples obtained by prolonged incubation is very low, 48 hours of incubation is recommended for routine diagnosis (Bolton *et al.*, 1988; NMKL Method, vol. 119; ISO 10272-1, 2006).

2.5.1.5. Identification on solid medium

On Skirrow or other blood-containing agars, characteristic *Campylobacter* colonies are slightly pink, round, convex, smooth and shiny, with a regular edge. On charcoal-based media such as mCCDA, the characteristic colonies are greyish, flat and moistened, with a tendency to spread, and may have a metal sheen.

2.5.1.6. Confirmation

Only a few biochemical tests including catalase production, indoxyl acetate hydrolysis, H_2S production and hippurate hydrolysis are useful for differentiation between *Campylobacter* species. A pure culture is required for species identification tests but a preliminary confirmation can be obtained by cell

morphology on microscopy examination of suspect colony material. The confirmatory tests for presence of thermophilic campylobacters are given in Table 3. Problems in identifications are related with the fact that many of these tests give variable results for different strains that belong to the same species. For example, misidentification of *C. jejuni* as *C. coli* is common due to the difficulties in performing the hippurate hydrolysis test (On and Holmes, 1995; Siemer *et al*., 2005). The results of confirmation are validated using the positive and negative controls together with the study samples.

Table 3. Confirmatory tests for thermophilic *Campylobacter* spp. (ISO 10272-1 and 10272-2, 2002; ISO 10272-1: 2006 (E); Euzeby, 2006)

Confirmatory test	Result for thermophilic *Campylobacter*
Morphology	Small curved bacilli
Motility	Characteristic (highly motile and cork-screw like)
Oxidase	Positive Exception is *C. gracilis* which is oxidase negative
Catalase	Positive *C. upsaliensis* is negative or slightly positive
Glucose (TSI)	Negative
Lactose (TSI)	Negative
Sucrose (TSI)	Negative
Gas (TSI)	Negative
Nitrate reduction	Positive Exception is *C. jejuni* subsp. *doylei*
Hippurate hydrolysis	Negative Exceptions are *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* which are hippurate positive
Indoxyl acetate	Positive *C. lari* is negative
H_2S production (TSI)	Negative Exceptions are: *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* Traces of blackening may occur in the presence of *C. coli*
Growth at 25 °C	Negative Exceptions are: *C. fetus* subsp. *fetus*; *C. fetus* subsp. *veneralis* and *C. hyointestinalis* subsp. *hyointestinalis*
Aerobic growth at 41.5 °C	Negative

TSI = triple sugar iron agar

2.5.1.7. Identification of *Campylobacter* to the species level

Among the *Campylobacter* spp. growing at 41.5 ºC, the most frequently encountered species from samples of animal origin are *C. jejuni* and *C. coli*. However, low frequencies of other species (*C. lari*; *C. upsaliensis* and some others) have been described. The characteristics given in Table 3 permit their differentiation. Generally, *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* can be differentiated from other *Campylobacter* species on the basis of the hydrolysis of hippurate as this is the only hippurate-positive species. The presence of hippurate-negative *C. jejuni* strains has been reported (On and Holmes, 1995; Steinhauserova *et al*., 2001; Siemer *et al*., 2005).

Detection of hippurate hydrolysis: Placing a colony with heavy inoculum in a tube containing 0.4 ml of a sodium hippuarate solution. Shaking in oder to mix thoroughly and incubation at 37 ºC for 4 hour. Carefully adding 0.2 ml of a ninhydrin solution on the top of the sodium hippurate solution. Interpretation after an additional incubation of 10 minute in water bath at 37 ºC. A dark violet colour indicates a positive reaction and a pale violet colour or no colour indicates a negative reaction (ISO 10272-1, 2006 E).

Detection of indoxyl acetate hydrolysis: Placing a colony from non-selective Columbia blood agar plate on an indoxyl acetate disc and adding a drop of sterile distilled water. If the indoxyl acetate is hydrolysed, a colour change to dark blue occurs within 5 to 10 minutes. No colour change indicates that hydrolysis has not taken place (ISO 10272-1, 2006 E).

Additionally, detection of catalase; detection of sensitivity to nalidixic acid and to cephalothin could be performed.

2.5.2. Molecular methods

Polymerase chain reaction (PCR)-based methods for the detection of *Campylobacter* in animal fecal samples and enriched meat samples have been described (On, 1996). Target genes used include those specific for the genus *Campylobacter* (*cadF, 16S* rRNA*, 23S* rRNA), those specific for *C. jejuni* (*HipO, ceuE, mapA*) and for *C. coli* (*ceuE*, putative aspartokinase). Some PCR methods can be used without a cultural step, reflecting improved cell concentration, better DNA purification, avoiding components

in food, feces or media that inhibit PCR reactions, as well as more sensitive detection, including enzyme-linked immunosornent assay (ELISA) and nested PCR. Hong *et al*. (2003) used PCR-ELISA for the *CeuE* gene for direct detection of *C. jejuni* and *C. coli* in carcass rinse, with a detection limit of 40 CFU/ml.
The rapid and sensitive detection of *C. jejuni* is necessary for the maintenance of a safe food/water supply, and the real-time PCR assay may provide a specific, sensitive and rapid method for quantitative detection of *C. jejuni* (Yang *et al*., 2003).

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

The subtyping of *Campylobacter* spp. remains an important requirement for epidemiological studies especially for tracing sources and routes of transmission of human infections; identifying and monitoring both temporally and geographically, specific strains with important phenotypic charachteristics; developing strategies to control organisms within the food production chain (Newell *et al*., 2000).

2.6.1. Phenotyping methods

Two serotyping schemes have been developed for *Campylobacter* serotyping, the Penner scheme and the Lior scheme (Penner and Hennessy, 1980; Lior *et al*., 1982). Both techniques give high numbers of untypable strains and are time consuming and technically demanding. The major disadvantages of serotyping are the high number of untypeable strains and limited commercial availability. Other phenotyping methods for differentiating between *Campylobacter* isolates include biotyping (Lior, 1984) and phage typing (Khakhira and Lior, 1992). A modified and extended Penner scheme, in combination with phage typing, is used by the UK Health Protection Agency Laboratory of Enteric Pathogens, to provide a relatively economic and rapid method for use in surveillance of human infection (Newell *et al*., 2000).

2.6.2. Genotyping methods

Tracing the sources and understanding epidemiology of *Campylobacter* is increasingly done by molecular typing (de Boer *et al*., 2000; Nielsen

et al., 2000; Wassenaar and Newell, 2000). Various molecular subtyping methods have been developed including pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) analysis (Hilton *et al*., 1997). Additionally, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the flagella (*flaA* and *flaB*) genes and amplified fragment length polymorphism (AFLP) are useful for epidemiological studies (Newell *et al*., 2000a).
A widely used method for molecular typing of *C. jejuni* is pulsed-field gel electrophoresis, PFGE (Gibson *et al*., 1995; Hänninen *et al*., 2000; Kärenlampi *et al*., 2003). It appers to be a highly discriminatory method especially when used with the two restriction enzymes, *Sma*I and *Sac*II/*Kpn*I (Gibson *et al*., 1997; Hänninen *et al*., 1998; Michaud *et al*., 2001). Using the pulsed-field gel electrophoresis in typing of *Campylobacter* strains has increased the accuracy of epidemiological investigations (Hänninen *et al*., 1998; Moore *et al*., 2001; Hänninen *et al*., 2003). Ribotyping method has been shown to be less discriminatory than PFGE or AFLP (Ge *et al*., 2006). Dingle *et al*. developed a multilocus sequence typing (MLST) scheme for *C. jejuni*, which has been shown to be a valuable tool for studying the diversity and population genetics of *Campylobacter* isolates (Dingle *et al*., 2001; Kärenlampi *et al*., 2007). Microarray-based comparative genomic hybridization (CGH) method has recently been introduced for strain typing (Taboada *et al*., 2004) as well as for comparisons of the gene expression profiles (Gaynor *et al*., 2004).

In conclusion, methods particularly useful for epidemiological studies are: PFGE, MLST, PCR-RFLP of the flagella (*flaA* and *flaB*) genes and AFLP (Hänninen *et al*., 2000; Newell *et al*., 2000a; Dingle *et al*., 2001; Hänninen *et al*., 2003; Kärenlampi *et al*., 2007). Several methods that have been used to study the genotypes of strains of human and poultry origin have indicated that same genotypes are common in human patients and in poultry but that in both groups unique genotypes are also identified. These studies suggest that humans are infected either directly by poultry or that genotypes circulating in the environment have a common source that infects both humans and poultry (Rautelin and Hänninen, 2000). In the study of Rautelin and Hänninen (2000) in the Helsinki area a large variety of PFGE genotypes were identified. Five common genotypes and their variants persisted among human patients, and they accounted for 36-61% of *C. jejuni* strains during a three-year study period, suggesting stability for a restricted group of infecting strains. Important finding of this study was that the same genotypes were also identified among strains

from poultry during the same sampling period. Rautelin and Hänninen (2000) suggested that more precise data on the association of poultry and human infections may be obtained by combining case-control and poultry studies on restricted geographic areas and by using genotyping methods. Wassenaar *et al*., (2000) reported that *Campylobacter* spp. are known to show much greater genomic plasticity than other bacteria such as the *Enterobacteriaceae* – with evidence for changes in gene order, as well as relatively frequent loss and acquisition of DNA. Therefore, to minimize misinterpretation of typing data, it is advisable to use more than one method of typing for epidemiological studies. Swaminathan *et al*. (2000) found both PFGE and *flaA* gene typing to be useful in outbreak investigations (Swaminathan *et al*., 2000).

Increasing interlaboratory collaboration to standardize and harmonize the subtyping techniques in use and the new technologies under development, combined with rapidly improving numerical analysis software and information technology, will allow the establishment of Internet databases for subtype profiles. Such databases will be invaluable tools in the timely monitoring of worldwide changing trends in *Campylobacter* infections (Newell *et al*., 2000). In the USA, since 1995, many public health laboratories have become involved under PulseNet subtyping activities using standardized molecular subtyping methodology which allow the comparison of isolates from different parts of the country enabling the recognition of nationwide outbreaks. In 2000 PulseNet included 32 state public health laboratories and the public health laboratories in New Yourk City, N.Y., and Los Angeles County, California (Swaminathan *et al*., 2000).

2.7. Antimicrobial susceptibility of *Campylobacter* spp.

C. jejuni and *C. coli* show variable susceptibilities to many antimicrobial agents. They are resistant to penicillins, most cephalosporins, trimethoprim and sulfamethoxazole but usually susceptible to erythromycin, clindamycin, amoxicillin-clavulanic acid, imipenem, and aminoglycosides (Reina *et al*., 1994). The in vitro susceptibilities of 478 *Campylobacter jejuni* and *Campylobacter coli* strains isolated from Finnish subjects in 2002-2004 resulted in good activity of erythromycin and telithromycin (macrolides) against campylobacters indicating the possible use of these antibiotics for treatment of campylobacteriosis in humans (Schönberg-Norio *et al*., 2006).

Antimicrobial resistance in *Campylobacter* spp. is increasing, and the most alarming is the increasing resistance to fluoroquinolones. Fluoroquinolone resistant *C. jejuni* was recognized during the late 1980s in Europe (Nachamkin *et al*., 2002). Fluoroquinolones offer an effective therapy against most of the enteric pathogens with ciprofloxacin being used extensively to treat acute bacterial diarrhea (Gibreel *et al*., 1998). Since the 1990s, a significant increase in the prevalence of resistance to macrolides and fluoroquinolones among *Campylobacter* spp. has been reported and this is recognised as an emerging public health problem in many European countries (Engberg *et al*. 2001). Entry of these isolates into the food chain could represent a significant threat to public health. An increasing number of *Campylobacter* isolates resistant to fluoroquinolones are being cultured from both clinical and food samples in several European countries as well as in Canada and the United States (Engberg *et al*., 2001). The development of antimicrobial resistance in pathogenic bacteria is a matter of increasing concern. The increasing rates of human infections caused by antimicrobial resistant strains of *Campylobacter* makes clinical management of cases of campylobacteriosis more difficult (Piddock, 1995; Travers and Barza, 2002). Antimicrobial resistance has emerged among campylobacters mainly as a consequence of the use of antimicrobial agents, especially fluoroquinolones, macrolides and tetracyclines in food animal production. Approval and use of fluoroquinolones in poultry in the Netherlands (Piddock, 1995), Spain (Velazquez *et al*., 1995) and the USA (Smith *et al*., 1999) were followed by increases in fluoroquinolone resistance in *Campylobacter* spp. from poultry and from human clinical cases. In the study of Hakanen *et al*. (2003) the multidrug resistance was found to be significantly associated with resistance to ciprofloxacin and the study of Rautelin *et al*. (2003) showed that of the four different fluoroquinolones studied, ciprofloxacin was the least active (MIC (90), 64 μg/ml). Since campylobacteriosis is transmitted to humans particularly via food of animal origin, the presence of antimicrobial resistant *Campylobacter* isolates in broiler chickens and the widespread emergence of multidrug resistance among *Campylobacter jejuni* are of great concern.

Most cases of human *Campylobacter* enteritis do not require treatment. Nevertheless, in severe or recurrent cases where antibiotics are required, susceptibility testing is important to ensure appropriate and timely treatment (Avrain *et al*. 2003; Rautelin *et al*. 2002; Van Looveren *et al*., 2001). Antimicrobial treatment is appropiate for systematic *Campylobacter* infections in which erythromycin or fluoroquinolones are

often recommended (Jacobs-Reitsma, 1997; Piddock *et al*., 2000; Smith *et al*., 2000; Aarestrup and Engberg, 2001; Engberg *et al*., 2001). Serious systematic infection may also be treated with an aminoglycoside such as gentamicin (Skirrow and Blaser, 2000). Tetracyclines have been suggested as an alternative choice in the treatment of clinical *Campylobacter* enteritis, but in practice they are rarely used. However, macrolides remain the agents of choice, and resistance rates to erythromycin remain comparatively low (Nachamkin *et al*., 2000).

2.7.1. Antimicrobial susceptibility testing

Several laboratory methods, including disc-diffusion, broth microdilution, agar dilution and the E-test have been applied to determine in vitro susceptibility profiles of *Campylobacter* to a range of antimicrobial agents. An approved method for *Campylobacter* susceptibility testing was not available until May 2002, all data generated prior to this date were obtained using non-standard methods (Silley, 2003). Clinical and Laboratory Standards Institute (CLSI) SubCommittee on Veterinary Antimicrobial Susceptibility Testing has recently approved an agar dilution protocol as a valid method (McDermott *et al*., 2004). Compared with the conventional agar dilution method, the E-test trends to give rise to lower minimal inhibitory concentrations (MICs) for sensitive strains and higher MICs for resistant strains (Moore *et al*., 2005). There are no recommended antibiotic breakpoint concentrations for *Campylobacter* spp. A breakpoint is used to separate sensitive strains from resistant strains and is thus crucial to any determination of antibiotic resistance. CLSI uses microbiological, pharmacokinetic and clinical data to establish breakpoints, without such considerations it is not possible to determine the clinical sensitivity and resistance (Silley, 2003).

Molecular techniques offer a mechanistic approach of assessing antimicrobial resistance among bacterial isolates. In a study of quinolone-resistant *Campylobacter* a majority of isolates were shown to possess a common mutation in the *gyrA* gene, whereby many isolates demonstrated a Thr-86-Ile substitution in the A-subunit of DNA gyrase A (Wang *et al*., 1993; Piddock *et al*., 2003). Molecular methods can facilitate analysis of organisms that may be sub-lethally damaged and difficult to grow. These strategies also offer the possibility of screening large numbers of isolates for a specific mutation within a single assay. The disadvantages of using

molecular detection methods include the failure to detect resistance if a new, unexpected or rare resistance mechanism is present or if in a resistant organism no target-specific mutations occur (Moore *et al*., 2005).

2.7.2. Antimicrobial susceptibility monitoring

Campylobacter spp. is included in the National Antimicrobial Resistance Monitoring System (NARMS) in the USA, and in the national monitoring programs for antibiotic resistance in EU countries. With the exception of Spain and Estonia, all EU member states have provided antimicrobial susceptibility data on *Campylobacter* isolates obtained from foods and animals in 2005, through monitoring programs. All countries used agar dilution (MIC) methods for antimicrobial susceptibility testing of *Campylobacter* isolates (EFSA, 2006). In Estonia the antimicrobial susceptibility monitoring program started in 2006.

2.8. Control strategies for *Campylobacter* spp.

A longitudinally integrated approach to control campylobacters along the entire food chain should be adopted for foods of animal origin, in particular, for poultry (Moore *et al*., 2005). Control measures should be directed primarily to the prevention of colonization of *Campylobacter* in food animals by the implementation of biosecurity measures, Good Hygiene Practices (GHP) and husbandry practices which should be incorporated in Hazard Analysis of Critical Control Point (HACCP) -based risk management systems (Whyte *et al*., 2002; Rosenquist *et al*., 2003).

2.8.1. Monitoring programs for *Campylobacter* spp.

In the USA the Food Safety and Inspection Service (FSIS) Chicken Monitoring Program for *Campylobacter* began in October 1998. This program aims to monitor the presence and levels of *C. jejuni/coli* in all types of raw whole poultry carcasses processed in plants operating under federal inspection. Poultry plants are sampled randomly; initially, 120 monthly samples are being taken and an increase in sampling is planned (Ransom *et al*., 2000).

Campylobacter is notifiable in *Gallus gallus* in Finland and Norway, and in all animals in Belgium, Estonia, Latvia, Lithuania, Slovenia, Spain, The Netherlands and Switzerland. In foods, *Campylobacter* is notifiable in Austria, Belgium, the Czech Republic, Estonia, Italy, Latvia, The Netherlands, Slovakia, Slovenia, Spain and Norway (EFSA, 2006). Monitoring programmes for *Campylobacter* in broilers have been implemented in Austria, Denmark, Finland, Italy, Norway, The Netherland and Sweden (EFSA, 2005). The majority of samples of these programs is collected at slaughter phase and analysed bacterilogically. Samples collected in this context are taken either at the slaughterhouse or at the farm, or in both locations. In Denmark, the programme is financed by the poultry industry alone and in Sweden the programme is co-financed by the poultry industry, the Swedish Board of Agriculture and by the European Commission. In Norway, an official action plan against *Campylobacter* spp. was established in 2001 and Finland implemented their control programme in 2004. In Denmark, the incidence of human campylobacteriosis dropped 5% in 2002 from the previous year and further 19% in 2003, possibly as a result of the control programme and intervention (Wingstrand *et al*., 2006). In Norway, the *Campylobacter* monitoring programme includes also intervention programmes as well as in Iceland (Hofshagen and Kruse, 2003; Riersen *et al*., 2003; Stern *et al*., 2003; Hofshagen and Kruse, 2005). An action plan against thermophilic *Campylobacter* spp. in Norwegian broilers was implemented in May 2001. The action plan consists of three parts: a surveillance program including all Norwegian broiler flocks slaughtered before 50 days of age, a follow-up advisory service on farms delivering flocks positive for *Campylobacter* spp., and surveys of broiler meat products at the retail level. Every flock is sampled and 10 fecal swabs per flock are taken 4-8 days prior to slaughter, and re-sampled at the slaughter line. Flocks that are positive for *Campylobacter* based upon the first sampling are slaughtered at the end of the day and the carcasses are either subjected to heat-treatment or are frozen for a minimum of five weeks. Farms delivering flocks that are identified as positive in the surveillance programme will receive consultation regarding hygienic conditions and sanitary measures that will help prevent flocks becoming contaminated with *Campylobacter*. The action plan in Norway is a successful collaboration between academia, regulatory agencies, and the poultry industry that has resulted in a significant reduction in the number of broiler carcasses positive for *Campylobacter* spp. on the market (Hofshagen and Kruse, 2005).

The programs share common principles and in general do focus on: 1) high level of biosecurity at the farm level to prevent flocks from being infected and 2) logistic slaughter i.e. slaughtering positive flocks at the end of the day to prevent cross-contamination at the slaughterhouse.

Furthermore, carcasses from positive flocks may be frozen or subjected to heat treatment. Denmark, Norway and Sweden have all experienced a decrease in the number of *Campylobacter* positive broiler flocks. This may, in part, be explained by the implemented control strategies (EFSA, 2005).

2.8.2. Biosecurity

The most efficient measures for preventing *Campylobacter* contamination of broilers are biosecurity measures and farm practices aimed to prevent the introduction of *Campylobacter* into flocks (Rosenquist *et al*., 2003). Control of *Campylobacter* contamination on the farm may reduce contamination of carcasses, poultry and meat products at the retail level (Kapperud *et al*., 1992; Rosenquist *et al*., 2003). Hygienic barriers should include: 1) strict hygienic routines to be applied when the farm workers enter the rearing room, 2) use of all-in all-out practices, 3) prevent the entry of insects, rodents and wild birds, 4) have in place a program for rodent control, 5) active pest control, 6) exclude animals other than the chicks from the grow-out house, 7) prevent contact with non-authorized personnel, 8) avoid contaminated equipment and transport crates. Poultry houses should be constructed with a functioning hygienic barrier between the inside and outside environments at all times during operation. The house shall be cleaned and disinfected between flocks. Houses and their surroundings should be constructed with hard or concrete surfaces (Van de Giessen *et al*., 1998; National Advisory Committee on Microbiology, 1994). Earlier epidemiological studies indicate that strict hygiene control reduces intestinal carriage of *Campylobacter* in food producing animals (Humphrey *et al*., 1993; Kapperud *et al*., 1993). The drinking water should fulfil the requirements of potable quality and chlorination may be necessary to prevent re-contamination. A nipple drinker system may prevent the fecal contamination of drinking water (Pearson *et al*., 1993; National Advisory Committee on Microbiology, 1994; EFSA Journal, 2004).

2.8.3. Chemical and physical treatments

Prior to the use of chemical and physical decontamination measures the application of good hygienic practices during all processing steps helps to ensure that the contamination of broiler carcasses remains as low as possible. Most important are procedures that keep the fecal spread to an absolute minimum (EFSA Journal, 2004). Cloacal plugging with rayon fibre tampons prior to stunning significantly reduced carcass contamination (Musgrove *et al*., 1997). At processing the potential options to reduce the levels of *Campylobacter* on food animal carcasses include irradiation, chemical decontamination, steam pasteurization and hot water immersion (Molins *et al*., 2001; Whyte *et al*., 2001; Whyte *et al*., 2003). Chemical treatments that reduce *Campylobacter* counts on carcasses include washing of carcasses in electrolysed or chlorinated water (Kapperud *et al*., 1993; Patterson, 1995; Yang *et al*., 2001; Park *et al*., 2002). Electrolysed water treatment during washing of carcasses in combination with chlorine resulted in a 3 $\log_{10}$ reduction of the *Campylobacter* contamination compared to a reduction of 1 $\log_{10}$ obtained by washing in deionised water (Park *et al*., 2002). Immersion of carcasses in 10% sodium triphosphate (TSP) has shown effective microbial decontamination performance (Whyte *et al*., 2001). Campylobacters are sensitive to heat and are inactivated by exposure to pasteurization temperatures (Smibert, 1984). D-value for *C. jejuni* in ground chicken heated at 49 ºC was approximately 20 minutes and at 57 ºC approximately 0.8 minutes (Blankenship and Craven, 1982). Dipping in hot, 80 ºC, water for 20 sec, reduced numbers of *Campylobacter* on carcasses by 2-3 $\log_{10}$ cycles, but as the negative consequence, damaged as well the appearance of the carcasses (Corry *et al*., 2003). Freezing of *Campylobacter*-contaminated carcasses reduces counts by 10 to 100 fold or more and is used in intervention programs in Denmark, Norway, and Iceland (Hofshagen and Kruse, 2003; Reiersen *et al*., 2003). Forced-air ventilation in the cooling operation may be useful during broiler processing, as campylobacters are relatively sensitive to drying (Doyle and Roman, 1982).

The optimal NaCl concentration for growth of *C. jejuni* is 0.5%. Campylobacters are sensitive to higher concentrations of NaCl. A concentration of 2% NaCl in broth held at 30 ºC or 35 ºC is bactericidal (Hänninen, 1981). Campylobacters are sensitive to drying and to standard concentrations of common disinfecting agents including sodium hypochlorite phenolic compounds, iodophors, quaternary ammonium compounds, 70% ethanol and glutaralaldehyde (Wang *et al*., 1983). Modified atmospheres

and vacuum packaging have little inhibitory effect on the survival of the microaerophilic *Campylobacter* spp. In the study of Hänninen *et al.* (1984) and Stern *et al.* (1986) *Campylobacter* organisms survived well both on beef and chicken packaged in oxygen permeable wrap or in various modified atmospheres or in a vacuum when stored at 4 °C.

2.8.4. Other possible control measures

Other possible control measures to eliminate or reduce the contamination of birds with *Campylobacter* include vaccination (Cawthraw *et al.*, 2003), the use of competitive exclusion (Mead *et al.*, 1996), improving genetic resistance of birds (Laisney *et al.*, 2004), and the use of probiotics, bacteriocins or phages (Morishita *et al.*, 1997; Atterbury *et al.*, 2003; Svetoch *et al.*, 2003). Efficiency, costs and applicability of these measures in large-scale production remain to be determined.

2.8.5. Distribution system and consumers

Three factors are critical in control of *Campylobacter* in food distribution: 1) prevention of cross-contamination of other foodstuffs, 2) adequate temperature control and 3) proper packaging. Application of HACCP principles is as important in distribution as in production and processing phases. Consumers and food handlers should be made aware of the role that they play in reducing the incidence of *Campylobacter* infection by preventing cross-contamination in kitchens or in food preparation areas (Humphrey *et al.*, 2001). Three important factors for consumers are: 1) washing and sanitizing of hands, cutting boards, utensils and containers before and after contact with raw poultry and other foods or their ingredients to prevent cross-contamination to fresh produce or ready-to-eat foods, 2) keeping raw and cooked foods separate, and 3) keeping hot foods hot (>60 °C) and cold foods cold (<4.4 °C) (National Advisory Committee on Microbiology, 1994). Consumers are important stakeholders in the food chain and as such they share equal responsibility. Among other things, each of the consumers should learn to understand and apply the basic rules of food hygiene. They should be able to discriminate between hygienic and unhygienic practices and participate in improving food safety in the community.

2.9. Legislation

The general food law of the EU aims to ensure a high level of protection of human life and health, taking into account the protection of animal health and welfare, plant health and the environment. This integrated "farm to fork" approach is now considered a general principle in EU food safety policy. Food law, both at national and EU level, establishes the rights of consumers to safe food and to accurate and honest information (Regulation EC/178/2002). Commission Regulation (EC) No 178/2002 lays down general food safety requirements, according to which food must not be placed on the market if it is unsafe. The use of microbiological criteria should form an integral part of the implementation of HACCP-based procedures and other hygiene control measures. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs constitutes that foodstuffs should not contain microorganisms or their toxins or metabolites in quantities that present an unacceptable risk for human health. According to Article 4 of Regulation (EC) No 852/2004, food business operators are to comply with microbiological food processing and food safety criteria. This should include testing against the values set for the criteria through the taking of samples, the conduct of analyses and the implementation of corrective actions, in accordance with food law and the instructions given by the competent authority.

According to the Directive 2003/99/EC on monitoring of zoonoses and zoonotic agents, Article 4.2 says monitoring of zoonotic microorganisms shall take place at the stage or stages of the food chain most appropriate to the zoonosis and zoonotic agent concerned. Article 4.3 says that monitoring shall cover zoonoses and zoonotic agents listed in Annex I, Part A: brucellosis, *Campylobacter*, listeriosis, salmonellosis etc. This directive implemented the monitoring of broiler flocks from 1 January 2005, with the European Food Safety Authority (EFSA) as the agency responsible for combining and reporting data collected by the EU Member States. Monitoring programmes are implemented to identify trends in *Campylobacter* infections and evaluate the feasibility of control programmes (Wagenaar *et al*., 2006).

Finally, legislation alone is not able to guarantee the quality and safety of the food. Food hygiene legislation and detailed microbiological standards are of low value if the legislation is hard to apply in practice. Most important is the responsibility of animal farmers, food processors and

consumers during the whole chain of food handling operations. The quality of raw materials, the hygienic environment within the food processing enterprise, the processing standards applied, the attitude of food enterprise personnel and education of people working in different stages in the food production chain are all of crucial importance.

3. AIMS OF THE STUDY

The aims of the present study were:

1. To determine *Campylobacter* spp. in raw retail poultry meat in Estonia in order to provide data for understanding the significance of poultry as a potential source of human *Campylobacter* infection in Estonia (I, II, III).

2. To serotype and PFGE genotype *Campylobacter* isolates originating from raw retail poultry meat to understand the distribution and diversity of serotypes and PFGE genotypes in Estonia (II).

3. To determine the antimicrobial susceptibility of the isolated *Campylobacter* strains in order to compare it to respective levels in other EU countries and to understand the problem severity in Estonia (II, III).

4. MATERIALS AND METHODS

4.1. Collection of samples (I, II, III)

In all, 90 fresh chicken meat samples were collected directly at packaging unit of an Estonian small-scale poultry meat plant in 2000 (Table 4). Between January 2002 and December 2003, a total of 580 raw broiler chicken (396 Estonian, 184 imported) and 30 turkey (imported) meat samples were collected from retail stores of Tallinn and Tartu in Estonia. Chicken meat samples obtained from Tallinn were packed and sold both fresh and frozen. All chicken meat samples from market halls of Tartu were sold fresh and unpackaged. The samples were stored chilled (2-4 °C) at retail and obtained during second marketing day at the Tartu market halls. Samples from Tartu were collected into sterile plastic bags which were transported to the laboratory after being placed in a portable cooler at a temperature 4-6 ºC and microbiological analyses were carried out during the same day (Study I and II).

The study in 2005 and 2006 included a total of 1254 fresh fecal samples of chickens from a large Estonian chicken farm containing altogether 60 unconnected flocks in separate housings (20 000 birds per flock) and 264 chicken cecal contents at a slaughterhouse level. Furthermore, a total of 340 fresh chicken meat samples from three food stores in Tartu were analysed. All samples were collected on a monthly basis; fecal samples from the farm between September 2005 and June 2006, cecal samples at a slaughterhouse between July 2006 and October 2006 and meat samples at retail level between September 2005 and September 2006. Fresh chicken meat samples were collected into sterile plastic bags which were transported to the laboratory after being placed in a portable cooler at a temperature of 4-6 ºC and microbiological analyses were carried out during the same day (Study III).

One loopful (10 µl) of fecal material or intestinal contents from the caecum was taken, and the material was transferred into tubes containing 10 ml of Preston enrichment broth (Oxoid; Hampshire, UK). The tubes with enrichment broths were stored at 4 ºC and transported immediately to the laboratory. Enrichment broths were incubated at 42 ± 0.5 ºC for 24 h in microaerobic conditions. Analyses for campylobacters were carried out at the State Veterinary and Food Laboratory, Tartu, Estonia and at the

laboratory of the Department of Food Science and Hygiene of Estonian University of Life Sciences, Tartu, Estonia.

4.2. Isolation of *Campylobacter* spp. (I, II, III)

The isolation of *Campylobacter* from poultry meat samples was carried out in two laboratories in 2002 and 2003. Altogether 290 samples at the Department of Food and Environmental Hygiene, University of Helsinki were analysed using the following method. One hundred milliliters of peptone (0.1%)–saline (0.85%) solution was added to the sample (e.g. broiler leg) in a plastic bag, and the sample was massaged by hand for one minute. Twenty milliliters of the suspension was added into 80 ml of *Campylobacter* enrichment broth (Lab M, Bury, Lancashire, UK) and enriched at 37 ºC for 24 h and 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Microaerobic conditions were produced in jars by using Oxoid gas-generating kits according to the manufacturer instructions (Oxoid, Basingstoke, Hampshire, UK).

The Estonian Veterinary and Food Laboratory in Tartu analysed 320 of the meat samples for *Campylobacter*, in 2002 and 2003, using the method of the Nordic Committee on Food Analysis (NMKL Method, vol. 119), which includes enrichment in Preston broth. The addition of 25 g of sample (minced meat or skin and muscle of breast, carcass, thigh, wing) to 250 ml Preston enrichment broth (Oxoid) followed by the sample being stomached for 60 s. Incubation was carried out at 42 ± 0.5 ºC for 24 h under microaerobic conditions.

In both methods, after 24 h and 48 h incubation a loopful of the enrichment broth was plated on mCCDA (Oxoid), and examined for typical growth after 48 h. Typical grayish, campylobacter-like colonies growing on mCCDA plates were streaked on Brucella blood agar (Oxoid), and confirmed by gram staining, motility analysis, oxidase and catalase test as campylobacters. The isolate from each positive sample was identified as *C. jejuni* as being positive or *C. coli* as being negative in hippurate hydrolysis test. Additionally, an indoxyl acetate hydrolysis test was performed for hippurate negative isolates, and the isolates negative in this test were regarded as *Campylobacter* spp. After the original isolation, the strains were stored at -70 °C in glycerol broth (15% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).
In 2005 and 2006 the study included 105 *Campylobacter jejuni* isolates

from a total of 1254 fresh fecal samples of chickens from a large Estonian chicken farm containing altogether 60 unconnected flocks in separate housings (20 000 birds per flock) and from 264 chicken cecal contents at a slaughterhouse level. Twenty-six additional isolates from 340 randomly purchased fresh chicken meat samples from three food stores in Estonia were analysed (Table 8). All the samples were collected monthly. Fecal samples from the farm were collected between September 2005 and June 2006, cecal samples at a slaughterhouse between July 2006 and October 2006 and meat samples at retail level were collected between September 2005 and September 2006. All 131 isolates were identified as *C. jejuni*.

One loopful (10 µl) of fecal material or intestinal contents from the caecum was taken aseptically, and the material was placed into tubes containing 10 ml of Preston enrichment broth (Oxoid; Basingstoke, Hampshire, England). The tightly capped tubes with enrichment broths were stored at 4 °C and transported immediately to the laboratory. Enrichment broths were incubated at 42 ± 0.5 °C for 24 h in microaerobic conditions. Analyses for campylobacters were carried out at the Veterinary and Food Laboratory in Tartu and at the Laboratory of the Department of Food Science and Hygiene of Estonian University of Life Sciences, Tartu, Estonia. Fresh poultry meat samples were analysed for campylobacters at the Veterinary and Food Laboratory in Tartu using the method of Nordic Committee on Food Analysis as described above.

4.3. Serotyping (II)

A total of 54 *C. jejuni* isolates (chosen arbitrarily) were serotyped using commercial *Campylobacter* antisera according to the manufacturer instructions (Denka Seiken, Tokyo, Japan). Before the serotyping test, the isolates were cultured on Brucella blood agar (Oxoid) plates at 37 °C for 48 h in microaerobic conditions. The manufacturer´s instructions were followed (Annex 1). Serotyping for 54 *C. jejuni* isolates was performed at the laboratory of the Department of Food and Environmental Hygiene, University of Helsinki.

4.4. Genotyping (II)

PFGE typing was performed for 70 *Campylobacter* isolates, representing one isolate from each positive sample. As described previously, *in situ* DNA was isolated and characterized by PFGE (Gibson *et al*., 1994; Hänninen *et al*., 1998). The DNA was digested with *Sma*I or *Kpn*I (New England Biolabs, Beverly, Mass.) (20 U per sample), and the restriction fragments were separated with ramped pulses of 1 to 30 s and 1 to 25 s for 19 h, respectively (Annex 2). The computer software program BioNumerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0%). The dendrogram was constructed using the unweighted pair-group method with arithmetic averages. Genotyping for 70 *Campylobacter* isolates was performed at the laboratory of the Department of Food and Environmental Hygiene, University of Helsinki.

4.5. Antimicrobial susceptibility testing (II, III)

Campylobacter isolates obtained from 2002 and 2003 (Study II) were tested by the disc diffusion method against ampicillin (25 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), and tetracycline (10 µg) (Oxoid), and by the E-test (AB Biodisk, Solna, Sweden) against ampicillin, ciprofloxacin, erythromycin, and tetracycline.

Campylobacter isolates were first grown on blood agar plates and were transferred in 5 ml of Mueller-Hinton (MH) broth (Oxoid), and incubated at 37 °C for 24 h under microaerobic conditions. Inoculum from the MH broth was diluted and a turbidity equivalent of a 0.5 McFarland standard was adjusted in physiological peptone-saline water and the growth suspension was spread on the MH blood agar plates (Oxoid, supplemented with 7% horse blood), the disks or E-test strips containing antimicrobial compounds were laid on the plates. The plates were incubated at 37 °C for 24 h in microaerobic conditions. The diameter of the growth inhibition zone was measured according to the recommendations of Clinical and Laboratory Standards Institute (CLSI, 2004). Minimal Inhibitory Concentration (MIC) values were determined by E-test according to the instructions given by the manufacturer (AB Biodisk). *C. jejuni* 143483 was used as control strain in the antimicrobial susceptibility testing (Hakanen *et al*., 2002).

The following zone diameter (mm) and MIC breakpoints for resistance were applied: ampicillin ≤ 13 mm and MIC ≥ 32 µg/ml, ciprofloxacin ≤ 26 mm and MIC ≥ 4 µg/ml, erythromycin ≤ 26 mm and MIC ≥ 32 µg/ml, gentamicin ≤ 12 mm, nalidixic acid ≤ 26 mm, and tetracycline ≤ 31 mm and MIC ≥ 16 µg/ml (DANMAP, 2004; CLSI, 2004). Antimicrobial susceptibility testing for *Campylobacter* isolates, obtained from 2002 and 2003, was performed at the laboratory of the Department of Food and Environmental Hygiene, University of Helsinki.

All 131 *C. jejuni* isolates, obtained from 2005 and 2006 (Study III), were tested for MIC by a broth microdilution method based VetMIC™ test (National Veterinary Institute; Uppsala, Sweden) against ampicillin, enrofloxacin, erythromycin, gentamicin, nalidixic acid and oxytetracycline. *Campylobacter* isolates were first cultured on Brucella blood agar (Oxoid) and incubated at 37 °C for 48 h. A loopful (1µl) of bacterial growth was transferred into 10 ml of cation-adjusted Mueller-Hinton (CAMHB) broth (Oxoid) and then incubated at 37 °C for 24 hours to achieve a level of around 10^8 CFU/ml. The bacterial suspension was diluted to 10^6 CFU/ml. One hundred microliters (µl) of bacterial suspension was inoculated into each well of microtitre plates. The plates were incubated at 37 °C for 40 h in microaerobic conditions. The MIC was read as the lowest concentration completely inhibiting visible growth of campylobacters in accordance with the instructions given by the test manufacturer. Control of the purity of the bacterial suspension was carried out by plating 10 µl of bacterial suspension on Brucella agar. The density of the bacterial suspension was controlled according to the guidelines of the Estonian Veterinary and Food Laboratory, and colony counts from 50 to 250 per plate were accepted (EELA, 2004; Veterinaar- ja Toidulaboratoorium, 2005). *C. jejuni* ATCC 33560 was used as a control strain in the antimicrobial susceptibility testing. The following MIC breakpoints for resistance were applied: ampicillin 32 µg/ml, enrofloxacin 1 µg/ml, erythromycin 16 µg/ml, gentamicin 8 µg/ml, nalidixic acid 32 µg/ml and oxytetracycline 4 µg/ml (EELA, 2004; Veterinaar- ja Toidulaboratoorium, 2005). The MIC testing for 131 *C. jejuni* isolates, obtained from 2005 and 2006, was carried out at the laboratory of the Department of Food Science and Hygiene of the Estonian University of Life Sciences, Tartu, Estonia.

4.6. Statistical analysis (I, III)

The chi-square test was used for statistical analyses for the *Campylobacter* spp. prevalence data in 2000 and 2002 (Article I). Related with antimicrobial susceptibility testing in 2005 and 2006 all individual results were recorded using MS Excel 2003 software (Microsoft Corporation; Redmond, WA, USA), and the statistical analysis was performed with the Statistical Package for Social Sciences 13.0 for Windows (SPSS Inc.; Chicago, IL, USA). Non-parametric Spearman's rank order correlation coefficients with two-tailed p-values and odds ratios (ORs) were calculated for bivariate cross-correlations between resistances to the six antimicrobials analysed as well as between antimicrobials and multiresistance, which was defined as resistance to three or more unrelated antimicrobials simultaneously. Furthermore, a non-parametric Mann-Whitney independent samples test was conducted to compare the level of antimicrobial resistance between multiresistant and non-multiresistant strains (Article III).

5. RESULTS

5.1. *Campylobacter* spp. on poultry meat products (I-III)

The precise results of *Campylobacter* spp. on poultry meat samples are shown in Tables 4, 5, 6, 7 and 8. In 2000 and 2002, altogether 279 samples (90 originated from small-scale and 189 from large-scale company) were analysed (Tables 4 and 5). Of the raw chicken products, 15.8% were positive for *Campylobacter*. The proportion of *Campylobacter* positive samples in the products (breasts, carcasses, thighs and wings) of the small-scale company (35.6%) were significantly higher than in those originated from the large-scale company (6.3%) ($P < 0.001$). The chicken carcasses and wings (28 and 31.3%) had significantly higher contamination level than breasts and thighs (0 and 0%) ($P < 0.001$).

Table 4. *Campylobacter* spp. positive samples on fresh chicken products[a] of an Estonian small-scale company in 2000

Month of collection	Sampling site	No. of positive samples/total no. of samples (positive %)
January	Carcasses	0/5 (0)
February	Carcasses	0/5 (0)
March	Carcasses	3/7 (42.9)
April	Carcasses	3/7 (42.9)
May	Carcasses	5/7 (71.4)
June	Carcasses	6/9 (66.7)
July	Carcasses	5/9 (55.6)
August	Wings	6/10 (60)
September	Wings	4/10 (40)
October	Thighs	0/6 (0)
November	Breasts	0/6 (0)
December	Breasts	0/9 (0)
Total		**32/90 (35.6)**

[a]samples collected directly at the end of slaughterline

Table 5. *Campylobacter* spp. positive samples on fresh chicken products[a] collected from market halls of Tartu in 2002

Month of collection	Sampling site	No. of positive samples/total no. of samples (positive %)
January	Minced meat	0/30 (0)
February	Legs	0/10 (0)
March	Carcasses	0/5 (0)
April	Carcasses	0/5 (0)
May	Carcasses	0/7 (0)
June	Carcasses	0/7 (0)
July	Carcasses	1/9 (11.1)
August	Wings	7/23 (30.4)
September	Wings	4/24 (16.7)
October	Thighs	0/39 (0)
November	Breasts	0/15 (0)
December	Breasts	0/15 (0)
Total		**12/189 (6.3)**

[a]an Estonian large-scale company

In all, 189 (year 2002), 131 (year 2003) and 340 (year 2005 and 2006) poultry meat samples were collected and analysed from market halls of Tartu (Table 5, 6 and 8). All samples collected in 2002 originated from one Estonian large-scale company. From the fresh chicken products of Estonian origin 29% and from imported frozen raw poultry products 46.4% were positive for *Campylobacter* in 2003. From fresh chicken products of Estonian origin no positive samples were found in January, February and March. In our study in 2002 the *Campylobacter* positive samples of fresh chicken meat from Tartu markets were collected in July, August and September when 11.1%, 30.4% and 16.7% of samples were positive, respectively (Table 5). Our study in 2003 showed that the most *Campylobacter* positive samples of fresh chicken meat of Estonian origin collected from Tartu markets were obtained in June, July, August and November when 37.5%, 50%, 75% and 62.5% of samples were positive, respectively (Table 6). In the later study period the only positive samples were collected in October 2005, and in June/July 2006 (Table 8).

Table 6. *Campylobacter* spp. positive samples on domestic and imported poultry meat products obtained from market halls of Tartu in 2003

Month of collection	Sampling site	Country[a]	No. of positive samples/total no. of samples (positive %)
January	Chicken carcasses	EE	0/7 (0)
February	Chicken legs	EE	0/10 (0)
March	Chicken legs	EE	0/5 (0)
	Chicken wings	US	2/5 (40)
April	Chicken carcasses	US	0/6 (0)
	Chicken wings	DK	3/4 (75)
May	Chicken wings	DK	2/2 (100)
	Chicken wings	EE	0/8 (0)
June	Chicken carcasses	EE	3/8 (37.5)
July	Chicken carcasses	EE	4/8 (50)
August	Chicken carcasses	EE	6/8 (75)
September	Turkey meat	HU	9/15 (60)
October	Turkey meat	HU	13/15 (86.7)
November	Chicken wings	EE	1/4 (25)
	Chicken thighs	DK	0/7 (0)
	Chicken legs	EE	4/4 (100)
December	Chicken legs	DK	3/5 (60)
	Chicken legs	GE	0/10 (0)
Estonian[b]			**18/62 (29)**
Imported[c]			**32/69 (46.4)**
Total			**50/131 (38.2)**

[a]EE, Estonia; US, United States; DK, Denmark; HU, Hungary; GE, Germany
[b]fresh products
[c]frozen products

A total of 610 poultry meat samples were collected in 2002 and 2003 from Estonian retail outlets (Table 7). The proportion of *Campylobacter* positive samples in the products sold in Tartu was significantly higher ($P < 0.001$) than in those collected from Tallinn retail outlets 16.9% and 3.3%, respectively. The most positive samples (18.3%) were obtained from Tartu Turg. From the fresh chicken products of Estonian origin and from imported frozen raw poultry products, 9.1% and 15.9% were positive for *Campylobacter* in 2002 and 2003, respectively.

Table 7. *Campylobacter* spp. positive samples on poultry products at the retail level in Tallinn and Tartu during 2002 and 2003

City/Store	No. of positive samples/no. of total samples (positive %)		
	Estonian[a]	Imported[b]	Total
Tallinn			
A	2/38 (5.3)	NS	2/38 (5.3)
B	0/18 (0)	NS	0/18 (0)
C	0/2 (0)	NS	0/2 (0)
D	1/6 (16.7)	NS	1/6 (16.7)
E	3/32 (9.4)	NS	3/32 (9.4)
F	0/10 (0)	1/95 (1.1)	1/105 (1.0)
G	0/3 (0)	1/18 (5.6)	1/21 (4.8)
H	NS	0/21 (0)	0/21 (0)
Total			**8/246 (3.3)**
Tartu			
I	0/4 (0)	NS	0/4 (0)
J	0/3 (0)	0/11 (0)	0/14 (0)
K	0/6 (0)	NS	0/6 (0)
L	30/270 (11.1)	32/69 (46.4)	62/339 (18.3)
M	0/4 (0)	NS	0/4 (0)
Total			**62/367 (16.9)**
Altogether	**36/396 (9.1)**	**34/214 (15.9)**	**70/610 (11.5)**

[a]fresh products
[b]frozen products
NS, no samples

A total of 1518 fresh fecal and cecal samples at an Estonian chicken farm and at slaughterhouse level were analysed from September 2005 to September 2006. Altoghether, 105 *Campylobacter* positive samples were obtained. Furthermore, 26 samples (7.6%) from 340 randomly purchased fresh chicken meat samples at the retail level in Estonia were positive (Table 8).

Table 8. *Campylobacter* spp. positive samples on fresh broiler chicken leg samples of Estonian origin collected from retail outlets of Tartu in 2005 and 2006

Sampling month/year	No. of positive samples/ total no. of samples	Positive %
September 2005	0/40	0
October 2005	16/45	35.6
November 2005	0/45	0
December 2005	0/30	0
January 2006	0/20	0
February 2006	0/20	0
March 2006	0/20	0
April 2006	0/20	0
May 2006	0/20	0
June 2006	4/20	20
July 2006	6/20	30
August 2006	0/20	0
September 2006	0/20	0
Total:	**26/340**	**7.6**

5.2. Serotypes and genotypes (II)

Serotypes

Eleven Penner serotypes were obtained among 54 *C. jejuni* isolates. Of these isolates, 22% were nontypeable. The most common serotypes O:1,44; O:21, and O:55 accounted for 28%, 13%, and 13% of the isolates, respectively. The isolates from chicken (n = 37) included ten serotypes, and the frequent serotypes were O:1,44 (32%) and O:21 (19%). The isolates from turkey (n = 17) belonged to three serotypes: O:55 (29%), O:1,44 (18%), and O:18 (12%) (Table 9).

Genotypes

The PFGE genotyping of 70 *Campylobacter* isolates yielded 29 *Sma*I and 34 *Kpn*I PFGE types (Table 9). The DNA of five isolates was not digested by *Sma*I. Combination of the macrorestriction patterns resulted in 37 PFGE types. Of these, 33 PFGE types consisted of *C. jejuni* isolates (91%), 2 *C. coli* isolates (6%), and 2 *Campylobacter* spp. isolates (3%) (Article II).

Table 9. Distribution of *Campylobacter jejuni* (*n* = 54) serotypes and *Campylobacter* spp. (*n* = 70) PFGE genotypes isolated from raw retail poultry from Estonia

Country	Serotype (number of certain serotype)	Pulsotypes	
		SmaI	KpnI
Denmark	O:1,44 (4); O:21 (3); O:41 (1)	6, 7, 23	6, 7, 23, 31, 32
Finland	O:1,44 (1)	26	26
United States	O:1,44 (1); NT[b] (2)	4, 5, 10	4, 5, 22
Hungary	O:1,44 (3); O:18 (2); O:55 (5); NT[b] (7)	1, 12, 13, 14, 15, 16, 17, 18, 19, 20	12, 13, 14, 15, 16, 17, 18, 20, 35
Estonia	O:1,44 (6); O:2 (2); O: 4-complex (2); O:11 (1); O:12 (3); O:21 (4); O:27 (1); O:38 (1); O:55 (2); NT[b] (3)	1, 2, 3, 8, 9, 10, 11, 21, 22, 24, 25, 27, 28, 29	1, 2, 3, 8, 9, 10, 11, 21, 22, 24, 25, 27, 29, 30, 33, 34, 36

[a]underlined PFGE type has been detected in poultry originated from more than one country

[b]NT, nontypeable

5.3. Antimicrobial susceptibility (II, III)

In 2002 and 2003 by disc diffusion method, resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 66%, 66%, 44%, 34%, and 14% of the *Campylobacter* isolates (*n* = 70), respectively. Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 44%, 44%, 22%, 19%, and 17% of the Estonian isolates (*n* = 36) and in 88%, 88%, 68%, 50%, and 12% of the imported isolates (*n* = 34), respectively. All isolates were susceptible to gentamicin. Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 50%, 50%, 27%, 23%, and 14% of the chicken isolates (*n* = 48), respectively. Two *C. coli* isolates from chicken showed resistance to ampicillin, ciprofloxacin, and nalidixic acid. One isolate of *Campylobacter* spp. from chicken was resistant to ampicillin,

and the other isolate to ciprofloxacin and nalidixic acid. Of the turkey isolates (n = 22) all were resistant to ciprofloxacin and nalidixic acid, 82% to tetracycline, and 59% to ampicillin. Resistance occurred in 57 isolates (81%) out of 70 tested to at least one of the antimicrobials.
Fifteen isolates (21%) were resistant to one, 30 isolates (43%) to two, and 12 isolates (17%) to three antimicrobial agents. The resistance of *Campylobacter* isolates to two antimicrobials showed a combination of ampicillin and ciprofloxacin (9%), ampicillin and erythromycin (4%), and ciprofloxacin and tetracycline (30%). The resistance of isolates to three antimicrobials showed a combination of ampicillin, ciprofloxacin, and erythromycin (4%), and ampicillin, ciprofloxacin, and tetracycline (13%). The highest level of resistance recorded was to ciprofloxacin (66%) followed by tetracycline (44%), ampicillin (34%), and erythromycin (14%). Results of disk diffusion method and the E-test were similar and all isolates resistant or susceptible by the disk diffusion method showed the same results by E-test. Antimicrobial resistance level (Table 10) was especially high to ciprofloxacin (44 isolates MIC ≥ 32 µg/ml), tetracycline (23 isolates MIC ≥ 256 µg/ml), and ampicillin (22 isolates MIC ≥ 256 µg/ml).

Resistance of isolates of Estonian origin (n = 36) to two unrelated antimicrobials was mainly to a combination of ciprofloxacin/nalidixic acid and tetracycline (8/36 and 22.2%). Three isolates showed a resistance combination of ampicillin and erythromycin, and two isolates of ampicillin and ciprofloxacin/nalidixic acid. We found no simultaneous resistance, of isolated *C. jejuni* strains of Estonian origin, to three or more unrelated antimicrobial agents in 2002 and 2003.

Table 10. MICs for ampicillin (AM), ciprofloxacin (CIP), erythromycin (ERY), and tetracycline (TC) of *Campylobacter* spp. isolates from raw retail poultry meat in Estonia[a] in 2002 and 2003

Anti-microbial agents	No. of isolates with MIC (µg/ml)[b]												
	≤ 0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥ 256
[c]AM				6	12	12	5	5	6	1	1		22
[c]CIP	5	11	4	2	1	1		1	1	44			
[c]ERY		1	8	17	16	6	8	2	2	1			9
[c]TC	13	9	6	6	3	1		1	3	3	2		23

[a] The MIC values for the isolates were evaluated according to the Danmap (2004) and Clinical and Laboratory Standards Institute (2004). Solid vertical lines indicate breakpoints between susceptible and resistant isolates.

[b] The E-test values between two-fold dilutions were rounded up to the next upper two-fold value before the categorization according to manufacturer instructions (AB Biodisk, Solna, Sweden).

[c]70, the number of *Campylobacter* isolates

In 2005 and 2006 resistance to one or more antibiotics was detected in 104/131 of the isolates (79.4%). Twenty isolates (15.3%) were resistant to three unrelated antimicrobials, thirteen isolates (10%) to four unrelated antimicrobials and three isolates (2.3%) to all tested antimicrobials. Enrofloxacin and nalidixic acid were regarded as one group of antimicrobials. Resistance of isolates to three unrelated antimicrobials was mainly to a combination of enrofloxacin/nalidixic acid, erythromycin and oxytetracycline (4.6%). Resistance of isolates to four unrelated antimicrobials was mainly to a combination of enrofloxacin/nalidixic acid, erythromycin, gentamicin and oxytetracycline (8.4%). Three isolates were resistant to five unrelated antimicrobials, comprising a combination of ampicillin, enrofloxacin/nalidixic acid, erythromycin, gentamicin, and oxytetracycline (2.3%). Table 11 shows that the highest frequency of resistance was to nalidixic acid and enrofloxacin (75.6% and 73.3%, respectively), followed by oxytetracycline (32.1%), erythromycin (19.8%), gentamicin (19.1%) and ampicillin (7.6%). Multidrug resistance (to three or more unrelated antimicrobials) was significantly ($p<0.01$) associated with enrofloxacin

and nalidixic acid resistance. The level of antimicrobial resistance was higher for nalidixic acid in multiresistant *C. jejuni* strains than in non-multiresistant strains (Mann-Whitney test, p=0.026), while resistance for other antimicrobials was not statistically different (p>0.05) between multi- and non-multiresistant strains.

Table 11. Antimicrobial sensitivity of *C. jejuni* isolates (*n* = 131) from broiler chickens collected in 2005 and 2006 in Estonia

Antimicrobial agents[a]	Antimicrobial concentration range (µg/ml) VetMIC™Camp	Breakpoints (µg/ml)	No. of resistant strains (%)
Am	0.5-64	32	10 (7.6)
Ef	0.03-4	1	96 (73.3)
Em	0.12-16	16	26 (19.8)
Gm	0.25-8	8	25 (19.1)
Nal	1-128	32	99 (75.6)
Tc	0.25-32	4	42 (32.1)

[a]antimicrobial agents: Am, Ampicillin; Ef, Enrofloxacin; Em, Erythromycin; Gm, Gentamicin; Nal, Nalidixic acid; Tc, Oxytetracyclin

6. DISCUSSION

6.1. *Campylobacter* spp. in raw poultry meat (I-III)

Present studies, initially planned to perform on campylobacters in Estonian poultry products, resulted in detection of high *Campylobacter* contamination levels both in Estonian and imported poultry meat products. High rates of contamination in Estonian small-scale company in 2000 were related to many cross-contamination possibilities, for example the use of water tanks for rinsing of carcasses instead of modern water rinsing systems. Water tanks have previously shown to induce the extensive cross-contamination through contaminated rinsing water and production environment (Bashor *et al.*, 2004; Purnell *et al.*, 2004). Manual procedures in slaughterhouses instead of automated systems are causing problems in hygiene as well. The large-scale poultry meat plant owns many poultry farms and follows good hygiene practices both at farm as well as at the meat processing levels. Air chilling has been suggested to be more efficient than water chilling for decreasing counts of *Campylobacter* and lowering the *Enterobacteriaceae* contamination rates because of the drying effects (Sanches *et al.*, 2002; Rosenquist *et al.*, 2003). The fact that an effective quality-control programme and air chilling systems had been implemented in the large-scale poultry processing plant probably accounted for the lower contamination levels found in the large-scale plant than in the small-scale plant in the present study. One possible reason for differences in occurrence of *Campylobacter* could be that the products from the small-scale plant were taken directly from the line and the samples from large-scale plant were studied after two days of slaughter. Storage at retail may have decreased the number of viable *Campylobacter* cells on chicken meat in our study in 2000 and 2002. Our results indicated that the chicken carcasses and wings were contaminated with *Campylobacter*, but there was no contamination of breasts, legs, minced meat and thighs. The skin on chicken wings is full of wrinkles and has larger feather follicles than the skin on many other parts of the chicken carcass. Open follicles, crevices and wrinkles on the skin offer bacteria more opportunities to persist after rinsing procedure (Berndtson *et al.*, 1992; Chantarapanont *et al.*, 2003). The results of our study indicated that raw poultry products of Estonian origin are contaminated by *Campylobacter* species and the problem appeared to be more severe in small-scale operation. The high *Campylobacter* contamination observed in present study may indicate that

the prevalence of human campylobacteriosis in Estonia is greater than the 124 cases (9.2 cases per 100 000 inhabitant) reported by the Estonian Health Protection Inspectorate in 2006 as well in the year 2005 (Health Protection Inspectorate, 2006).

The proportion of *Campylobacter* spp. positive samples on fresh chicken products of Estonian origin was 9.1% compared to 15.9% obtained from imported frozen raw poultry products at the retail level in Tallinn and Tartu during 2002 – 2003. Frozen storage has been shown to reduce substantially the number of viable campylobacters (Hänninen, 1981; Blaser *et al*., 1983; Stern and Kazmi, 1989). This may relate to the freeze-damaged cells encountered on frozen carcasses (Jorgensen *et al*., 2002). However, laboratory studies have suggested that low numbers of campylobacters may survive for several weeks during frozen storage (Hänninen, 1981; Svedhem *et al*., 1981). It is possible that high prevalence in imported frozen chicken meat at Estonian retail level could be due to the high *Campylobacter* contamination rates during processing at the plant. However, the cross-contamination of the poultry products at the retail level due to the fact that the products were sold unpackaged cannot be excluded.

Countries in Europe, especially in temperate zones, observe a seasonal distribution of human *Campylobacter* infection with a well-defined summer peak (Friedman *et al*., 2000). A distinct seasonality is also observed in New Zealand with a peak incidence in the warmer months of the year (Brieseman, 1990).

The study of Rautelin and Hänninen (2000) showed that the seasonal peak of human *Campylobacter* infection is from June to September when 12-45% of patients acquire their infection in Finland. Reports on Estonian human campylobacteriosis by the Estonian Health Protection Inspectorate in 2006 shows that the most *Campylobacter* human infections occured from June to August when 34.7% *Campylobacter* infection cases were registered in Estonia. Eighty one (65.3%) patients with campylobacteriosis were hospitalized and 3.2% of human *Campylobacter* infection cases were obtained during traveling in abroad. Still there was no well-defined seasonal peaks of human *Campylobacter* infection in 2006 in Estonia (Health Protection Inspectorate, 2006). A two-year national surveillance study in Denmark at broiler chicken farm level showed that most *Campylobacter* positive samples were found in July, August and September, while the lowest number of positive samples was found in January, February, March

and April (Wedderkopp *et al*., 2001). This is in agreement with results in Norway, where *Campylobacter* colonisation was highest from August to November (Kapperud *et al*., 1993). It is well established that poultry products are a vehicle for foodborne campylobacteriosis (EFSA Journal, 2004; Hänninen *et al*., 2000; Neimann, 2001; Domingues *et al*., 2002) as well as it is ascertained that intestinal colonisation usually leads to contamination of the final product, which cannot be prevented in the processing plant (Stern *et al*., 2003). Fecal contamination of carcasses during the slaughter of food animals is virtually inevitable, causing contamination with foodborne pathogenic bacteria such as *Campylobacter*. Seasonal peak level from June to September in the proportion of positive samples detected in our study I and II on domestic chicken meat samples from a small-scale Estonian company (Table 4) and Tartu market halls (Table 5) was detected. The peak level period is similar as seen in the Nordic countries such as Sweden, Finland, Denmark and Norway (Kapperud, 1994; Rautelin and Hänninen, 2000; Wingstrand *et al*., 2006). When the results of imported meat samples are included the seasonal peak is not so distinct and campylobacters are detected in samples over the whole year.

The raw poultry products obtained in Tartu markets showed higher *Campylobacter* contamination in comparison with those collected in Tallinn retail outlets. One possible reason for differences of prevalences could be differences in transportation times and in sample charachteristis. For the samples collected from Tallinn the laboratory analyses were made in Helsinki and all products were sold in package. In Tartu town the analyses were performed almost immediately after sampling and majority of products were sold unpacked. Due to the fact that *Campylobacter* are quite sensitive to environmental stresses the longer transportation time may have decreased the number and detection of *Campylobacter* on chicken meat collected from Tallinn retail outlets. However, more severe contamination of the poultry products at the retail level in Tartu may be associated with the fact that the general hygiene level in Tartu Turg, were most samples were collected, was low during that time and products were sold unpackaged. Packaging can reduce the contamination levels of poultry products with *Campylobacter* spp. *Campylobacter* originating from raw poultry meat can contaminate retail counters and cause cross-contamination of primarily not contaminated poultry meat as well other foods if hygienic procedures are not adequate. This emphasizes the need to follow the strict hygienic conditions and GHPs within entire food chain.

6.2. Serotypes and genotypes (II)

Our studies showed high serotype and genotype diversity among *Campylobacter* isolates from raw retail poultry meat in Estonia. Nine of the eleven *C. jejuni* serotypes obtained were common in poultry products of Estonian origin, and five in those imported to Estonia. The serotype distribution did not show association with the origin of the sample. The most common serotypes were O:1,44; O:21 and O:55, accounting for 54% of the isolates. Serotype distribution differences occurred for chicken and turkey isolates. The chicken isolates had two common serotypes (O:1,44 and O:21) out of ten, whereas turkey isolates belonged to only three different serotypes (O:1,44; O:18 and O:55). In the studies in Denmark (Nielsen and Nielsen, 1999) and New Zealand (Devane *et al*., 2005), the serotype O:1,44 was also one of the most common in poultry products, and this serotype seems to have global distribution among strains isolated from human *Campylobacter* infections (Nielsen *et al*., 1997; Vierikko *et al*., 2004; Devane *et al*., 2005; Miller *et al*., 2005). The most frequently isolated serotype in chicken meat in New Zealand was O:21 (Devane *et al*., 2005), the second most common serotype in our study. The presence of serotypes O:2, O:4-complex and O:12, common to both chickens and human patients (Petersen *et al*., 2001; Saito *et al*., 2005), occurred in only 13% of the isolates studied.

Serotyping of *C. jejuni* showed that 22% of the isolates were nontypeable, and seven of the nontypeable isolates originated from turkey meat imported from Hungary. By using the same commercial serotyping set as in our study, Rautelin and Hänninen (1999) found 14% of the isolates, and in a Danish study, using their own antisera, 16% of the isolates nontypeable remained (Nielsen and Nielsen, 1999) revealing the need to improve the present serotyping methods. One reason for nontypeability is the low production of capsular antigens responsible for the serotype specificity of *C. jejuni*, another reason could be new serotypes not accounted for in the present test (Jacobs-Reitsma *et al*., 1995).

Genotyping of the 70 *Campylobacter* isolates from 2002-2003 showed *Kpn*I to be a more discriminatory enzyme, yielding 34 PFGE types compared to 29 obtained by *Sma*I. Furthermore, the DNA of five strains was not digested by *Sma*I. The genotypes of the isolates from the poultry products of different countries were not overlapping, except *Sma*I PFGE types 1 (isolates from Estonia and Hungary) and 10 (isolates from Estonia and

USA), and *Kpn*I PFGE type 22 (isolates from Estonia and USA). Our results, as well as the data from several previous studies (Gibson *et al*., 1994; Hänninen *et al*., 1998; Wassenaar and Newell, 2000), however, emphasize the utility of two restriction enzymes, such as *Sma*I *and Kpn*I, in PFGE typing studies of *Campylobacter*. In our study the majority of the isolates sharing a similar PFGE genotype originated from one country. The association of genotypes with country of origin requires further studies using a larger collection of isolates.

We found several serotypes within one PFGE type. For example, the PFGE type 4 contained the serotypes O:11, O:55 and nontypeable isolates, PFGE type 27 contained O:4-complex, O:38 and a nontypeable isolate. Furthermore, within one serotype, several PFGE types were found. For instance, the common serotypes of our study, O:1,44; O:21 and O:55, contained up to 12, 3 and 2 different PFGE types. Similar results have been found in other studies (Perko-Mäkelä *et al*., 2002) as well.

6.3. Antimicrobial susceptibility (II, III)

Period 2002 and 2003 (II)

An important finding of our study was the recognition of a high number (81%) of *Campylobacter* isolates with increased antimicrobial resistance. Antimicrobial resistance level was especially high to ciprofloxacin (44 isolates MIC ≥ 32 µg/ml), tetracycline (23 isolates MIC ≥ 256 µg/ml), and ampicillin (22 isolates MIC ≥ 256 µg/ml). The resistance to antimicrobials, except erythromycin, was higher in isolates from imported poultry products than in those originating from Estonia. The *Campylobacter* isolates from turkey meat had a higher resistance to ampicillin, ciprofloxacin, nalidixic acid, and tetracycline than those from chicken meat. All isolates resistant or susceptible by the disk diffusion method showed the same results by E-test.

Ciprofloxacin resistance was high among isolates from both imported (88% of the isolates) and domestic products (44% of the isolates). Furthermore, 100% of the turkey and 50% of the broiler isolates showed resistance to ciprofloxacin. All isolates with resistance to ciprofloxacin were also resistant to nalidixic acid. A study in Spain (Sáenz *et al*., 2000) showed very high prevalence (98%) of ciprofloxacin resistance in *Campylobacter* isolates from broiler intestinal samples. The study by Endtz *et al*. (1991) showed a link for the first time between veterinary fluoroquinolone use

and increasing fluoroquinolone resistance in poultry and human isolates of *Campylobacter*. Later studies have confirmed their results (Smith *et al*., 2000; Engberg *et al*., 2001). Enrofloxacin and flumequine, both fluoroquinolone group antimicrobials, are accepted for poultry in Estonia (Anonymous, 2005), possibly explaining the high level of resistance detected among Estonian isolates.

Various studies typically find tetracycline resistance among poultry isolates. Ledergerber *et al*. (2003) reported a much lower (12%) tetracycline resistance and contrary Ge *et al*. (2003) found a higher resistance (82%) among poultry than the rates established in our study (44%). Neverthless, we found a higher resistance for turkey isolates (82%) than in the Belgian resistance study (37%) (Van Looveren *et al*., 2001). Tetracycline (doxycycline) is also accepted for treatment of poultry in Estonia (Anonymous, 2005).

Ampicillin is a widely used antimicrobial in veterinary medicine. Resistance to ampicillin in broiler isolates, 23%, was at a similar level and resistance in turkey isolates, 59%, was higher than found in the Belgian study, 24% and 33%, respectively (Van Looveren *et al*., 2001). Ampicillin is not recommended for the treatment of *Campylobacter* infections due to the high incidence of resistance to this drug among human isolates (Navarro *et al*., 1993). Amoxicillin is accepted for use in veterinary medicine in Estonia (Anonymous, 2005).

Campylobacter isolates displayed the lowest resistance frequency against erythromycin (14%). All resistant isolates were *C. jejuni* and they were either from Danish or Estonian chicken products. All turkey isolates were susceptible to erythromycin. Belgium, Ireland and Switzerland (Fallon *et al*., 2003; Ledergerber *et al*., 2003; Van Looveren *et al*., 2001) also reported a low erythromycin resistance. Erythromycin is considered as a first line choice for the treatment of *C. jejuni* infections and low resistance among retail meat isolates supports this common policy of antimicrobial use. Additionally, similar to Ge *et al*. (2003) and Van Looveren *et al*. (2001), none of the chicken and turkey isolates showed resistance to gentamycin.

We found a high level (60%) of multidrug (two or three antimicrobial agents) resistant isolates in our study period of 2002 and 2003. Fallon *et al*. (2003) found 30% of the isolates resistant to two or more antimicrobials.

In our study, 69% of isolates consisted of two or three antimicrobials originated from poultry products imported from Denmark or Hungary. All turkey isolates were resistant to two (59%) or three (41%) antimicrobials. Multiresistant isolates consisted of a combination of ampicillin, ciprofloxacin, erythromycin or tetracycline.

In general, the PFGE genotypes and antimicrobial susceptibility profiles correlated, except for PFGE type 16. Seven isolates of this PFGE type showed resistance to ampicillin, ciprofloxacin and tetracycline, whereas four isolates were resistant to ciprofloxacin and tetracycline but sensitive to ampicillin. All of these eleven isolates were from turkey meat originating from Hungary and obtained during the same period. These results may indicate that in a multiresistant bacterial population with identical genotype, the resistance patterns may be different.

Period 2005 and 2006 (III)

In the period of 2005 and 2006, an important finding was the high number (79.4%) of antimicrobial-resistant *C. jejuni* isolates, 36 (27.5%) of which exhibited multiresistance (resistance to three or more unrelated antimicrobials). Resistance was especially high to enrofloxacin, 96 isolates showed MIC ≥ 1 µg/ml which was the breakpoint between the sensitive and resistant isolates (CLSI, 2004). In the present study, two different fluoroquinolones were studied. Cross-resistance between the different fluoroquinolones has been previously documented (Rautelin *et al*., 2002; Griggs *et al*., 2005), as their modes of action are similar (inhibition of DNA gyrase). Most of the *Campylobacter* strains for which enrofloxacin MICs were high were also not inhibited by low concentrations of nalidixic acid. Gentamicin and erythromycin resistance was rather high among our *C. jejuni* strains, 19% and 19.8%, respectively. The reason for this is still unknown, but could be associated with the veterinary use of latter antimicrobial agents in broiler chicken production. High MICs of both macrolides and fluoroquinolones for isolates pose a problem and because erythromycin is considered as a first-line choice of treatment for human *C. jejuni* infections, this resistance has an important public health impact.

We found a high proportion of multidrug-resistant isolates (27.5%). All of these isolates were resistant to enrofloxacin and all, except one, resistant to nalidixic acid. Hakanen *et al.* (2003) noted that 20% of the human isolates associated with traveling were resistant to three or more antimi-

crobials. Multiresistant isolates consisted of a combination of all tested antimicrobials. Our results showed that multidrug resistance was significantly associated with enrofloxacin and nalidixic acid resistance (correlation coefficient 0.372 and 0.310, $p<0.01$). These findings suggest that the use of fluoroquinolones may select multiresistant strains since resistance to erythromycin, gentamicin or oxytetracycline was exceptional without simultaneous resistance to fluoroquinolones. A recent study on antimicrobial resistance of *Escherichia coli* at a farm where no antimicrobial treatment of the birds was performed during one year before the sampling showed that the resistance to tetracycline, gentamycin and streptomycin persisted but all isolates were susceptible to enrofloxacin (Smith *et al*., 2007). Thus multiresistant strains may reflect the past history of antimicrobial usage during a longer period. This phenomenon may partly explain a rather high number of multiresistant strains in our study as well.

7. CONCLUSIONS

1. *Campylobacter* spp. positive samples on fresh chicken products of the small-scale company (35.6%) were significantly more prevalent ($P < 0.001$) than on those originated from the large-scale company (6.3%). The chicken carcasses and wings (28% and 31.3%) had significantly more positive samples ($P < 0.001$) than chicken breasts and thighs (0% and 0%).

2. Proportion of *Campylobacter* positive samples on fresh chicken products of Estonian origin was 9.1% compared to 15.9% obtained from imported frozen raw poultry products at the retail level in Tallinn and Tartu during 2002 – 2003. Higher proportion of *Campylobacter* positive samples on imported frozen poultry products may indicate the presence of high *Campylobacter* contamination at primary production level.

3. Compared to raw poultry products collected in Tallinn retail outlets, more commonly *Campylobacter* spp. positive samples were obtained from products collected from Tartu markets. One possible reason for differences in positive sample proportions could be differences in transportation time of samples to the laboratories, which for the samples collected from Tallinn was several hours longer (laboratory analyses were made at the University of Helsinki) than in Tartu were analyses were performed almost immediately after sampling. However, more severe contamination of the poultry products at the retail level in Tartu may be associated with the fact that the general hygiene level in Tartu Turg, were most samples were collected, was low during that time and products were sold unpackaged.

4. Analysis of seasonality of *Campylobacter* positive samples indicated that the seasonal peak of *Campylobacter* on chicken meat was from June to October.

5. Our studies showed high serotype and genotype diversity among *Campylobacter* isolates from raw retail poultry meat in Estonia. The serotype distribution did not show association with the origin of the sample. The genotyping of the 70 *Campylobacter* isolates showed *Kpn*I to be more discriminatory, yielding 34 PFGE types compared to 29 obtained by *Sma*I. PFGE with the enzymes *Kpn*I and *Sma*I for digestion proved to be discriminatory, repeatable and reproducible. In practice use of the enzyme *Kpn*I is sufficiently discriminatory. PFGE had good typeability and it was a useful tool in molecular typing of isolates from foods. In our study the majority of the isolates sharing a similar PFGE genotype originated from

one country. The association of genotypes with country of origin requires further studies using a larger collection of isolates.

6. Our antimicrobial susceptibility studies of *Campylobacter* strains resulted in high resistance patterns for several antimicrobials. High MICs of both erythromycin and ciprofloxacin pose a problem and because erythromycin is considered as a first-line choice of treatment for human *C. jejuni* infections, the resistance has an important public health impact. Multidrug resistance in Estonian broiler chicken isolates was one of the highest reported in latest studies of broiler chicken *Campylobacter* isolates all over the world. Our findings in 2005 and 2006 suggest that the use of fluoroquinolones may select multiresistant strains since resistance to erythromycin, gentamicin or oxytetracycline was exceptional without simultaneous resistance to fluoroquinolones.

In summary, this study which was the first of its kind performed in Estonia, revealed that there are several areas where further studies are required. More studies to monitor the potential *Campylobacter* levels and the reasons for changes in contamination levels with time are needed in Estonia. Furthermore, similar *Campylobacter* spp. control programs used in the Nordic countries could be applied in Estonia. The general focus of those programs is to high level of biosecurity at the farm level to prevent flocks from being infected and to logistic slaughter i.e. slaughtering positive flocks at the end of the day to prevent cross-contamination at the slaughterhouse. Furthermore, carcasses from positive flocks may be frozen or subjected to heat treatment. Additionally, more effective cooperation between human medicine and veterinary medicine in *Campylobacter* research is needed in Estonia in order to have the best knowledge of *Campylobacter* infection trends and finally to prevent or decrease human *Campylobacter* infections. Multiresistant strains may reflect the past history of antimicrobial usage during a longer period. This phenomenon may partly explain a rather high number of multiresistant strains in our study as well. The widespread emergence of multiresistant isolates poses a threat to humans and limits therapeutic medication. In Estonia, more restricted use of antimicrobial agents, especially fluoroquinolones, in food animal production should be implemented. Antimicrobial susceptibility studies need to be continued to find the trends in levels of *Campylobacter* resistance as well as the mechanisms for resistance and potential to decrease the *Campylobacter* resistance in Estonia. Research based risk assessment, risk management and risk communication has to be performed in Estonia in relation with *Campylobacter* spp. in food production chain.

8. SUMMARY IN ESTONIAN

KAMPÜLOBAKTERITE ESINEMINE EESTIS KODULINDUDEL JA TOORETES LINNULIHATOODETES, TÜVEDE TÜPISEERIMINE JA ANTIBIOOTIKUMIDELE TUNDLIKKUSE MÄÄRAMINE

8.1. Sissejuhatus

Kampülobakterid on üle maailma levinud nii loomade kui inimeste populatsioonis. Kuigi patogeeni ülekande teatud aspektid on seniajani teadmata, on toimunud suur edasiminek nende reservuaaride ja infektsiooni üldise leviku välja selgitamisel. Zoonoosid ehk loomadelt inimestele ülekanduvate haiguste uurimine on oluline mitte ainult nende ohtlikkuse ja sageda esinemise tõttu, vaid ka seepärast, et nad on sageli ettearvamatud ning raskesti diagnoositavad. Eelnev kehtib nii kampülobakterite kui ka teiste zoonootiliste patogeenide suhtes. Perekonna *Campylobacter* mitmed liigid on sageli mets- ja koduloomade (veised, lambad, sead, kitsed, närilised, kassid, koerad ja linnud) maosooletrakti kommensaalideks. Mitmed uuringud on näidanud, et inimestel põhjustavad haigestumist eelkõige loomsetest reservuaaridest pärinevad bakterid. Enamik kampülobakteritest põhjustatud haigustest tekib sporaadiliselt ja nakatumise taust ei ole sageli teada. Nakkusallikateks peetakse tavaliselt linnuliha, toorest või alaküpsetatud liha, pastöriseerimata piimatooteid ning kuumtöötlemata toite, kui on toimunud saastumine töötlemisprotsessis. Toorpiim on samuti sagedane haigestumise põhjustaja. Kampülobakterite probleemi lahendamiseks on olemas nii kohesed kui ka pikemaajalised vastumeetmed. Kohestest meetmetest tuleb märkida pastöriseerimist, toiduainete korralikku läbiküpsetamist (sisetemperatuur tootes vähemalt 72 °C) ning ristsaastumise vältimist igal tasandil. Toiduainete külmutamine vähendab oluliselt toodete kontaminatsiooniastet, kuid mõnede bakterite eluvõime võib püsida mitmeid kuid. Pikemaajalised meetmed hõlmavad tekitajate saastumisastme vähendamist koduloomade ja lindude seas; protseduuride väljatöötamist, mis hävitavad patogeeni enne toidu tarbijani jõudmist ning avalikkuse toiduhügieenialast teavitamist.

8.2. Kirjanduse ülevaade

Kampülobakterid on kõverdunud, S-kujulised või spiraalsed kepid, vanemates kultuurides võivad nad esineda ka kerakujuliste vormidena. Nad on gram-negatiivsed, polümorfsed, mikroaerofiilsed (3–5% O_2) ja oksüdaas-positiivsed mikroorganismid. Nad ei moodusta eoseid, ei fermenteeri ega oksüdeeri süsivesikuid. Kampülobakteritele on iseloomulik kiire ringliikumine tänu üksikule polaarsele viburile (Nachamkin, 1999). Mõõtmetelt on nad väikesed, nende pikkus on 0,5–5,0 µm ning läbimõõt 0,2–0,9 µm.

Perekond *Campylobacter* esindajad kuuluvad sugukonda *Campylobacteraceae* ja tänapäeval on teada nende 17 kampülobakterite liiki ning 6 alamliiki (Euzeby, 2006). Inimeste maosooletraktiga on kõige rohkem seotud järgnevad liigid: *C. jejuni*, *C. coli*, *C. lari*, *C. upsalaensis* ja *C. hyointestinalis* (Hänninen *et al.,* 2000). *C. jejuni, C coli* ja *C. lari* on termotolerantsed ning kasvavad temperatuuridel, mis jäävad vahemikku 30 °C kuni 45 °C. *C. jejuni* ja *C. coli* on enteraalsed bakterid ja inimestele patogeensed. Mõnede juhul on enteriidi põhjustajaks olnud lisaks eelmainitud liikidele ka *C. lari*. *Campylobacter* spp. on paljudes riikides põhiliseks inimeste bakteriaalsete enteraalsete haigestumiste põhjustajaks (Hänninen *et al*., 2003). Lääne Euroopas, USA-s, Kanadas, Austraalias ning Uus-Meremaal on inimestel kampülobakteritest põhjustatud haigestumiste arv pidevalt suurenenud. Paljude autorite andmeil ületab kampülobakterite leid soolestiku talitlushäirete puhul sageli patogeensete ešerihhiate, šigellade ja isegi salmonellade leiu (Tauxe, 1992). *C. jejuni* ja *C. coli* võivad inimestel esile kutsuda raske haigestumise, pikaajalise töövõimetuse ja mõnikord lõpeb haigus surmaga. USA-s tehtud uuringute põhjal kannatab igal aastal vähemalt üks protsent Ameerika Ühendriikide elanikkonnast *Campylobacter* infektsioonist põhjustatud tervise häirete all. Tänapäeval on lisandunud lisaks eelpool mainitud probleemidele ka kampülobakterite suurenenud resistentsus antimikroobsete ainete suhtes. Lähtuvalt eeltoodust avaldas Rahvatervishoiu Veterinaarmeetmete Teaduskomitee (*SCVMPH - Scientific Committee on Veterinary Measures relating to Public Health*) 2000 aastal seisukoha toiduga levivate zoonooside kohta, kus *Campylobacter* spp. kuulutati toiduga levivate zoonootiliste patogeenide seas rahvatervishoiu seisukohalt kõige olulisemaks toidumürgistusi põhjustavaks zoonootiliseks patogeeniks (SCVMPH, 2000). Rahvatervishoiu Veterinaarmeetmete Teaduskomitee 2003 aasta *Campylobacter* spp. alases otsuses nimetati fluorokinoloonide kasutamist loomakasvatuses väga oluliseks terviseriskiks inimestele.

Campylobacter spp. on looduses laialdaselt levinud ning seda ka

põllumajandusloomade populatsioonis. Ta on kommensaalne organism ning teda isoleeritakse sageli veistelt, lammastelt, sigadelt ning kodulindudelt (Friedman *et al*., 2000). Kodulinnud on kampülobakteritele kõige sagedasemad peremeesorganismid ja selle põhjuseks peetakse lindude kõrgemat kehatemperatuuri (Skirrow, 1977). Erinevad epidemioloogilised uuringud on näidanud, et enamik linnufarme on tabandatud *C. jejuni*'ga. Mikroobide intestinaalne kolonisatsioon põhjustab sageli lõpptoodete saastumise, mida on tapamaja tasandil väga raske ennetada.

Põllumajanduslindude tapamajades teostatud uuringud näitasid, et linnurümpade saastumine kampülobakteritega oli tingitud fekaalsest kontaminatsioonist (Berndtson *et al*., 1992; Mead *et al*., 1995; Ono ja Yamamoto, 1999; Stern *et al*., 2003). Sulgede eemaldamisel kasutatakse tapamajades sule folliikulite avanemise soodustamiseks kupatamist, see aga ja sulgede kitkumise protseduur on bakteriaalse ristsaastumise allikateks (Bailey *et al*., 1987). Tapmise ajal võib soolesisaldis sattuda rümba pinnale, saastunud lihaga aga kantakse haigustekitajad inimeseni. Saastunud linnulihaga on kampülobakterioosi haigestumise risk inimesel kõrgeim (Oosterom, 1984).

Linnuliha roll toidupõhise kampülobakterioosi levikus on väga oluline ning seda on tõestanud mitmed teadusuuringud (Kapperud *et al*., 1992; Hänninen *et al*., 2000; Neimann, 2001; Domingues *et al*., 2002). Potentsiaalseteks nakkusallikateks tuleb lugeda ka teisi tooreid ja loomse päritoluga toitusid. *Campylobacter spp*. on isoleeritud toorpiimast, sealihast, veiselihast, lambalihast, karploomadest ja kalalihast (Hudson *et al*., 1999; Jakobs-Reitsma, 2000; Duffy *et al*., 2001).

Kampülobakteritest tingitud haigused esinevad peamiselt sporaadiliste juhtudena, enamasti suvel ja on põhjustatud eeskätt toiduainete ebapiisavast kuumtöötlemisest. Enamasti on inimeste haigestumine kampülobakterioosi tingitud alaküpsetatud kanaliha ja teiste lihtoitude tarbimisest, toorpiima ja töötlemata vee joomisest ning lemmikloomadega (kassid, koerad), kes on sageli eelpool mainitud bakterite kandjateks (WHO, 1994), kokkupuutest, Kampülobakteritest põhjustatud enteriiti esineb kõikidesse vanusegruppi kuuluvatel inimestel, kuid kõige sagedamini haigestuvad väikelapsed ja 20 kuni 40 aastased täiskasvanud (Skirrow, 1977). Haiguspuhangute kevadel ja suvel sagedasemate esinemiste põhjuseks on enamasti kontamineeritud piima ja joogivee tarbimine. Patsiendid, kes on tabandunud *C. jejuni* või *C. coli* infektsioonist, võivad haigestuda sümptomaatiliselt või põdeda

asümptomaatiliselt. Haiguse sümptomitest prevaleerivad palavik, kõhuvalu ja kõhulahtisus (sageli verine). Haigusele iseloomulikke sümptomeid võib täheldada mitme päeva kuni mitme nädala jooksul. Paranemine toimub tavaliselt spontaanselt, kuid püsib suur tõenäosus reinfektsiooniks. Kampülobakteritest tingitud haigestumise korral võivad sümptomid sarnaneda vahel ägeda apenditsiidi sümptomitega ja lõppeda mittevajaliku operatsiooniga. Ekstraintestinaalse infektsioonina või kroonilise järelhaigusena võivad tekkida bakterieemia, artriidid, bursiidid, meningiit, endokardiit, peritoniit, pankreatiit, kuseteede infektsioonid, abort, neonataalne sepsis. Tuhande intestinaalinfektsiooni kohta esineb bakterieemiat pooleteisel korral ja kõrgeim tõenäosus haigestuda on vanematel inimestel. HIV-positiivsetel patsientidel võivad esineda püsiv kõhulahtisus ja bakterieemia ning nende ravi võib osutuda väga raskeks. *C. jejuni* on ka arvatavasti Guillain-Barre sündroomi (GBS) põhjustajaks, mis iseloomustub perifeerse närvisüsteemi paralüütilise kahjustusega (Nachamkin, 1999). Hinnanguliselt umbes ühel kolmandikul Guillain-Barre sündroomiga patsientidel tekivad vastavale haigusele omased sümptomid üks kuni kolm nädalat pärast *C. jejuni* põhjustatud enteriiti (Jay, 1997). Penneri serotüpiseerimise skeemis on üle neljakümne kaheksa *C. jejuni* serotüübi ning serotüüpi numbriga 19 seostatakse GBS-iga. See tüvi omab oligosahhariidide struktuuri, mis on identne peremehe GM 1 gangliossiidide terminaalsete tetrasahhariididega. Kuna gangliossiidid on närvikoepinna komponendid, siis *C. jejuni* oligosahhariidse struktuuri poolt esilekutsutud antikehade produktsioon võib põhjustada antineuraalseid kahjustusi (Jay, 1997). Minimaalne nakatumisannus kampülobakterite puhul ei ole selgelt määratletud, kuid on tõestatud, et ligikaudu 500 mikroorganismi võib põhjustada kampülobakterioosi (Friedman *et al*., 2000).

C. jejuni ja *C. coli* on mitmetes varasemates uuringutes osutunud resistentseks penitsilliinide, tsefalosporiinide, trimetoprim-s*ulfamethoxazole* suhtes. Haigustekitajad on osutunud tundlikuks erütromütsiini, klindamütsiini, imipeneemi, aminoglükosiidide ja amoksitsilliini suhtes (Reina et al., 1994). Tuhande üheksasaja kaheksakümnendatel aastatel võeti eeskätt gramnegatiivsetest bakteritest põhjustatud haigusi põdevate patsientide raviks kasutusele suukaudselt manustatavad fluorokinoloonid: tsiprofloksatsiin, norfloksatsiin ja ofloksatsiin. Mainitud ravimite kasutamise tõttu veterinaar- ja humaanmeditsiinis, ilmnes peagi kampülobakterite tüvede kõrge resistentsus fluorokinoloonide suhtes (Nachamkin *et al*., 2002). Kampülobakterenteriitide ravis on peale kinoloonide kasutusel ka makroliidid (Rautelin *et al*., 2000). Uurimustööde tulemusena on leitud, et kampülobakterid omavad unikaalset võimet resistentsuse tekkimises

kinoloonrühma preparaatide suhtes (Gootz ja Martin, 1991; Smith *et al*., 1999), millest tingituna on Soomes selle preparaatide kasutamine lindude raviks olnud juba mitmeid aastaid keelatud (Mattila *et al*., 1993). Tingituna kõrgest resistentsusest fluorokinoloonidele on humaanmeditsiinis kampülobakterioosi ravis alternatiivsete preparaatidena kasutusel erütromütsiin (makroliidid) ja doksütsükliin (tetratsükliinide ravimgrupp) (Kaijser *et al*., 1994; Rautelin ja Hänninen, 2000).

8.3. Uurimistöö eesmärgid

1. Määrata Eestis müüdavate toorete linnulihatoodete saastatus termofiilsete kampülobakteritega, et selgitada linnuliha kui kampülobakterite potentsiaalse siirutaja roll inimesele (I, II, III).

2. Kampülobakterioosialaste epidemioloogiliste uuringute täiendamiseks ning termofiilsete kampülobakterite sero- ja genotüüpilise jaotumuse ning diversiteedi mõistmiseks teostada isoleeritud kampülobakterite tüvede sero- ja genotüpiseerimine (II).

3. Määrata isoleeritud kampülobakterite tüvede tundlikkus antibiootikumidele, et saadud tulemusi võrrelda teiste Euroopa Liidu maadega, saada selgust probleemi tõsidusest Eestis ja pakkuda välja võimalusi promleemide lahendamiseks (II; III).

8.4. Uurimistöö materjal ja metoodika

Uuritavaks materjaliks olid põhiliselt nii Eestis toodetud, kui Eestisse imporditud toored linnuliha tooted.

Proovid asetati vedelikku mitteläbilaskvasse steriilsesse kilekotti ning transportimiseks kasutati külmakotti, kus temperatuur oli ligikaudu +7 ºC. Proovid analüüsiti Tartu Vetrinaar- ja Toidulaboratooriumis ning Helsingi Ülikooli Loomaarstiteaduskonna Keskkonna- ja Toiduhügieeni osakonna laboratooriumis. *Campylobacter* spp. avastamiseks kasutati NMKL meetodit (NMKL Method, vol. 119), mis sisaldab eelrikastamist Prestoni puljongis. Proov (25 g) kaaluti, asetati steriilsesse kilekotti ning lisati 250 ml Prestoni rikastamipuljongit (Oxoid, Basingstoke, Hampshire, England). Proove muljuti ja loksutati 60 sekundi jooksul.

Uuritav materjal valati steriilsesse Shotti söötmepudelisse, mis suleti õhukindlalt ja inkubeeriti mikroaeroobsetes tingimustes temperatuuril 42 ± 0.5 °C 24 tundi. Rikastussöötmest külvati materjal 10 µl aasaga selektiivagarile (CCDA, *Charcoal Cefoperazone Deoxycholate Agar*, Oxoid). Petri tassid inkubeeriti mikroaeroobselt anaerostaadis, millesse paigutati kampülobakterite optimaalseks kasvuks vajaminevat gaasilist keskkonda tootvad reagentide kotid (Campy-Gen™, Oxoid). Inkubeerimine toimus temperatuuril 42 ± 0,5 °C juures 48 tundi. Kampülobakterite tüüpilised pesad külvati puhaskultuuri saamiseks *Brucella* agarile e. antibiootikumivabale *Campylobacter* söötmele (Oxoid). Tasse inkubeeriti mikroaeroobsetes tingimustes temperatuuril 42 ± 0,5 °C 24 tundi. Kontrolltüveks oli kõikide analüüsi etappide juures *C. jejuni* ATCC 29428. Pärast *Brucella* agaril inkubeerimist teostati kinnitustestid. Liikuvust määrati faas-kontrast mikroskoobi abil. *Brucella* agaril kasvavast värskest külvist võeti baktermass, mis segati lihapuljongiga (kampülobakterid kaotavad vees kiiresti liikumisvõime) ning mikroskoobi abil tehti kindlaks bakterite liikuvus. Kampülobakterid liikusid kiirete pöörlevate liigutustega. Oksüdaastest teostati kommertsiaalsete oksüdaas-tikkudega (Oxoid) ning kokkupuutes kampülobakterite kolooniatega ilmnes positiivne reaktsioon (tiku reagentidega ots värvus tumelillaks). Tsütokroom c test ehk oksüdaastest seisneb selles, et tsütokroom c (cyt c) on heemi sisaldav ensüüm, mis katalüüsib elektronide ülekannet hapnikule, mille tagajärjel tekib vesi ja oksüdeeritud cyt c. Oksüdaastest põhineb *p*-fenüleendiamiini kasutamisel. Tsütokroom c oksüdeerib värvitu dimetüül-*p*-fenüleendiamiini violetseks indofenoolsiniseks. Katalaastest teostati 3% H_2O_2-ga. Alusklaasile kanti värsket baktermassi ning sellele peale asetati tilk 3% H_2O_2-te. Juhul, kui oli tegemist *C. jejuni, C. coli* või *C. lari´*ga, toimus kiire hapnikumullikeste eraldumine, mis viitas positiivsele reaktsioonile. Katalaas lagundab vesinikperoksiidi veeks ja hapnikuks. Bakterid, kes ei ole võimelised kasutama hapnikku, ei produtseeri katalaasi ning katalaastest osutub negatiivseks. Teostati värvimine Grami järgi, mis kampülobakterite puhul oli negatiivne. Gramreaktiivsuse võti peitub grampositiivsete ja gramnegatiivsete bakterite rakukesta erisuguses ehituses. Grampositiivsete bakterite paks peptidoglükaankiht ei lase moodustunud kristallvioletti-joodi värvikompleksi etanooliga välja pesta, sest see on suurem kui rakku sisenenud kristallvioleti molekul. Gramnegatiivsetest bakteritest pestakse kristallviolett-joodi kompleks alkoholiga välja, sest gramnegatiivsete bakterite rakukesta lipopolüsahhariidne välismembraan on alkoholi mõjul muutunud läbilaskvaks ja peptidoklükaankiht on õhuke. Kampülobakterid olid väikesed S- või V-kujuliselt kõverdunud kepid (sageli meenutavad

lendavat kajakat). Kontrolliti kasvu esinemist aeroobsetes tingimustes: veriagarile (Oxoid) kanti CCDA agarilt tüüpilised pesad ning inkubeeriti temperatuuril 37 °C 24 tundi. Kampülobakterite puhul kasv aeroobsetes tingimustes puudus.
Biokeemiline identifitseerimine teostati kampülobakterite tüvede liigilise koosluse määramise eesmärgil. Teostati hipuraat-test ja nalidiksiinhappe tundlikkuse test. Hipuraatide hüdrolüüs: aasatäis kampülobakterite kolooniaid emulgeeriti 0,4 ml naatriumhipuraadi lahuses ning inkubeeriti termostaatkapis üleöö temperatuuril 37 °C. Lahust segati ning seejärel lisati ettevaatlikult (segunemise vältimiseks) 0,2 ml ninhüdriini lahust ja inkubeeriti vesivannil kümme minutit. Positiivse reaktsiooni korral muutus lahus tumesiniseks ja negatiivse reaktsiooni korral helesiniseks. *C. jejuni* andis positiivse reaktsiooni ning *C. coli* ja *C. lari* negatiivse reaktsiooni. *Brucella* agaril säilitati külve umbes ühe nädala jooksul temperatuuril +4 °C mikroaeroobses keskkonnas. Puhaskultuur (vähemalt 1 koloonia igast positiivsest proovist) säilitati glütserooli puljongis (15% [v/v] glütserooli 1%-ses [w/v-mahukaal] proteoos-peptoonis) temperatuuril -70 °C.

Campylobacter jejuni serogruppide määramisel passiivse hemaglutinatsiooni meetodiga (Denka Seiken Co., LTD, Jaapan) koosnes analüüsimine neljast etapist: sensibiliseeritud bakteriaalse antigeeni lahuse valmistamine, fikseeritud punaliblede ettevalmistamine, sensibiliseeritud rakkude ettevalmistamine ja passiivne hemaglutinatsioonireaktsioon e. PHA-test (*passive hemaglutination*).
I etapp:
1,5 ml tsentrifuugi tuubi viidi 0,25 ml soolalahust. Tikupea suurune bakterite mass susbendeeriti soolalahuses ning suspensioonile lisati 0,25 ml ekstraheeritud reagente 1 ja 2 (mõlemat 0,25 ml). Suspensiooni korralikuks läbisegamiseks kasutati tuubi-loksutit (vorteks) ning seejärel inkubeeriti 10 minutit toatemperatuuril. Seejärel lisati 0,25 ml ekstraheeritud reagenti 3, segati korralikult ja tsentrifuugiti viis minutit 7000 p/min (rootori pöörde juurdes minutis) ning seejärel kasutati supernatanti kui antigeeni lahust sensibiliseerimaks kanatibude punaliblesid.
II etapp:
Test-tuubi dispenseeriti ühe analüüsi kohta 0,5 ml fikseeritud punaliblesid test-tuubi (analüüside arv X 0,5 ml) ning seejärel lisati võrdväärne kogus PBS-i ja tsentrifuugiti 10 minutit 3000 p/min.. Seejärel eemaldati supernatant ning resuspendeeriti punalibled PBS-is (testide arv X 0,5 ml).

III etapp:
Igasse 1,5 ml tsentrifuugi tuubi, mis sisaldas 0,5 ml sensibiliseerimiseks vajaminevat bakteriaalset antigeeni lahust**,** lisati 0,5 ml fikseeritud punaliblesid ja inkubeeriti 37 °C juures 30 minutit ning inkubeerimise ajal toimus sage materjali loksutamine. Järgnevalt tsentrifuugiti 30 sekundit 6000 p/min. ja seejärel eemaldati supernatant. Etapi lõpetamiseks lisati 1,0 ml puhvrit ning kasutades tuubi-loksutit resuspendeeriti sadet.
IV etapp
Mikroplaadi süvendisse tilgutati üks tilk konkreetset antiseerumit ning kõrval olevasse kaevu tilgutati üks tilk kontrollseerumit (spontaanse aglutinatsiooni rektsiooni kontrolliks) Igasse kaevu pipeteeriti 25 µl sensibiliseeritud rakkude suspensiooni ning kasutades tuubi-loksutit segati korralikult. Viimase etapi lõpetamiseks inkubeeriti mikroplaati niiskusboksis 30 minutit toatemperatuuril ning seejärel hinnati aglutinatsiooni reaktsiooni.

Campylobacter jejuni ja *Campylobacter coli* DNA-tüpiseerimine teostati kasutades pulseeriva välja geel-elektroforeesi (PFGE). PFGE analüüsiks kasvatati sügavkülmast võetud kampülobakterite isolaate ühe ööpäeva jooksul Brucella agaril (Oxoid) ning seejärel tehti ümberkülv Brucella agarile ning plaate inkubeeriti ööpäeva jooksul mikroaeroobsetes tingimustes temperatuuril 37 °C. Endogeense nukleaasi inaktiveerimiseks töödeldi bakterirakud formaldehüüdiga ning DNA valmistati ette vastavalt Maslow *et al.,* (1993) poolt kirjeldatule. DNA lõhustati *Sma*I ja *Kpn*I ensüümidega 20 ühikut proovi kohta (New England Biolabs Inc.). DNA fragmendid eraldati 1%-ses agaroosgeelis, mis asetati 0,5 X TBE puhvrisse (45 mmol Tris, 45 mmol boorhape, 1 mmol EDTA) 200 V juures ning restriktsiooni fragmendid eraldati 19 tunni jooksul pulsatsioonisagedustega 1-30 sekundit ja 1–25 sekundit, vastavalt ensüümile (*Sma*I ja *Kpn*I). DNA fragmentide eraldamiseks kasutati *Gene Navigator* aparaati (Pharmacia LKB Biotehnology AB, Uppsala, Sweden). Genotüüpide hindamisel kasutati arvutipõhist tarkvara (*BioNumerics*, versioon 3,0; *Applied Maths*, Kortrijk, Belgia) ning visuaalset analüüsi. Omavahel tihedalt seotud genotüüpideks loeti genotüübid, mis erinesid üksteisest 1–3 fragmendi võrra.

Tundlikkuse määramiseks antibakteriaalsete ainete suhtes kasutati aastatel 2002 ja 2003 kogutud isolaatide puhul Kirby-Baueri disk-difusiooni meetodit ning epsilomeeter testi (*E-test, AB Biodisk, Solna, Sweden*) ning hinnati inhibitsioonitsooni ulatust. Aastatel 2005 ja 2006 kogutud tüvede

antibiootikumide tundlikkuse määramisel kasutati minimaalse inhibeeriva kontsentratsiooni määramise testi (*VetMIC™ test, National Veterinary Institute; Uppsala, Sweden*).

8.5. Katsete tulemused ja arutelu

Linnulihatoodete saastatus

Aastatel 2000 ja 2002 uuriti kokku 279 proovi, millest 90 koguti Eesti väikese võimsusega lihakäitlemise ettevõttest ning 189 proovi koguti Eesti suure võimsusega lihakäitlemise ettevõtte toodangust. Eesti suure võimsusega lihakäitlemise ettevõtte toodangu proovid koguti Tartu linna turgudelt. Kõikide antud perioodil kogutud proovide näol oli tegemist jahutatud toodetega, mida säilitati +4 °C kuni +7 °C juures. Aastatel 2000 ja 2002 võrreldi suure võimsusega Eesti lihakäitlemisettevõtte (tabel 5) ning väikese võimsusega Eesti lihakäitlemisettevõtte linnulihatoodete (tabel 4) saastatust termofiilsete kampülobakteritega. Analüüsi tulemused näitasid, et saastumise protsendid olid vastavalt 6,3% ja 35,6% ning seega oli Eesti väikese võimsusega lihakäitlemisettevõtte toodang võrreldes Eesti suure võimsusega lihakäitlemisettevõtte linnuliha toodanguga oluliselt ($P < 0{,}001$) rohkem saastunud termofiilsete kampülobakteritega. Suure võimsusega käitlemisettevõttel olid heade tootmistavade (*GHP - Good Hygiene Practices*) rakendamiseks paremad tingimused ning rakendati efektiivseid kvaliteedi kontrolli programme. Kõrged kontaminatsiooni näitajad Eesti väikese võimsusega käitlemisettevõtte toodangus olid seotud toodete mitmete ristsaastumise võimalustega. Ristsaastumise näitena võiks esitada rümpade automaatsete jahutussüsteemide asemel ebahügieeniliste jahutusvee vannide kasutamise, mis võimaldasid saastunud jahutusvee kaudu mikroobse kontaminatsiooni levikut (Bashor *et al.*, 2004; Purnell *et al.*, 2004). Uurimise käigus võeti väikese võimsusega käitlemisettevõttest rümpade loputusvee proovid ning laboratoorse analüüsi tulemusena selgus, et kõik proovid (n = 10) olid saastunud termofiilsete kampülobakteritega. Lisaks eeltoodule oli väikese võimsusega käitlemisettevõtetes hügieeni probleemiks ka manuaalsete protseduuride kasutamine automaatsete süsteemide asemel. Eesti suure võimsusega linnuliha käitlemisettevõtte omanduses olid mitmed linnufarmid ning head hügieeni tavad kasutusel nii farmi-, tapamaja- kui tootmise tasandil. Vesijahutuse asemel õhkjahutuse kasutamine tapamajades võimaldab oluliselt alandada jahutatud linnulihatoodete kontaminatsioniastet enterobakteritega (Oosterom *et al.*, 1983; Sanches *et al.*, 2002; Rosenquist *et al.*, 2003). Efektiivsete kvaliteedi

kontrolli programmide ning rümpade õhkjahutuse kasutamine võimaldab oluliselt vähendada kontaminatsiooni kampülobakteritega, mida ilmekalt tõestavad ka antud uurimuse tulemused. Antud perioodil teostatud uuringus leiti samuti, et broilerite rümbad ja tiivatükid osutusid oluliselt ($P < 0{,}001$) rohkem saastunuks (28% ja 31,3%), kui broilerite rinna- ja kintsutükid (0% ja 0%). Tiivatükkidel on nahapind kurrulisem ja sulefolliikulid suuremad, mis loob bakteritele loputamise järgselt nahapinnale püsima jäämise suuremad võimalused. Avatud sulefolliikulid, naha kurrud ja kriimustused võimaldavad kampülobakteritel nahapinnale kinnituda ning püsima jääda ka pärast intensiivset rümpade loputamist (Berndtson et al., 1992; Chantarapanont *et al*., 2003). Folliikulite sulgumisel jahutamisprotseduuri jooksul on tõenäoline, et folliikulitesse jäävad püsima ka mõned sinna eelnevalt sattunud mikroorganismid (Cason *et al*., 1999).

Aastatel 2002 ja 2003 uuriti kokku 610 linnuliha proovi. Eesti päritoluga toodete proove oli 396 (64,9%) ning importtoodangu proove 214 (35,1%). Laboratoorseks uurimiseks võeti erinevaid jahutatud ja külmutatud linnuliha tooteid: kanarümbad, kanatiivad, kanahakkliha, rinna- ja kintsutükid ning kalkuni koivad. Eesti päritolu toodetest osutusid kampülobakterite suhtes positiivseteks 36 proovi (9,1%) ning importtoodetest 34 (15,9%). Eesti päritoluga tooted olid jahutatud ning välismaise päritoluga tooted külmutatud. Külmutamise protsessis kampülobakterid enamasti hukuvad, mõned saavad subletaalseid kahjustusi, kuid säilitavad eluvõime. Kampülobakteritele subletaalseid kahjustusi põhjustavad tegurid on madal temperatuur, osmootne stress ning toitainete puudus (Humphrey, 1994). Eelnevad uuringud on tõestanud, et isegi väga väikese arvu kampülobakterite esinemine toidus võib põhjustada inimeste haigestumise (Kapperud *et al*., 1992). Külmutatud importtoodete kõrgemad saastumise näitajad võrreldes Eesti päritolu jahutatud linnuliha toodetega, on tingitud kõrgest algkontaminatsioonist, mille tulemusel külmutamine ei hävita kõiki kampülobaktereid. Eesti jahutatud linnuliha toodete saastumise protsent (9,1%) on madalam kui enamikus arenenud riikides, kus kontaminatsiooni näitajad ulatuvad isegi üle viiekümne protsendi (Dominguez *et al*., 2002). Toore ja külmutatud linnuliha ostmine jaekaubandusest ning selle tarbimine pärast ebapiisavat kuumtöötlemist on olulisteks riskifaktoriteks sporaadilise *Campylobacter* infektsiooni tekkes (erinevuse määr = 2,42; P = 0,042) (Kapperud *et al*., 1992).

Efektiivsete kvaliteedi kontrolli programmide ning tapamajades rümpade õhkjahutuse rakendamine tingib madalama kampülobakteritega saastumise,

mida tõestavad ka antud uurimistöö tulemused (I). Võrreldes Eesti toodetega olid importtooted rohkem saastunud ning tuginedes eeltoodud tulemuste analüüsile, võib kinnitada, et kodumaiste linnuliha toodete tarbimine on ohutum, kusjuures tõhustada tuleb importtoodete ja nende tootjate kontrolli.

Septembrist 2005 kuni Septembrini 2006 (13 kuud) koguti ja analüüsiti kokku 340 Eesti päritolu jahutatud broileriliha proovi. Termofiilsete kampülobakterite suhtes osutus positiivseks 26 proovi (7,6%), millest 16 proovi saadi oktoobris (35,6%), neli juunis (20%) ning kuus juulis (30%). Ülejäänutel kuudel kogutud proovide analüüsimisel selgus, et termofiilseid kampülobaktereid proovides ei esinenud.

Kampülobakterite sero- ja genotüüpiline jaotumus

Eestis jaekaubandusest kogutud toorest linnulihast isoleeritud *Campylobacter* spp. tüved osutusid sero- ja genotüüpselt jaotuselt mitmekesiseks. Viiekümne neljast *Campylobacter jejuni* tüvest identifitseeriti 11 erinevat serotüüpi, millest 9 seondusid eelkõige Eesti toodetest isoleeritud ning 5 importtoodetest isoleeritud tüvedega. Serotüüpilise jaotumuse ja toodete päritolu (müügipunktide) vahel ei esinenud selget seost. Töös kasutatud serospetsiifilise seerumiga (Denka Seiken Co., LTD, Jaapan) osutusid 12 tüve mitte serotüpiseeritavateks ning kolm tüve kuulusid kompleks-serotüüpi. Kõige rohkem tüvesid (54%) kuulusid serotüüpidesse O:1,44; O:21 ja O:55, vastavalt 28%, 13% ja 13%. Eesti päritolu toodetest identifitseeriti kaheksa erinevat *Campylobacter jejuni* serotüüpi, üks kompleks serotüüp ning kolm tüve osutusid mitte serotüpiseeritavateks. Broilerilihast identifitseeriti kõige rohkem *Campylobacter jejuni* tüvesid, mis kuulusid serotüüpidesse O:1,44 (32%) ning O:21 (19%). Tüved, mis kuulusid serotüüpi O:1,44 isoleeriti Eesti, Taani, Ameerika Ühendriikide, Ungari ja Soome päritoluga linnulihast ning seega omas antud serotüüp ulatusliku geograafilise leviku. Erinevates riikides teostatud varasemad uuringud (Nielsen *et al*., 1997; Vierikko *et al*., 2004; Devane *et al*., 2005 ja Miller *et al*., 2005) on samuti tõestanud isoleeritud kampülobakterite tüvede kuulumise serotüüpi O:1,44. Ungari päritolu kalkunilihast isoleeritud *Campylobacter jejuni* tüved (n = 17) jaotusid kolme erinevasse serotüüpi: O:55 (29%), O:1,44 (18%) ning O:18 (12%). Serotüüpidesse O:2, O:4-kompleks ning O:12, mis eelnevate uuringute alusel on osutunud omasteks nii lindudele kui inimestele, kuulus meie uuringute alusel kokku 13% kampülobakterite isolaatidest.

Seitsmekümne *Campylobacter*'i isolaadi genotüpiseerimine andis 29 *Sma*I ning 34 *Kpn*I PFGE tüüpi, mis osutas restriktsiooniensüümi *Kpn*I

suuremale genotüpiseerimise võimele. Veelgi enam osutas *Kpn*I kasuks fakt, et viie *Campylobacter´*i isolaadi DNA ei lõhustunud üldse *Sma*I ensüümi kasutades. Üldiselt võib öelda, et erinevate riikide toodangust isoleeritud kampülobakterite tüved ei kattunud genotüüpiliselt koosluselt üksteisega, väljaarvatud *Sma*I PFGE-tüüp 1, millesse kuulusid nii Eesti kui Ungari tüved ning *Kpn*I PFGE-tüüp 22, millesse kuulusid nii Eesti kui Ameerika Ühendriikide toodangust pärit kampülobakterite tüved. Enamik tüvedest, mis kuulusid samasse PFGE genotüüpi pärinesid konkreetse riigi toodetest. Makrorestriktsiooni kombinatsioon andis 37 PFGE-tüüpi, millest 33 koosnesid *C. jejuni* (89%), kaks *C. coli* (5,5%) ning kaks *Campylobacter* spp. isolaatidest (5,5%). Uuringutega leiti, et ühte PFGE-tüüpi kuulunud kampülobakterite tüved kuulusid sageli erinevatesse serotüüpidesse ning vastupidi.

Kampülobakterite tüvede tundlikkus antibiootikumidele

Antud uurimistöös teostati kampülobakterite antibiootikumidele tundlikkuse määramine aastatel 2002 ja 2003 isoleeritud ning aastatel 2005 ja 2006 isoleeritud tüvedega. Mainitud varasemal perioodil (aastatel 2002 ja 2003) isoleeritud kampülobakterite tüvedega (n = 70) teostati antibiootikumide tundlikkuse määramine ampitsilliini, erütromütsiini, gentamütsiini, nalidiksiinhappe, tetratsükliini ning tsiprofloksatsiini suhtes. Resistentsete tüvede protsent ampitsilliinile, erütromütsiinile, nalidiksiinhappele, tetratsükliinile ja tsiprofloksatsiinile oli vastavalt 19,4%, 16,6%, 44,4%, 22,2% ja 44,4%. Linnulihast isoleeritud kampülobakterite tüved olid absoluutselt tundlikud vaid gentamütsiinile. Kahte erinevasse antibiootikumi rühma kuuluvate antibiootikumide suhtes esines tüvede üheaegne resistentsus põhiliselt kombinatsioonis nalidiksiinhape/tsiprofloksatsiin ja tetratsükliin (22,2%). Uurimisperioodil 2002 a. ja 2003 a. ei isoleeritud ühtegi multiresistentset tüve (resistentne vähemalt kolme erinevasse rühma kuuluva antibiootikumi suhtes). Disk-difusiooni ja E-testi tulemused kattusid niivõrd, et kõik tüved, mis osutusid tundlikuks disk-difusiooni testiga osutusid tundlikeks ka E-testi tulemuste alusel.

Aastatel 2005 ja 2006 isoleeritud *Campylobacter´*i tüvede antibiootikumi tundlikkuse määramisel olid tulemused märksa murettekitavamad, sest 36 isolaati (27,5%) osutusid multiresistentseteks ehk olid resistentsed kolme või enama erinevasse rühma kuuluva antibiootikumi suhtes. Ühe või rohkema antibiootikumi suhtes esines resistentsus 104 isolaadil (79,4%). Kakskümmend isolaati (15,3%) osutusid resistentseteks kolme erinevasse rühma kuuluva antibiootikumi suhtes, 13 isolaati (10%) nelja erinevasse rühma kuuluva antibiootikumi suhtes ning kolm isolaati olid resistentsed kõigi

testitud antibiootikumide suhtes. Resistentsust antibiootikumidele eraldi hinnates selgus, et kõige rohkem oli resistentseid tüvesid enrofloksatsiini ja nalidiksiinhappe suhtes, kus vastavalt 73,3% ja 75,6% kampülobakterite tüvedest osutusid resistentseteks. Järgnesid tetratsükliini (32,1%), erütromütsiini (19,8%), gentamütsiini (19,1%) ning ampitsilliini (7,6%) suhtes resistentseks osutunud tüved. Võrreldes mitte multiresistentsete tüvedega oli multiresistentsete *C. jejuni* tüvede antibiootikumide suhtes resistentsuse tase nalidiksiinhappe ja enrofloksatsiini korral oluliselt kõrgem (Mann-Whitney test, p=0,026). Resistentsus teistele antimikroobsetele ühenditele ei olnud multiresistentsete ja mitte multiresistentsete tüvede võrdluses oluliselt erinev (p>0,05). Seega aastatel 2005 ja 2006 toimunud uuringud osutasid võimalusele, et fluorokinoloonide kasutamine linnukasvatuses võib esile kutsuda multiresistentsete tüvede tekke. Kampülobakterite antibiootikumidele resistentsete tüvede tundlikkuse määramise uurimuse tulemused on olulised, sest kemoterapeutikumi valik bakteriaalse infektsiooni korral sõltub diagnoosist ja tekitaja antibiootikumi tundlikkusest. Õige kemoterapeutikumi annustamine ja raviskeemist kinnipidamine on mikrobiaalse nakkuse ravi ning polüresistentsete mikroobitüvede tekke vältimise aluseks. Teadustööde tulemusena on selgunud, et termofiilsetel kampülobakteritel on unikaalne võime muutuda resistentseks kinoloonrühma preparaatide suhtes (Gootz *et al*., 1991). Uuringute tulemusi aluseks võttes soovitati lindude haiguste profülaktikas mitte kasutada söötades antibiootikume, kusjuures eriti taunitavaks peeti kinoloonrühma antibiootikumide kasutamist. Humaanmeditsiinis on kampülobakterioosi ravis kinoloonidele alternatiivseteks preparaatideks erütromütsiin ja doksütsükliin (Kaijser *et al*., 1994). Profülaktilistel eesmärkidel antibiootikumide kasutamine lindude söödasegudes on paljudes maades (nt. Põhjamaad) keelatud, kuna on leitud, et nende sisaldumine söödasegudes on otseseks põhjuseks bakterite polüresistentsete tüvede tekkes. Antud uurimuse tulemused osutasid kaudselt faktile, et lindude söödasegudes kasutatakse antibiootikume, mida kinnitab eeskätt aastatel 2005 ja 2006 lindude roojast ja linnulihatoodetest suurel arvul resistentsete tüvede isoleerimine. Riiklikku seiret korraldavatele veterinaarinspektoritele peaksid uurimuse tulemused selgitama loomakasvatusettevõttes antibiootikumide kasutamise põhimõtteid ning olema aluseks antibiootikumide jääkainete määramise proovivõtu plaanide koostamisel.

Uurimistöö tulemusena leiti, et tuleb tõhustada veterinaar- ja humaanmeditsiini spetsialistide koostööd rahva tervishoiu parendamisel. Head hügieeni tavad peavad leidma rakendamist nii tootmise, jaekaubanduse kui

tarbija tasandil, mis võimaldab paljude enteraalsete haiguste ennetamist. Tõrjeprogrammide rakendamisel on vajalik kõikide linnufarmide pidevad uuringud ning lindude tapmise eelselt tuleb välja selgitada linnukarjade tabandumine. Lindlates tuleb rakendada väga rangeid bioohutuse võtteid. Ristsaastumise ja *Campylobacter* spp. kontaminatsiooni vähendamise või vältimise eesmärgil tuleb kampülobakterite suhtes negatiivsed karjad tappa eraldi positiivsetest ning soovitavalt tapmispäeva esimesel poolel kampülobakterite suhtes negatiivsed ning teisel poolel positiivsed karjad. Kodulindude tapamajades tuleb suuremat tähelepanu pöörata sellele, et tapmisele järgnevad protseduurid minimeeriks soolesisaldise sattumise rümpadele. Üheks võimaluseks oleks nt. tapaliinide töökiiruse vähendamine ning kasutatava puhta loputusvee koguse suurendamine. Oluline võimalus kontaminatsiooni vähendamiseks oleks ka intensiivsema ning pikemaajalise rümpade õhkjahutamise kasutamine.

Väga oluline on antud uurimistöö informatsiooni edastamine turustamisotstarbelise toidutoorme ja toidu käitlejatele, kes on kohustatud muutma või täiendama tehnoloogilisi skeeme ja leidma teisi lahendusi, et välistada tarbijale potentsiaalselt ohtliku tooraine või toidu turustamist.

8.6. Kokkuvõte ja järeldused

1. Eesti väikese võimsusega ettevõtte linnuliha toodang oli võrreldes suure võimsusega ettevõtte toodanguga oluliselt ($P < 0{,}001$) rohkem kampülobakteritega saastunud. Lindude rümbad ja tiivatükid (vastavalt 28% ja 31.3%) olid oluliselt ($P < 0{,}001$) sagedamini saastunud, võrreldes rinna- ja kintsutükkidega (0% ja 0%).

Eesti päritolu jahutatud linnulihatoodete saastumine termofiilsete kampülobakteritega oli 9,1% kusjuures külmutatud impordtoodetel ulatus see näitaja 15,9%-ni. Imporditud linnuliha kõrgem saastumine võis olla tingitud algtootmise kõrgest kontaminatsioonist termofiilsete kampülobakteritega.

Võrreldes Tallinnast kogutud proovidega osutusid Tartu turgudel võetud linnuliha proovid oluliselt rohkem saastunuteks termofiilsete kampülobakteritega. Selline oluline erinevus võis olla tingitud proovide laboratooriumisse toimetamise erinevatest transpordiaegadest. Tartust võetud proovid toimetati analüüside teostamiseks laboratooriumi praktiliselt

kohe pärast proovide võtmist, kuid Tallinnast võetud proovid analüüsiti Helsingi Ülikooli Toidu- ja Keskkonnahügieeni laboris, mis tähendas mitmete tundide võrra pikemat aega analüüsi alguseni.

Hooajaliselt isoleeriti Eestis müüdud tooretelt linnulihatoodetelt termofiilseid kampülobaktereid kõige sagedamini juunist kuni novembrini.

Meie termofiilsete kampülobakterite uuringud, mis sellises ulatuses olid Eesti veterinaarmeditsiinis esimesed, peavad jätkuma, et täiendavate uuringutega selgitada välja *Campylobacter* spp. levimust ja esinemissagedust põllumajandusloomade karjade tasandil. Uuringute jätkumine on vajalik ka edasiste *Campylobacter* spp. kontaminatsiooni suundumuste välja selgitamisel.

2. Uuringutest saadud tulemused osutasid toiduainetest isoleeritud kampülobakterite tüvede sero- ja genotüüpilisele mitmekesisusele.

Olulist seost serotüüpilise jaotumuse ja proovide päritolu vahel antud uuringutes ei leitud.

Seitsmekümne *Campylobacter* 'i isolaadi genotüpiseerimine näitas, et restriktsiooniensüüm *Kpn*I on võrreldes *Sma*I-ga suurema genotüüpide eraldamisvõimega. Nimelt, andis restriktsiooniensüüm *Kpn*I meie uuringutes 34 PFGE tüüpi võrreldes *Sma*I restriktsiooniensüümi 29 genotüübiga. PFGE analüüs *Kpn*I ja *Sma*I ensüümidega tõestas head kampülobakterite isolaatide tüpiseerimise ning katsete korratavuse võimet.

Selge seos oli genotüüpilise jaotumuse ja proovide päritolu vahel, kuid genotüüpide ja geograafilise piirkonna seotus vajab ulatuslikemaid uuringuid.

3. Antud uurimuse tulemused näitasid toiduainetest isoleeritud kampülobakterite tüvede kõrget resistentsust praktiliselt kõikide uuringus kasutatud antimkroobsete ühendite suhtes. Kõrged minimaalsed inhibeerivad kontsentratsioonid (MIK) makroliidide ja fluorokinoloonide suhtes osutavad tõenäoliselt võimalikele tervishoiualastele probleemidele tingituna asjaolust, et erütromütsiin ja teatud fluorokinoloonid on inimeste kampülobakteritest põhjustatud infektsioonide ravis esimesteks valikpreparaatideks.

Teostatud uuringute tulemuste eriti murettekitavaks faktiks tuleb aga pidada multiresistentsete kampülobakterite tüvede kõrget arvu, 36 isolaati ehk 27,5% isoleeritud kampülobakterite tüvedest, osutus multiresistentseks. Aastatel 2005 ja 2006 toimunud uuringud osutasid selgelt faktile, et fluorokinoloonide kasutamine võib esile kutsuda multiresistentsete tüvede tekke.

Campylobacter spp. antibiootikumidele tundlikkuse uuringud peavad jätkuma, et selgitada välja resistentsuse suundumusi ning seonduvaid resistentsusmehhanisme ja võimalusi termofiilsete kampülobakterite resistentsuse/multiresistentsuse vähendamiseks Eestis.

8.7. Ettepanekud

Antud uuringutega, mis olid *Campylobacter* spp. esimesed sellise ulatusega uuringud Eestis, jõuti järeldusele, et on veel mitmeid valdkondi, mis vajaksid täiendavaid uuringuid. Vajalik on jätkata uuringutega linnufarmide tasandil ning leida teaduslikud põhjendused saastumisastme muutustele erinevatel aastaaegadel ning aastatel. Eestis on vaja rakendada *Campylobacter* spp. monitooringu programmi kogu toiduahela ulatuses. *Campylobacter* spp. kontrolli programmide loomisel ja rakendamisel tuleks lähtuda Põhjamaade senisest kogemusest. Põhjamaades rakendatavate *Campylobacter* spp. kontrolli programmide üldine tähelepanu on suunatud bioohutuse tagamisele linnufarmide tasandil, et vältida karjade nakatumist. Teise olulise strateegiana tuleks rakendada lindude logistilist tapmist ehk *Campylobacter* positiivsed karjad tapetakse kas päeva lõpul või täiesti eraldi päevadel. Positiivsetest karjadest pärit lindude rümbad tuleks kuumtöödelda või rakendada nende töötlemisel vähemalt viie nädalast rümpade külmutamist. Inimestel esinevate *Campylobacter* spp. nakkusjuhtude arvu vähendamiseks või infekstiooni ennetamiseks, samuti *Campylobacter* infektsiooni suundumuste alaste teadmiste hankimisel, tuleks veterinaarmeditsiini spetsialistidel arendada koostööd humaanmeditsiini teadlaste ning klinitsistidega. Patogeenide multiresistentsed tüved peegeldavad pikema perioodi jooksul toimunud antibiootikumide kasutamist, mis antud uuringutes andsid arvukalt multiresistentseid isolaate. Multiresistentsete isolaatide ulatuslik esiletulek on kõrgeks riskiks inimeste tervisele ning limiteerib kampülobakteritest põhjustatud infektsioonide ravimisel antibiootikumteraapia rakendamist. Eestis on toiduloomade tasandil vaja rakendada senisest märksa karmimat antibiootikumide kasutamise

poliitikat, seda eriti fluorokinoloonide osas. Jätkata tuleb mikroobide antibiootikumidele tundlikkuse uuringutega, et määrata kampülobakterite resistentsuse tekke tendentse, resistentsuse mehhanisme ning võimalusi toidupatogeenide resistentsuse vähendamiseks Eestis. Rakendada tuleb *Campylobacter* spp. teaduspõhist riskihindamist, riskiohjamist ja riskikommunikatsiooni ning seda kogu toidutootmise ahela ulatuses.

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I

PUBLICATIONS

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Prevalence of *Campylobacter* in raw chicken meat of Estonian origin

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Introduction

Campylobacter are the most common registered bacterial causes of human intestinal infections in many developed countries (HÄNNINEN et al., 2003). In industrialized countries, including Western Europe, USA, Canada, Australia and New Zealand, the rate of human *Campylobacter* infections has been increasing steadily.

Campylobacter spp. are widespread in nature, not only in wildlife but also among food animals. *Campylobacter* are commensal organisms routinely found in cattle, sheep, swine, and avian species (FRIEDMAN et al., 2000). The avian species are the most common host for *Campylobacter*, probably because of their higher body temperature (SKIRROW, 1977). Monitoring studies indicate that most chicken flocks are colonised with *C. jejuni*. Intestinal colonisation usually leads to contamination of the final product, which cannot be prevented in the processing plant (ANONYMOUS, 1994). Studies carried out in slaughterhouses have shown that the main source of the spread of *C. jejuni* on poultry carcasses is their intestinal contents (BERNDTSON et al., 1992; MEAD et al., 1995; ONO and YAMAMOTO, 1999; STERN et al., 2003). The scalding procedure is used to open the feather follicles to facilitate feather removal. The potential for bacterial cross-contamination during scalding and picking is well known (BAILEY et al., 1987). During slaughter, intestinal contents can contaminate meat surfaces, and in this way the consumer can be exposed. The risk of infection is particularly high if poultry is involved (OOSTEROM, 1984).

It is well established that poultry products are a vehicle for foodborne campylobacteriosis and they are suspected to be an important source of infection (KAPPERUD et al., 1992; HÄNNINEN et al., 2000; NEIMANN, 2001; DOMINGUES et al., 2002). Other foods (mainly of animal origin) must be considered as potential sources of infection. *Campylobacter* have also been isolated from such food items as raw milk, pork, beef, lamb, and seafood (HUDSON et al., 1999; JAKOBS-REITSMA, 2000; DUFFY et al., 2001).

In 2004, 124 human campylobacteriosis cases were reported by the Estonian Health Protection Inspectorate (ANONYMOUS, 2004a). Estonia has no existing, continuous *Campylobacter* monitoring that is following "from farm to fork" concept and more research is needed to address this problem. The main aim of present project was to study the prevalence of thermophilic *Campylobacter* in raw chicken meat of Estonian origin. Before 2000, when the present study was launched, there were no data on the prevalence of *Campylobacter* spp. in raw chicken meat of Estonian origin. *Campylobacter* spp. were not included in Estonian food monitoring system until July 2004.

Materials and methods

Sampling

Altogether, 279 samples of Estonian raw chicken meat (breasts, carcasses, legs, minced meat, thighs and wings) were analysed during 2000 and 2002. Of these, 90 were collected directly from the end of the slaughter line of a small-scale poultry meat plant and 189 from traditional market halls of Tartu town. All chicken meat samples from the market halls had originated from the slaughterhouse of one Estonian large-scale poultry meat plant, where each day an average of 23,000 chickens were being slaughtered. Except for unloading and shackling, all processing points in the large-scale poultry plant were automated, and air chilling was being used. Manual procedures were used at almost in all processing points in the small-scale company. The small-scale poultry company was orientated to egg production, and both hens and chicken were killed and retailed. In the present study, *Campylobacter* contamination was studied only in chicken meat. Different samples (breasts, carcasses, thighs and wings) were collected from the small-scale and large-scale company at

the same period of year. Minced meat and leg samples of small-scale company were not available. Samples were transported to the laboratory after being placed in a portable cooler at a temperature 4-6 ºC and microbiological analyses were carried out immediately in the Estonian State Veterinary and Food Laboratory in Tartu. *C. jejuni* ATCC 29428 was used as control strain.

Isolation and identification of *Campylobacter* spp.

Campylobacter were detected using the NCFA method (ANONYMOUS, 1990), which includes the enrichment in Preston broth. A 25 g sample of poultry meat was placed in a sterile plastic bag, and 250 ml Preston enrichment broth (Oxoid, Basingstoke, Hampshire, England) was added. The sample was stomached for 60 s using a Lab-Blender 400 stomacher. The enrichment broth was incubated in sealed jars for 24 h at 42±0.5ºC in microaerobic conditions. Preston enrichment media was plated out on selective mCCDA (modified Charcoal Cefoperazone Deoxycholate Agar, Oxoid). The plates were incubated microaerobically at 42±0.5ºC and examined for growth after 48 h. Typical colonies were streaked on Brucella agar (Pronadisa, Madrid, Spain), and verified to the species level by the use of Gram-stain, phase contrast microscopy for motility, oxidase and catalase test, hippurate hydrolysis, and susceptibility to nalidixic acid (30 µl/ml). Agar plates were incubated under microaerobic conditions produced by the Campy-Gen™ atmosphere generation system (Oxoid). At least one colony from each sample was stored in glycerol broth (15% [vol/vol] glycerol in 1% [wt/vol] proteose peptone) at -70°C.

Statistical analyses

The chi-square test was used for statistical analyses.

Results and discussion

Of the raw chicken products of Estonian origin, 15.8% were positive for *Campylobacter* (Table 1). The prevalence of *Campylobacter* in the products (breasts, carcasses, thighs and wings) of the small-scale poultry meat plant (35.6%) was significantly higher than in those originated from the large-

scale company (8.1%) ($P < 0.001$). According to continuous *Campylobacter* research project, this tendency of contamination of Estonian small-scale and large-scale company products is similar to that reported in internal report of Estonian Science Foundation (ANONYMOUS, 2004b).
The large-scale company has better facilities for meeting good production standards and has implemented effective quality-control programmes. High rates of contamination in Estonian small-scale company were related with many cross-contamination possibilities, for example the use of water tanks for rinsing of carcasses instead of modern water rinsing systems (BASHOR et al., 2004; PURNELL et al., 2004). Manual procedures in slaughterhouses instead of automated systems are causing problems in hygiene as well. The large-scale poultry meat plant owns many poultry farms that follow good hygiene practices at the company and farm level. Air chilling has been suggested to be more efficient than water chilling for killing *Campylobacter* cells because of the drying effects (OOSTEROM et al., 1983; SANCHES et al., 2002; ROSENQUIST et al., 2003). The fact that an effective quality-control programme and air chilling systems had been implemented in the large-scale poultry processing plant probably accounts for the lower contamination levels found in the large-scale plant than in the small-scale plant in this study.

The prevalence of *Campylobacter* in the chicken carcasses and wings (28 and 31.3%) were significantly higher than that found in breasts and thighs (0 and 0%) ($P < 0.001$) (Table 1). Our results indicated that the chicken carcasses and wings were contaminated with *Campylobacter*, but there was no contamination of breast, legs, minced meat and thighs. The skin on chicken wings is full of wrinkles and has larger feather follicles than the skin on many other parts of the chicken carcass. Open follicles, crevices and wrinkles on the skin offer bacteria more opportunities to persist after rinsing procedure (BERNDTSON et al., 1992; CHANTARAPANONT et al., 2003).

Phenotypic tests used to differentiate between the *Campylobacter* strains at the species level showed that of 46 isolated *Campylobacter* strains, 35 (76%) were *C. jejuni* and 11 (24%) *C. coli*. In most other countries *C. jejuni* has accounted for more than 90% of the *Campylobacter* isolates from poultry (GILLESPIE et al., 2002; JØRGENSEN et al., 2002). Further studies are needed to determine the causes of the high incidence of *C. coli* in Estonian chicken products.

The results of this study indicate that raw poultry products of Estonian origin are contaminated by *Campylobacter* species. The problem appears to be more severe in small-scale operations. The high *Campylobacter* contamination observed in present study may indicate that the prevalence of human campylobacteriosis in Estonia is greater than the 124 cases reported by the Estonian Health Protection Inspectorate (ANONYMOUS, 2004a). More effective cooperation between human medicine and veterinary medicine is needed in Estonia in order to prevent *Campylobacter* infection. An Estonian *Campylobacter* control programme is currently under development, and additional studies of different foods, at farms and processing plants are needed.

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Zusammenfassung

In den Jahren 2000 und 2002 wurde rohes Hühnerfleisch estnischer Herkunft auf die Anwesenheit von thermophilen *Campylobacter* untersucht. Insgesamt wurden 279 Proben (90 Proben aus einem Kleinbetrieb und 189 Proben aus einem Großbetrieb stammend) analysiert. 15.8% aller rohen Hühnerfleischprodukte war mit *Campylobacter* kontaminiert. In den Produkten (Brust, Schlachtkörper, Oberschenkel und Flügel) der Kleinbetriebe wurde ein siginifikant höheres Vorkommen von *Campylobacter* (35.6%) festgestellt als in Großbetrieben (8.1%) ($P < 0.001$). Schlachtkörper und Flügel (28 und 31,3%) wiesen eine signifikant höhere Kontamination als Brust und Oberschenkel (0 and 0%) ($P < 0.001$) auf. Von insgesamt 44 *Campylobacter*-Stämmen wurden 76% als *C. jejuni* und 24% als *C. coli* identifiziert. In dieser Untersuchung konnte nachgewiesen werden, daß estnisches rohes Hühnerfleisch aus Kleinbetrieben stammend eine höhere *Campylobacter* Kontamination aufweist als gleiche Produkte aus Großbetrieben.

Summary

Thermophilic *Campylobacter* spp. contamination of raw chicken meat of Estonian origin produced in a small-scale and large-scale company was studied during 2000 and 2002. Altogether, 279 samples (90 originated from small-scale and 189 from large-scale company) were analysed. Of the raw chicken products, 15.8% were positive for *Campylobacter*. The prevalence of *Campylobacter* in the products (breasts, carcasses, thighs and wings) of the small-scale company (35.6%) were significantly higher than in those originated from the large-scale company (8.1%) ($P < 0.001$). The chicken carcasses and wings (28 and 31.3%) had significantly higher contamination level than breasts and thighs (0 and 0%) ($P < 0.001$). Of 44 *Campylobacter* strains, 76% have been identified as *C. jejuni* and 24% *C. coli*. In this study, Estonian raw chicken meat products of the small-scale company did show a higher prevalence of *Campylobacter* than similar products of the large-scale company.

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Table 1. **The prevalence of *Campylobacter* spp. in different chicken products of Estonian origin**

Sampling site	No. of positive samples/no. of total samples (%)		
	Small-scale company	Large-scale company	Total
Breasts	0/15 (0)	0/30 (0)	0/45 (0)
Carcasses	22/49 (44.9)	1/33 (3)	23/82 (28)
Legs	NS	0/10 (0)	0/10 (0)
Minced meat	NS	0/30 (0)	0/30 (0)
Thighs	0/6 (0)	0/39 (0)	0/45 (0)
Wings	10/20 (50)	11/47 (23.4)	21/67 (31.3)
Total	32/90 (35.6)	12/189 (6.3)	44/279 (15.8)

NS, no samples available

II

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PFGE genotyping and antimicrobial susceptibility of *Campylobacter* in retail poultry meat in Estonia

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Abstract

In the present study, the *Campylobacter* isolates from retail poultry meat in Estonia were sero- and genotyped, and the antimicrobial susceptibility was determined. Forty-eight chicken (36 Estonian, 12 imported) and 22 turkey (imported) *Campylobacter* isolates from 580 raw broiler chicken (396 Estonian, 184 imported) and 30 turkey (imported) meat samples were studied. Of the isolates, 64 were *C. jejuni*, 4 *C. coli*, and 2 *Campylobacter* spp. Penner serotyping of 54 *C. jejuni* isolates revealed 11 different serotypes, and 22% of the isolates were nontypeable by the commercial antisera. The most common serotypes O:1,44; O:21, and O:55 accounted for 28%, 13%, and 13% of the isolates, respectively. Differences in serotype distribution were seen for chicken and turkey isolates. Genotypic characterization of all *Campylobacter* isolates ($n=70$) was performed by pulsed-field gel electrophoresis (PFGE). *Sma*I and *Kpn*I yielded 29 and 34 PFGE types, respectively, revealing high diversity among isolates. The serotype distribution did not show an association with the origin of the sample, but the majority of the isolates sharing a similar PFGE genotype originated from one country. High levels of resistance to ciprofloxacin (66%), nalidixic acid (66%), tetracycline (44%), ampicillin (34%), and erythromycin (14%) were detected among the 70 *Campylobacter* isolates. The simultaneous resistance to two or three antimicrobial agents occurred in 60% of the isolates. The *Campylobacter* isolates from turkey meat had higher resistance to ampicillin, ciprofloxacin, nalidixic acid, and tetracycline than those from chicken meat. None of the chicken isolates were resistant to gentamicin, and no turkey isolates to erythromycin or gentamicin.

Keywords: *Campylobacter*; Poultry meat; PFGE; Serotyping; Antimicrobial susceptibility

1. Introduction

Campylobacter jejuni is the most common bacterial cause of human food-borne illnesses in developed countries (Altekruse et al., 1999; Friedman et al., 2000; Rautelin and Hänninen, 2000). Several epidemiological case-control studies have established that ingesting undercooked poultry products significantly increases the risk for acquisition of food-borne campylobacteriosis (Eberhart-Phillips et al., 1997; Studahl and Andersson, 2000; Kramer et al., 2000; Neimann et al., 2003; Schönberg-Norio et al., 2004). Slaughterhouse studies have shown that the main source of contamination of *C. jejuni* poultry carcasses is their intestinal contents (Wedderkopp et al., 2000; Newell et al., 2001; Berrang et al., 2004).

Serotyping is a widely used method for typing *C. jejuni* (Rautelin and Hänninen, 1999; Wassenaar and Newell, 2000). Two serotyping schemes have been developed for campylobacter subtyping (Penner and Hennessy, 1980; Lior et al., 1982). Tracing the sources and understanding the epidemiology of *Campylobacter* is increasingly done by molecular typing (de Boer et al., 2000; Nielsen et al., 2000; Wassenaar and Newell, 2000). A widely used method for molecular typing of *C. jejuni* is pulsed-field gel electrophoresis (PFGE) (Gibson et al., 1995; Hänninen et al., 2000; Kärenlampi et al., 2003). It appears to be a highly discriminatory method especially when used with the two restriction enzymes, *Sma*I and *Sac*II/*Kpn*I (Gibson et al., 1997; Hänninen et al., 1998; Michaud et al., 2001).

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Table 1
Distribution of *Campylobacter jejuni* serotypes isolated from raw retail poultry

Serotype	No. of isolates originating from different countries [a]				
	DK	EE	FI	HU	US
O:1,44	4	6	1	3	1
O:2		2			
O:4-complex		2			
O:11		1			
O:12		3			
O:18				2	
O:21	3	4			
O:27		1			
O:38		1			
O:41	1				
O:55		2		5	
NT [b]		3		7	2
Total	8	25	1	17	3

[a] Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; US, The United States.
[b] NT, nontypeable.

Erythromycin is the antimicrobial agent recommended for the treatment of human campylobacteriosis (Engberg et al., 2001). Antimicrobial resistance has emerged among *Campylobacter* mainly as a consequence of the use of antimicrobial agents, especially fluoroquinolones, macrolides, and tetracyclines in food animal production (Endtz et al., 1991; Jacobs-Reitsma, 1997; Piddock et al., 2000; Smith et al., 2000; Aarestrup and Engberg, 2001; Engberg et al., 2001).

The aims of the present study were to serotype and PFGE genotype *Campylobacter* isolates originating from raw retail poultry meat in Estonia, as well as to determine the antimicrobial susceptibility of the *Campylobacter* isolates to ampicillin, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, and tetracycline.

2. Materials and methods

2.1. Isolates

We studied 48 broiler chicken (8 Danish, 36 Estonian, 1 Finnish, and 3 U.S. origin) and 22 turkey (Hungarian origin) *Campylobacter* isolates from 580 raw broiler chicken (396 Estonian, 184 imported) and 30 turkey (imported) meat samples obtained from retail stores in Estonia between January 2002 and December 2003. Of the isolates, 64 were identified as *C. jejuni*, 4 *C. coli*, and 2 *Campylobacter* spp.

The isolation of *Campylobacter* was carried out in two laboratories. The Department of Food and Environmental Hygiene, University of Helsinki analysed altogether 290 samples using the following method. One hundred milliliters of peptone (0.1%)–saline (0.85%) solution was added to the whole sample (broiler leg) in a plastic bag, and the sample was massaged by hand for 1 min. Twenty milliliters of the suspension was added into 80 ml of *Campylobacter* enrichment broth (Lab M, Bury, Lancashire, UK) and enriched at 37 °C for 24 h and 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Microaerobic conditions were produced in jars by using Oxoid gas-generating kits according to the manufacturer's instructions (Oxoid, Basingstoke, Hampshire, UK).

The Central Veterinary and Food Laboratory in Tartu, Estonia analysed 320 of the samples for *Campylobacter* using the method of the Nordic Committee on Food Analysis (Anonymous, 1990), which includes enrichment in Preston broth. The addition of 25 g of sample (minced meat or skin and muscle of breast, carcass, thigh, wing) to 250 ml Preston enrichment broth (Oxoid) followed by the sample being stomached for 60 s. Incubation was carried out at 42±0.5 °C for 24 h under microaerobic conditions.

In both methods, after 24 h and 48 h incubation a loopful of the enrichment broth was plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid), and examined for typical growth after 48 h. Typical grayish, campylobacter-like colonies growing on mCCDA plates were streaked on Brucella blood agar (Oxoid), and confirmed by gram staining, motility analysis, oxidase and catalase test as campylobacters. The isolate from each positive sample was identified as *C. jejuni* as being positive or *C. coli* as being negative in hippurate hydrolysis test. Additionally, an indoxyl acetate hydrolysis test was performed for hippurate negative isolates, and the isolates negative in this test were regarded as *Campylobacter* spp. After the original isolation, the strains were stored at −70 °C in glycerol broth (15% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).

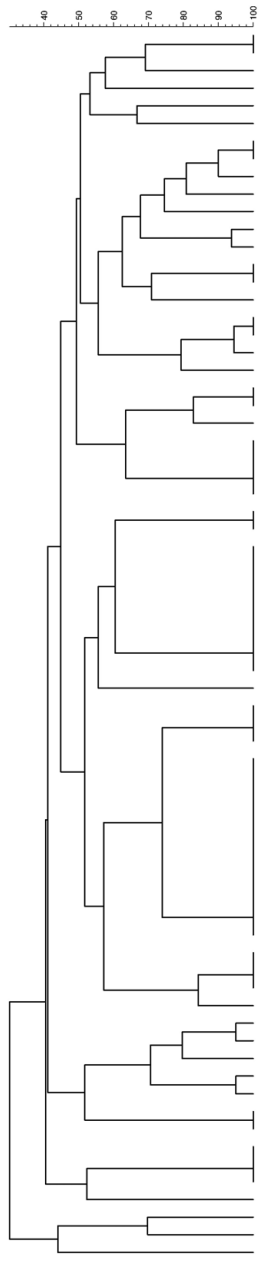
2.2. Serotyping

Arbitrarily chosen 54 *C. jejuni* isolates were serotyped using commercial *Campylobacter* antisera according to the manufacturer's instructions (Denka Seiken, Tokyo, Japan). Before the serotyping test, the isolates were cultured on Brucella blood agar (Oxoid) plates at 37 °C for 48 h in microaerobic conditions.

2.3. In situ DNA isolation and PFGE

PFGE typing was performed for 70 *Campylobacter* isolates, representing one isolate from each positive sample. As described previously, *in situ* DNA was isolated and characterized by PFGE (Gibson et al., 1994; Hänninen et al., 1998). The DNA was digested with *Sma*I or *Kpn*I (New England Biolabs, Beverly, Mass.) (20 U per sample), and the restriction fragments were separated with ramped pulses of 1 to 30 s and 1 to 25 s for 19 h, respectively.

Fig. 1. Combined dendrogram of *Sma*I and *Kpn*I macrorestriction patterns (MRP) of *Campylobacter* isolated from raw retail poultry meat in Estonia. Similarity analysis was performed using the Dice coefficient, and clustering was performed by the unweighted pair-group method with arithmetic averages (position tolerance, 1.0%). Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; US, United States. Species: CC, *Campylobacter coli*; CJ, *Campylobacter jejuni*; Csp. *Campylobacter* spp. [a]ND, not digested. [b]NT, nontypeable. [c]NP, not performed.



PFGE type	MRP *SmaI*[a]	MRP *KpnI*	Product	Country	Species	Serotype[b, c]
18	12	12	turkey	HU	CJ	O:18
18	12	12	turkey	HU	CJ	O:18
17	11	11	chicken carcass	EE	CJ	O:1,44
5	4	4	chicken wing	US	CJ	NT
14	ND	33	chicken carcass	EE	Csp.	NP
6	5	5	chicken wing	US	CJ	NT
13	10	10	chicken wing	EE	CJ	O:1,44
13	10	10	chicken wing	EE	CJ	O:1,44
34	28	10	chicken leg	EE	CJ	O:1,44
33	27	27	chicken leg	EE	CJ	O:27
35	10	36	chicken leg	EE	CJ	O:1,44
28	22	22	chicken leg	EE	CJ	O:1,44
36	10	22	chicken leg	US	CJ	O:1,44
12	9	9	chicken carcass	EE	CJ	O:2
12	9	9	chicken carcass	EE	CJ	O:2
32	26	26	chicken leg	FI	CJ	O:1,44
8	6	31	chicken wing	DK	CJ	O:1,44
8	6	31	chicken wing	DK	CJ	O:1,44
7	6	6	chicken wing	DK	CJ	O:1,44
9	6	32	chicken wing	DK	CJ	O:1,44
20	14	14	turkey	HU	CJ	O:1,44
20	14	14	turkey	HU	CJ	O:1,44
21	15	15	turkey	HU	CJ	O:1,44
27	21	21	chicken wing	EE	CJ	O:4-complex
27	21	21	chicken leg	EE	CJ	O:4-complex
27	21	21	chicken leg	EE	CJ	O:38
27	21	21	chicken leg	EE	CJ	NT
11	8	8	chicken wing	EE	CC	NP
11	8	8	chicken wing	EE	CC	NP
4	3	3	chicken wing	EE	CJ	NP
4	3	3	chicken carcass	EE	CJ	NP
4	3	3	chicken wing	EE	CJ	NT
4	3	3	chicken wing	EE	CJ	O:11
4	3	3	chicken wing	EE	CJ	O:55
4	3	3	chicken wing	EE	CJ	O:55
4	3	3	chicken wing	EE	CJ	NT
4	3	3	chicken wing	EE	CJ	NP
10	7	7	chicken wing	DK	CJ	O:41
2	1	1	chicken carcass	EE	CJ	O:12
2	1	1	chicken carcass	EE	CJ	O:12
2	1	1	chicken carcass	EE	CJ	O:12
16	1	35	turkey	HU	CJ	NP
16	1	35	turkey	HU	CJ	NP
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
1	ND	30	chicken carcass	EE	CJ	O:21
1	ND	30	chicken carcass	EE	CJ	O:21
1	ND	30	chicken carcass	EE	CJ	O:21
15	ND	34	chicken carcass	EE	CJ	O:21
22	16	16	turkey	HU	CJ	NT
25	19	16	turkey	HU	CJ	NT
24	18	18	turkey	HU	CJ	NP
19	13	13	turkey	HU	CJ	NP
26	20	20	turkey	HU	CJ	NP
3	2	2	chicken carcass	EE	CC	NP
3	2	2	chicken carcass	EE	CC	NP
29	23	23	chicken leg	DK	CJ	O:21
29	23	23	chicken leg	DK	CJ	O:21
29	23	23	chicken leg	DK	CJ	O:21
23	17	17	turkey	HU	CJ	NT
31	25	25	chicken leg	EE	CJ	NP
37	29	29	chicken leg	EE	CJ	NP
30	24	24	chicken leg	EE	Csp.	NP

Table 2
Distribution of *Campylobacter* PFGE genotypes from raw retail poultry with *Sma*I and *Kpn*I, according to country

Country	Number of strains	Number of PFGE genotypes		PFGE genotypes[a]	
		*Sma*I	*Kpn*I	*Sma*I	*Kpn*I
Denmark	8	3	5	6, 7, 23	6, 7, 23, 31, 32
Estonia	36[b]	14	17	1, 2, 3, 8, 9, 10, 11, 21, 22, 24, 25, 27, 28, 29	1, 2, 3, 8, 9, 10, 11, 21, 22, 24, 25, 27, 29, 30, 33, 34, 36
Finland	1	1	1	26	26
Hungary	22	10	9	1, 12, 13, 14, 15, 16, 17, 18, 19, 20	12, 13, 14, 15, 16, 17, 18, 20, 35
United States	3	3	3	4, 5, 10	4, 5, 22
Total	70	29	34		

[a] Underlined PFGE genotype has been detected in poultry that originated from more than one country.
[b] The DNA of five isolates was not digested by *Sma*I.

2.4. PFGE pattern analysis

The computer software program BioNumerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0%). The dendrogram was constructed using the unweighted pair-group method with arithmetic averages.

2.5. Antimicrobial susceptibility testing

All *Campylobacter* isolates were tested by the disc diffusion method against ampicillin (25 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), and tetracycline (10 μg) (Oxoid), and by the Epsilometer test (*E*-test) (AB Biodisk, Solna, Sweden) against ampicillin, ciprofloxacin, erythromycin, and tetracycline.

Campylobacter isolates were first grown on blood agar plates and were transferred in 5 ml of Mueller–Hinton (MH) broth (Oxoid), and incubated at 37 °C for 24 h under microaerobic conditions. Inoculum from the MH broth was diluted and a turbidity equivalent of a 0.5 McFarland standard was adjusted in physiological peptone–saline water and the growth suspension was spread on the MH blood agar plates (Oxoid, supplemented with 7% horse blood), the disks or *E*-test strips containing antimicrobial compounds were laid on the plates. The plates were incubated at 37 °C for 24 h in microaerobic conditions. The diameter of the growth inhibition zone was measured according to the CLSI (2004). MIC values were determined by *E*-test according to the instructions given by the manufacturer (AB Biodisk). *C. jejuni* 143483 was used as control strain in the antimicrobial susceptibility testing (Hakanen et al., 2002).

The following zone diameter (mm) and MIC breakpoints for resistance were applied: ampicillin ≤ 13 mm and MIC ≥ 32 μg/ml, ciprofloxacin ≤ 26 mm and MIC ≥ 4 μg/ml, erythromycin ≤ 26 mm and MIC ≥ 32 μg/ml, gentamicin ≤ 12 mm, nalidixic acid ≤ 26 mm, and tetracycline ≤ 31 mm and MIC ≥ 16 μg/ml (Anonymous, 2004; CLSI, 2004).

3. Results

3.1. Serotype distribution

Eleven serotypes were obtained from 54 *C. jejuni* isolates (Table 1, Fig. 1). Of the isolates, 22% (12/54) were nontypeable. The most common serotypes O:1,44; O:21, and O:55 accounted for 28%, 13%, and 13% of the isolates. The isolates from chicken meat ($n=37$) included ten serotypes, and the frequent serotypes were O:1,44 (32%) and O:21 (19%). The isolates from turkey meat ($n=17$) belonged to three serotypes: O:55 (29%), O:1,44 (18%), and O:18 (12%).

3.2. PFGE genotypes

The PFGE genotyping of 70 *Campylobacter* isolates yielded 29 *Sma*I and 34 *Kpn*I PFGE types (Table 2, Fig. 1). The DNA of five isolates was not digested by *Sma*I. Combination of the macrorestriction patterns resulted in 37 PFGE types (Fig. 1). Of these, 33 PFGE types were from *C. jejuni* (91%), 2 from *C. coli* (6%), and 2 from *Campylobacter* spp. isolates (3%).

3.3. Antimicrobial susceptibility

In the disc diffusion method, resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 66%, 66%, 44%, 34%, and 14% of the *Campylobacter* isolates ($n=70$). Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 44%, 44%, 22%, 19%, and 17% of the Estonian isolates ($n=36$) and in 88%, 88%, 68%, 50%, and 12% of the imported isolates ($n=34$), respectively. All isolates were susceptible to gentamicin.

Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 50%, 50%, 27%, 23%, and 14% of the chicken isolates ($n=48$). Two *C. coli* isolates from chicken showed resistance to ampicillin, ciprofloxacin, and nalidixic acid. One isolate of *Campylobacter* spp. from chicken was resistant to ampicillin, and the other isolate to ciprofloxacin and nalidixic acid. Of the turkey isolates ($n=22$) all were resistant to ciprofloxacin and nalidixic acid, 82% to tetracycline, and 59% to ampicillin.

Resistance occurred in 57 isolates (81%) out of 70 tested to at least one of the antimicrobials (Table 3). Fifteen isolates (21%) were resistant to one, 30 isolates (43%) to two, and 12 isolates (17%) to three antimicrobial agents. The resistance of *Campylobacter* isolates to two antimicrobials showed a combination of ampicillin and ciprofloxacin (9%), ampicillin and erythromycin (4%), and ciprofloxacin and tetracycline (30%). The resistance of isolates to three antimicrobials showed a combination of ampicillin, ciprofloxacin, and erythromycin (4%), and ampicillin, ciprofloxacin, and tetracycline (13%). The highest level of resistance recorded was to ciprofloxacin (66%) followed by tetracycline (44%), ampicillin (34%), and erythromycin (14%).

4. Discussion

Our studies showed high serotype and genotype diversity among *Campylobacter* isolates from raw retail poultry meat in

Table 3
MICs for ampicillin (AM), ciprofloxacin (CI), erythromycin (ERY), and tetracycline (TC) of *Campylobacter* isolated from raw retail poultry meat in Estonia

Country of origin[a]	No. of isolates	Antimicrobial agents	No. of isolates with MIC (μg/ml)[b]												
			≤ 0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥ 256
DK	8	AM				2	2				1				3
		CI					1					7			
		ERY			1	1	1	1							4
		TC	1	1			1			1			1		3
EE	36	AM				3	7	8	4	3	4	1	1		5
		CI	4	10	3	2		1				16			
		ERY			1	8	12	3	4	1	1	1			5
		TC	10	6	5	4	2	1			2	3	1		2
FI	1	AM													1
		CI			1										
		ERY							1						
		TC				1									
HU	22	AM					3	4	1	1					13
		CI								1	1	20			
		ERY		1	5	8	2	2	3	1					
		TC	2	1		1									18
US	3	AM				1				1	1				
		CI	1	1								1			
		ERY			1		1				1				
		TC		1	1						1				

The MIC values for the isolates were evaluated accordance to the Danmap (2004) and Clinical and Laboratory Standards Institute (2004). Solid vertical lines indicate breakpoints between susceptible and resistant isolates.

[a]Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; US, United States.

[b]The E-test values between two-fold dilutions were rounded up to the next upper two-fold value before the categorization according to manufacturer instructions (AB Biodisk, Solna, Sweden).

Estonia. Nine of the eleven *C. jejuni* serotypes obtained were common in poultry products of Estonian origin, and five in those imported to Estonia. The serotype distribution did not show association with the origin of the sample. The most common serotypes were O:1,44; O:21, and O:55, accounting for 54% of the isolates. Serotype distribution differences

occurred for chicken and turkey isolates. The chicken isolates had two common serotypes (O:1,44 and O:21) out of ten, whereas turkey isolates belonged to only three different serotypes (O:1,44; O:18 and O:55). In the studies in Denmark (Nielsen and Nielsen, 1999) and New Zealand (Devane et al., 2005), the serotype O:1,44 was also one of the most common in poultry products, and this serotype seems to have global distribution among strains isolated from human *Campylobacter* infections (Nielsen et al., 1997; Vierikko et al., 2004; Devane et al., 2005; Miller et al., 2005). The most frequently isolated serotype in chicken meat in New Zealand was O:21 (Devane et al., 2005), the second most common serotype in our study. The presence of serotypes O:2, O:4-complex, and O:12, common to both chickens and human patients (Fricker and Park, 1989; Hudson et al., 1999; Perko-Mäkelä et al., 1999; Petersen et al., 2001; Saito et al., 2005), occurred in only 13% of the isolates studied.

Serotyping of *C. jejuni* showed that 22% of the isolates were nontypeable, and seven of the nontypeable isolates originated from turkey meat imported from Hungary. By using the same commercial serotyping set as in our study, Rautelin and Hänninen (1999) found 14% of the isolates, and in a Danish study, using their own antisera, 16% of the isolates remained nontypeable (Nielsen and Nielsen, 1999) revealing the need to improve the present serotyping methods. One reason for nontypeability is the low production of capsular antigens responsible for the serotype specificity of *C. jejuni*, another reason could be new serotypes not accounted for in the present test (Jacobs-Reitsma et al., 1995).

The genotyping of the 70 *Campylobacter* isolates showed *Kpn*I to be more discriminatory, yielding 34 PFGE types compared to 29 obtained by *Sma*I. Furthermore, the DNA of five strains was not digested by *Sma*I. The genotypes of the isolates from the poultry products of different countries were not overlapping, except *Sma*I PFGE types 1 (isolates from Estonia and Hungary) and 10 (isolates from Estonia and USA), and *Kpn*I PFGE type 22 (isolates from Estonia and USA). Our results, as well as the data from several previous studies (Gibson et al., 1994; Hänninen et al., 1998; Wassenaar and Newell, 2000), however, emphasize the utility of two restriction enzymes, such as *Sma*I and *Kpn*I, in PFGE typing studies of *Campylobacter*. In our study the majority of the isolates sharing a similar PFGE genotype originated from one country. The association of genotypes with country of origin requires further studies using a larger collection of isolates, however.

We found several serotypes within one PFGE type (Fig. 1). For example, the PFGE type 4 contained the serotypes O:11, O:55, and nontypeable isolates, and PFGE type 27 contained O:4-complex, O:38, and a nontypeable isolate. Furthermore, within one serotype, several PFGE types were found. For instance, the common serotypes of our study, O:1,44; O:21, and O:55, contained up to 12, 3, and 2 different PFGE types.

An important finding of our study was the recognition of a high number (81%) of *Campylobacter* isolates with increased antimicrobial resistance. Antimicrobial resistance level was especially high to ciprofloxacin (44 isolates MIC $\geq$ 32 μg/ml), tetracycline (23 isolates MIC $\geq$ 256 μg/ml), and ampicillin (22 isolates MIC $\geq$ 256 μg/ml). The resistance to antimicrobials, except erythromycin, was higher in isolates from imported poultry products than in those originating from Estonia. The *Campylobacter* isolates from turkey meat had a higher resistance to ampicillin, ciprofloxacin, nalidixic acid, and tetracycline than those from chicken meat. All isolates resistant or susceptible by the disk diffusion method showed the same results by *E*-test.

Ciprofloxacin resistance was high among isolates from both imported (88% of the isolates) and domestic products (44% of the isolates). Furthermore, 100% of the turkey and 50% of the broiler isolates showed resistance to ciprofloxacin. All isolates with resistance to ciprofloxacin also tested resistant to nalidixic acid. A study in Spain (Saenz et al., 2000) showed very high prevalence 98% of ciprofloxacin resistance in *Campylobacter* isolates from broiler intestinal samples. The study by Endtz et al. (1991) showed a link for the first time between veterinary fluoroquinolone use and increasing fluoroquinolone resistance in poultry and human isolates of *Campylobacter*. Later studies have confirmed their results (Smith et al., 2000; Engberg et al., 2001). Enrofloxacin and flumequine, both fluoroquinolone group antimicrobials, are accepted for poultry treatment in Estonia (Anonymous, 2005), possibly explaining the high level of resistance detected among Estonian isolates.

Different studies typically find tetracycline resistance among poultry isolates. Ledergerber et al. (2003) reported a much lower (12%) tetracycline resistance but Ge et al. (2003) found a higher resistance (82%) among poultry than in our study (44%). Nevertheless, we found a higher resistance for turkey isolates (82%) than in the Belgian study (37%) (Van Looveren et al., 2001). Tetracycline (doxycycline) is also accepted for treatment of poultry in Estonia (Anonymous, 2005).

Ampicillin is a widely used antimicrobial in veterinary medicine. Resistance to ampicillin in broiler isolates, 23%, was at a similar level, and resistance in turkey isolates, 59%, was higher than found in the Belgian study (24% and 33%, respectively, Van Looveren et al., 2001). Ampicillin is not recommended, however, for the treatment of *Campylobacter* infections due to the high incidence of resistance to this drug among human isolates (Navarro et al., 1993). Amoxicillin is accepted for use in veterinary medicine in Estonia (Anonymous, 2005).

Campylobacter isolates displayed the lowest resistance frequency against erythromycin (14%). All resistant isolates were *C. jejuni* and they were either from Danish or Estonian chicken products. All turkey isolates were susceptible to erythromycin. Belgium, Ireland, and Switzerland (Fallon et al., 2003; Ledergerber et al., 2003; Van Looveren et al., 2001) also reported a low erythromycin resistance. Erythromycin is considered as a first line choice for the treatment of *C. jejuni* infections and low resistance among retail meat isolates supports this common policy of antimicrobial use. Additionally, similar to Ge et al. (2003) and Van Looveren et al. (2001), none of the chicken and turkey isolates showed resistance to gentamicin.

In our study we found a high level (60%) of multidrug (two or three antimicrobial agents) resistant isolates. Fallon et al. (2003) found 30% of the isolates resistant to two or more

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III

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Research Note

High Level of Antimicrobial Resistance in *Campylobacter jejuni* Isolated from Broiler Chickens in Estonia in 2005 and 2006

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ABSTRACT

The development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* is a matter of increasing concern. Because campylobacteriosis is transmitted to humans usually via food of animal origin, the presence of antimicrobial-resistant campylobacters in broiler chickens has important public health implications. The aim of our study was to analyze resistance patterns of *C. jejuni* isolated from fecal samples collected at a large Estonian chicken farm, from cecal contents collected at slaughterhouses, and from meat samples collected at the retail establishments in 2005 and 2006. A total of 131 *C. jejuni* isolates were collected over a 13-month period and tested by the broth microdilution VetMIC method (National Veterinary Institute, Uppsala, Sweden) to determine the MICs of various antimicrobials. Resistance to one or more antimicrobials was detected in 104 (79.4%) of the 131 isolates. High proportions of the isolates were resistant to enrofloxacin (73.3%) and nalidixic acid (75.6%). Multidrug resistance (resistance to three or more unrelated antimicrobials) was detected in 36 isolates (27.5%), all of which were resistant to enrofloxacin. Multidrug resistance was significantly associated with enrofloxacin resistance ($P < 0.01$), and the use of enrofloxacin may select for multiresistant strains.

Campylobacter species are the most common bacterial cause of human intestinal infections in many countries *(13, 14, 19, 31).* Studies carried out in slaughterhouses have indicated that the major source of *Campylobacter* contamination of poultry carcasses is their intestinal contents *(12, 28).* Poultry products are suspected to be an important source of human *Campylobacter* infections because of frequent contamination of poultry meat at the retail level *(18, 23).* Emerging antimicrobial resistance among *Campylobacter* isolates is mainly a consequence of the use of antimicrobial agents, especially fluoroquinolones, macrolides, and tetracyclines, in food animal production. In most cases of human *Campylobacter* enteritis, affected individuals recover spontaneously and do not require antimicrobial treatment. Antimicrobial treatment is appropriate for systemic campylobacteriosis, and erythromycin or fluoroquinolones often are recommended *(11, 25).* In vitro susceptibility testing of *Campylobacter* isolates is important in ensuring rapid and appropriate management of patients with foodborne campylobacteriosis *(15, 26, 29).* Enrofloxacin, flumequine, tetracycline, and amoxicillin are accepted therapeutic treatments for poultry in Estonia *(10),* but the use of these agents may induce resistance in *Campylobacter* isolates to these antimicrobials. Our study was conducted during a 1-year period in 2005 and 2006 to isolate campylobacters from a poultry production chain and to determine the prevalence of antimicrobial resistance of these isolates to ampicillin, enrofloxacin, erythromycin, gentamicin, nalidixic acid, and oxytetracycline.

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MATERIALS AND METHODS

***Campylobacter* isolates.** The study included 105 *Campylobacter* isolates from a total of 1,254 fresh fecal samples from chickens at a large Estonian chicken farm containing 60 unconnected flocks in separate housings (20,000 birds per flock) and from samples of cecal contents from 264 chickens at a slaughterhouse. Twenty-six additional isolates from 340 randomly purchased fresh chicken meat samples from three food stores in Estonia also were analyzed. All the samples were collected monthly. Fecal samples from the farm were collected between September 2005 and June 2006, cecal samples at a slaughterhouse were collected between July 2006 and October 2006, and meat samples at retail were collected between September 2005 and September 2006. All 131 isolates were identified as *Campylobacter jejuni.*

One loopful (10 μl) of fecal material or intestinal contents from the cecum was transferred into tubes containing 10 ml of Preston enrichment broth (Oxoid, Basingstoke, UK). The tubes were cooled to 4°C and transported immediately to the laboratory, where they were incubated at 42 ± 0.5°C for 24 h under microaerobic conditions. Analyses for campylobacters were carried out at the State Veterinary and Food Laboratory (Tartu, Estonia).

Fresh poultry meat samples were analyzed for campylobacters using the method of the Nordic Committee on Food Analysis

TABLE 1. *MICs for 131* Campylobacter jejuni *isolates from broiler chicken meat and fecal samples in Estonia*[a]

Antimicrobial agent[b]	No. of isolates with MIC (μg/ml) of:												
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
AMP					44	12	10	39	14	2	8	2 (2)	
ENX			5	16	14	7	3	86 (80)					
ERY			3	2	40	16	14	27	3	26 (26)			
GEN				13	51	26	11	5	25 (20)				
NAL								4	14	14	8	12	79 (64)
TET				69	8	9	3	4	5	2	31 (18)		

[a] MIC values for isolates were evaluated according to the VetMIC test kit manufacturer instructions (National Veterinary Institute, Uppsala, Sweden). Solid vertical lines indicate breakpoints between sensitive and resistant isolates. Numbers in parentheses are the number of *C. jejuni* strains with MIC values exceeding the VetMIC maximum concentration range.

[b] Antimicrobial agents: AMP, ampicillin; ENX, enrofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; TET, oxytetracycline.

(2), which includes an enrichment phase in Preston broth. Preston enrichment broth (250 ml) was added to a 25 g of meat sample, and the mixture was stomached for 60 s and then incubated at 42 ± 0.5°C for 24 h under microaerobic conditions.

After 24 h of incubation, a loopful of the enrichment culture from both fecal and meat samples was plated on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid), incubated under microaerobic conditions at 42 ± 0.5°C for 48 h, and examined for typical growth. Organisms growing on mCCDA plates were streaked on *Brucella* blood agar (Oxoid), and their identity was confirmed as *Campylobacter* by Gram staining, motility analysis, and oxidase and catalase tests. One randomly chosen colony from each positive sample was analyzed for hippurate hydrolysis, and hippurate-positive isolates were regarded as *Campylobacter jejuni.* After the original isolation, the strains were stored at −70°C in glycerol broth (15% [vol/vol] glycerol in 1% [wt/vol] Proteose Peptone).

Antimicrobial susceptibility testing. All 131 *C. jejuni* isolates were tested for MICs using the broth microdilution VetMIC test (National Veterinary Institute, Uppsala, Sweden) against ampicillin, enrofloxacin, erythromycin, gentamicin, nalidixic acid, and oxytetracycline. MIC testing was carried out at the laboratory of the Department of Food Science and Hygiene of the Estonian University of Life Sciences (Tartu, Estonia). *Campylobacter* isolates were first cultured on *Brucella* blood agar and incubated at 37°C for 48 h. A loopful (1 μl) of bacterial culture was transferred into 10 ml of cation-adjusted Mueller-Hinton broth (Oxoid) and then incubated at 37°C for 24 h to achieve around 10^8 CFU/ml. The bacterial suspension was then diluted to 10^6 CFU/ml, and 100 μl of this suspension was inoculated into each well of microtiter plates. The plates were incubated at 37°C for 40 h under microaerobic conditions. The MIC was the lowest concentration that completely inhibited visible growth of campylobacters, in accordance with the instructions given by the test manufacturer. Control of the purity of the bacterial suspension was carried out by plating 10 μl of bacterial suspension on *Brucella* agar. The density of the bacterial suspension was controlled according to the guidelines of the Estonian Veterinary and Food Laboratory, and counts of 50 to 250 colonies per plate were accepted *(3, 4). C. jejuni* ATCC 33560 was used as a control strain for antimicrobial susceptibility testing. The following MIC breakpoints for resistance were applied: ampicillin, 32 μg/ml; enrofloxacin, 1 μg/ml; erythromycin, 16 μg/ml; gentamicin, 8 μg/ml; nalidixic acid, 32 μg/ml; and oxytetracycline, 4 μg/ml *(3, 4).*

Statistical analysis. All individual results were recorded using MS Excel 2003 software (Microsoft Corporation, Redmond, Wash.), and statistical analysis was performed with the Statistical Package for Social Sciences 13.0 for Windows (SPSS Inc.; Chicago, Ill.). Nonparametric Spearman's rank order correlation coefficients with two-tailed *P* values and odds ratios were calculated for bivariate cross-correlations between resistances to the six antimicrobials analyzed and between antimicrobials and multiresistance, which was defined as resistance to three or more unrelated antimicrobials. The nonparametric Mann-Whitney independent samples test was conducted to compare the level of antimicrobial resistance between multiresistant and nonmultiresistant strains.

RESULTS

Table 1 shows the distribution of the 131 *C. jejuni* isolates based on their MICs. *C. jejuni* was detected in 105 (7%) of the broiler chicken fecal samples and in 26 (7.6%) of fresh chicken meat samples. Resistance to one or more antibiotics was detected in 104 isolates (79.4%). High proportions of the isolates were resistant to enrofloxacin (73.3%) and nalidixic acid (75.6%). The antimicrobial resistance phenotypes of the 131 *C. jejuni* strains are presented in Table 2. Twenty isolates (15.3%) were resistant to three unrelated antimicrobials, 13 isolates (10%) were resistant to four unrelated antimicrobials, and 3 isolates (2.3%) were resistant to all tested antimicrobials. Enrofloxacin and nalidixic acid were regarded as one group of antimicrobials. Isolates resistant to three unrelated antimicrobials were mainly resistant to a combination of enrofloxacin–nalidixic acid, erythromycin, and oxytetracycline (4.6%), and those resistant to four unrelated antimicrobials were mainly resistant to a combination of enrofloxacin–nalidixic acid, erythromycin, gentamicin, and oxytetracycline (8.4%). The highest frequency of resistance was to nalidixic acid and enrofloxacin (75.6 and 73.3%, respectively), followed by oxytetracycline (32.1%), erythromycin (19.8%), gentamicin (19.1%), and ampicillin (7.6%). Multidrug resistance was significantly associated ($P < 0.01$) with enrofloxacin and nalidixic acid resistance. The level of antimicrobial resistance was higher for nalidixic acid in multiresistant *C. jejuni* strains than in nonmultiresistant strains (Mann-Whitney test, $P = 0.026$), but resistance to

TABLE 2. *Antimicrobial resistance phenotypes among 131* Campylobacter jejuni *isolates from broiler chickens in Estonia in 2005 and 2006*

Antimicrobial resistance phenotype[a]	Fecal samples		Meat samples		Total	
	No.	%	No.	%	No.	%
AMP-ENX-ERY-GEN-NAL-TET	3	2.6			3	2.3
AMP-ENX-ERY-NAL-TET	1	1.0			1	0.8
AMP-ENX-ERY-GEN-NAL	1	1.0			1	0.8
ENX-ERY-GEN-NAL-TET	11	10.4			11	8.4
ENX-ERY-NAL-TET	4	3.8	6	23.1	10	7.6
ENX-GEN-NAL-TET	6	5.7			6	4.6
AMP-ENX-GEN-NAL	2	1.9			2	1.5
AMP-ENX-NAL-TET	1	1.0			1	0.8
ENX-NAL-TET	3	2.9	2	7.7	5	3.8
AMP-ENX-NAL	1	1.0			1	0.8
ENX-GEN-TET	1	1.0			1	0.8
ENX-NAL	41	38.9	13	50.0	54	41.2
GEN-TET	1	1.0			1	0.8
NAL-TET	1	1.0			1	0.8
AMP	1	1.0			1	0.8
NAL	3	2.9			3	2.3
TET	2	1.9			2	1.5
Resistant to an antimicrobial	83	79.0	21	80.8	104	79.4
Sensitive to all antimicrobials	22	21.0	5	19.2	27	20.6
Total	105		26		131	

[a] Antimicrobial agents: AMP, ampicillin; ENX, enrofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; TET, oxytetracycline.

other antimicrobials was not statistically different ($P > 0.05$) between multiresistant and nonmultiresistant strains.

DISCUSSION

The chickens sampled in the study were from a company that produces more than 90% of all commercial broilers in Estonia. Therefore, the isolates analyzed were considered representative of the pattern of antimicrobial resistance of *Campylobacter* in chickens in Estonia during 2005 and 2006. This study was not designed to assess the prevalence of *Campylobacter* in broilers produced in Estonia. However, prevalence of *Campylobacter*-positive samples in our study was relatively low compared with that detected in some other studies *(9, 18, 23).* An important finding was the high percentage (79.4%) of antimicrobial-resistant *Campylobacter* isolates, 36 (27.5%) of which exhibited multiresistance (resistance to three or more unrelated antimicrobials). Resistance was especially high to enrofloxacin (80 isolates had MICs ≥ 4 μg/ml). Development of resistance in *Campylobacter* isolates from food production animals that have been treated for infections with fluoroquinolones since the 1990s has been previously documented *(11).* Experimentally, resistance to fluoroquinolones develops rapidly and is persistent in *C. jejuni*–infected chickens treated with fluoroquinolones *(16, 22).* In countries such as Denmark, where the use of enrofloxacin to poultry has been prohibited since 2000, a decrease in the percentage of fluoroquinolone-resistant *C. jejuni* and *Campylobacter coli* isolates has been detected *(5).* In many studies, a connection between the use of fluoroquinolones in chickens and the evolution of fluoroquinolone resistance in *C. jejuni* has been documented (*16, 22*). Of the enrofloxacin-resistant strains, 39.6 and 27.1% were resistant to oxytetracycline and erythromycin, respectively. Enrofloxacin and flumequine, both fluoroquinolones, are permitted for use in poultry in Estonia *(10),* possibly explaining the high level of fluoroquinolone resistance detected among Estonian *C. jejuni* isolates. Unfortunately, we do not have data on previous antimicrobial usage associated either with the chicken farm or slaughterhouse samples.

In various studies, tetracycline resistance has been reported as common among poultry isolates. Ledergerber et al. *(21)* reported tetracycline resistance in 12% of the isolates examined, and Ge et al. *(15)* found a much higher level of resistance, 82% of the isolates examined. In our study, 32.1% of the isolates were resistant to oxytetracycline. Doxycycline is permitted for treatment for poultry in Estonia *(10).*

Reported ampicillin resistance among chicken *C. jejuni* isolates ranges from the same level as we found, less than 10% *(1, 20),* up to approximately 30% in some countries, e.g., France *(6, 8),* Belgium *(30),* and Ireland *(24).* This finding likely reflects the less common use of ampicillin in Estonia; ampicillin is not part of the therapeutic treatment of infectious diseases in poultry. Gentamicin resistance was high among our *C. jejuni* strains (19%), but in several other studies no resistance to this antimicrobial was detected *(6–8).* Erythromycin resistance also was higher (19.8%) among our *C. jejuni* strains than that reported in several other studies, e.g., from Belgium *(30),* Ireland *(13),* and Switzerland *(21).* Because erythromycin is considered a first-line treatment for human *C. jejuni* infections, resistance to this drug has important public health implications.

We found a high proportion of multidrug-resistant isolates (27.5%); all of these isolates were resistant to enrofloxacin, and all except one were resistant to nalidixic acid. Hakanen et al. *(17)* noted that 20% of the human isolates associated with traveling were resistant to three or more antimicrobials. Multiresistant isolates were resistant to a combination of tested antimicrobials. Multidrug resistance was significantly associated with enrofloxacin and nalidixic acid resistance (correlation coefficients of 0.372 and 0.310, $P < 0.01$). These findings suggest that the use of fluoroquinolones may select for multiresistant strains because resistance to erythromycin, gentamicin, or oxytetracycline was exceptional without simultaneous resistance to fluoroquinolones. In a recent study of antimicrobial resistance of *Escherichia coli* at a broiler chicken farm where no antimicrobial treatment were used on the birds during the year before samples were collected, resistance to tetracycline, gentamicin, and streptomycin persisted, but all isolates were susceptible to enrofloxacin *(27).* Thus, multiresistant

strains may reflect the past history of antimicrobial usage during a longer period. This phenomenon may partly explain the high number of multiresistant strains in our study.

In conclusion, multidrug resistance in Estonian broiler chicken *Campylobacter* isolates was one of the highest reported in similar studies of broiler chickens. The widespread emergence of multiresistant *Campylobacter* isolates poses a threat to human health and limits therapeutic options. In Estonia, more restricted use of antimicrobial agents, especially fluoroquinolones, in food animal production should be implemented.

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Scientific publications:

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Roasto, M., Tamme, T ja Juhkam K. „Toiduhügieen ja ohutus" (Food Hygiene and Safety – second, improved version). Teine, parandatud ja täiendatud trükk. Tartu: Eesti Maaülikool, 2006. 151 lk. ISBN 9985-9582-0-9, in estonian language;

Roasto, M., Tamme, T ja Juhkam K. „Toiduhügieen ja ohutus" (Food Hygiene and Safety). Tartu: Eesti Põllumajandusülikool, Halo Kirjastus (publisher), 2004. 237 lk. ISBN: 9985-9582-0-9, in estonian language.

3.3 Articles in conference proceedings

Roasto, M., Juhkam, K., Tamme, T., Praakle, K., Hörman, A., Hänninen, M.-L. (2007). Antimicrobial resistance in *Campylobacter jejuni* isolated from broiler chickens in Estonia during periods 2002-2003 and 2005-2006. XIII International Congress in Animal Hygiene ISAH-2007, Proceedings, Volume 2, 785-789**;**

Praakle, K., **Roasto, M**., Elias, P., Korkeala, H. and Hänninen, M.-L. (2004). Prevalence of *Campylobacter* spp. in retail poultry in Estonia. 50th International Congress of Meat Science and Technology. Conference proceedings. Helsinki, Finland;

Praakle, K., **Roasto, M.,** Hänninen, M-L., Korkeala, H. (2004). Thermophilic *Campylobacter* spp. in raw poultry products at the retail level in Estonia. Conference on Farm to Fork Food Safety: A Call for Common Sense. Conference proceedings. Athens, Greece;

Roasto, M., Elias, P., Praakle, K., Korkeala, H. and Hänninen, M.-L. (2004). The prevalence of thermophilic *Campylobacter* spp. in raw foodstuffs of Estonian origin sold at retail level of Tartu town. International Scientific Conference „Animals. Health. Food hygiene". Conference proceedings. Jelgava, Latvia, ISSN: 1407-1754. pp. 246-247;

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Roasto, M., Jõers, K., Spiridonova, I. (2002). Termofiilsed kampülobakterid, kampülobakterioos ja toiduainete saastumine. *Veterinaarmeditsiin*, 25-31.

Training activities:

VL.0700 Hygiene and Veterinary Control in Meat Industry – 3.75 ECTS;
VL.0644 General Food Hygiene – 9.0 ECTS;
VL.0325 Hygiene and Veterinary Public Health – 3.0 ECTS
VL.0434 Meat Inspection – 4.5 ECTS

In-service training:
NOVABA (Nordic-Baltic) research course – Hygiene of fish and fish products, Tartu, December 7-11, 1998;
NOVABA research course – Novel Food, Tartu, October 25-29, 1999;
NOVABA research course – Welfare of Slaughter Animals and Meat Quality, Tartu, February 26 – March 2, 2001;
Food microbiology and principles of PCR- diagnostics, Helsinki University, March 3 – March 7, 2003;
Retraining in food hygiene – Berlin, Freie Universitet, 24.08 – 07.09.2003;
Molecular typing methods in food microbiology and evaluation of the microbiological quality of water, Helsinki University, January 19 - January 23, 2004;
Microbial ecology of meat and microbiological hazards in meat industry, Latvia University of Agriculture, May 5 - May 6, 2004;
Detection of pathogens using the Roche LightCycler, Potsdam, Biotecon Diagnostics GmbH, Germany August 4 – August 5, 2004;
Study course „Teaching at University", Tartu, 13.05 – 11.06.2005, 3,0 ECTS;
Course „Production Animals Medicine", Tartu, 15.07.2006 – 17.07.2006;

Microbial Contaminants and Contamination Routes in Food Industry, Espoo, Finland, January 22-23, 2007;
Retraining program „Composeing distance learning course by WebCT instruments“, Tartu, 09.02.2007 – 02.03.2007, 1,5 ECTS;
Retraining program „Using the Statistical package STATISTICA“, Tartu Universirty, Tartu, 01.03.2007 – 09.03.2007;
Risk Assessment of Microbial Problems and Preventive Actions in Food Industry, Istanbul, Turkey, October 22-23, 2007;
SAFOODNET – 1st workshop of the SAFOODNET project "Detection and Identification, of Pathogenic Microorganisms", Veterinary Research Institute Brno, Czech Republic, December 10-12, 2007;
Study course: "The fundamentals of self-expression", Tartu, 06.02.2008 – 08.02.2008;
International study course "Methodology of research", Saku, Harjumaa, Estonia, 11.02.2008 - 12.02.2008.

APPROBATION
International and regional conferences and workshops

Oral presentations
Tartu, Estonia 2007. XIII International Congress in Animal Hygiene ISAH. Presentation: Antimicrobial resistance in *Campylobacter jejuni* isolated from broiler chickens in Estonia during periods 2002-2003 and 2005-2006".

Jelgava, Latvia 2004. International Scientific Conference "Animals. Health. Food Hygiene". Presentation: The prevalence of thermophilic *Campylobacter* spp. in raw foodstuffs of Estonian origin sold at retail level of Tartu town.

Tartu, Estonia 2004. International seminar of Nordic Council of Ministers "Enlargement of European Union and Food Safety in Estonia". Presentation: Thermophilic *Campylobacter* spp. as a food-borne pathogens.

Tartu, Estonia 2002. Conference of Veterinary Medicine. Presentation: Termofiilsed kampülobakterid, kampülobakterioos ja toiduainete saastumine.

Poster presentations

Helsinki, Finland 2004. 50th International Congress of Meat Science and Technology. Presentation: Prevalence of *Campylobacter* spp. in retail poultry in Estonia.

Athens, Greece 2004. Conference on Farm to Fork Food Safety: A Call for Common Sense. Presentation: Thermophilic *Campylobacter* spp. in raw poultry products at the retail level in Estonia.

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Ametikoht:	korraline lektor, toiduhügieeni ja -kontrolli osakonna juhataja
Sünniaeg:	05.02.1973
Haridus:	Kärdla Keskkool, 1991 Arkna Katsesovhoos-kool, veterinaarvelsker-seemendustehnik, 1992
Keelteoskus:	Eesti keel (suurepärane), inglise keel (suurepärane), vene keel (rahuldav)
Teenistuskäik:	Eesti Põllumajandusülikool, veterinaarmeditsiini eriala, 1998 erakorraline ja alates 1. sept. 2000 korraline assistent ning alates 1. sept. 2002 korraline lektor, õppetooli hoidja. Alates 01.10.2006 – Toiduteaduse ja -hügieeni osakonna juhataja, Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut.
Teadus- või akadeemiline kraad:	MSc. toiduteaduse erialal
Kraadi välja andnud asutus, aasta:	Eesti Põllumajandusülikool, 2001
Tunnustused:	Prof. Esko Nurmi nimeline stipendium, 2000 a. DeLaval'i nimelise stipendiumi laureaat (III taseme uurimistöö stipendium) 2006 a.

Teadusorganisatsiooniline ja –administratiivne tegevus:

Rahvusvahelise Loomatervishoiu Ühingu liige, alates 2007 a.;
Ameerika Mikrobioloogia Ühingu liige, alates 2006 a.;
Akadeemilise Põllumajanduse Seltsi liige, alates 2004 a.;
Eesti Loomaarstide Ühingu liige, alates 2000 a.
Vastutav isik liha- ja piimatehnoloogia magistriõppeprogrammi akrediteerimisel, Eesti Kõrghariduse Akrediteerimiskeskus. Otsus: täisakrediteering, 09.04.2007;
Vastutav isik Veterinaarmeditsiini ja loomakasvatuse instituudi toiduhügieenialase õppe akrediteerimisel. Järelhindamine 03.07.2007 – 05.07.2007, prof. Marcel Wanner, EAEVE president, prof. Marc Vandevelde, EAEVE ekspert. Otsus: esimese kategooria puudused toiduhügieenis on kõrvaldatud;
Veterinaarmeditsiini ja loomakasvatuse instituudi nõukogu liige 2005 – 2007;
Veterinaarmeditsiini eriala õppemetoodika komisjoni liige 2005 - 2007;
Veterinaarmeditsiini ja loomakasvatuse instituudi nõukogu liige 2008 …….
Veterinaarmeditsiini eriala õppemetoodika komisjoni liige 2008 ….
Liha- ja piimatoidutehnoloogia eriala õppemetoodika komisjoni liige 2008 ……..

Juhendamisel kaitstud magistri- ja doktoritööd:

Marko Breivel. 2006. Juhendajad lektor Mati Roasto ja lektor Kadrin Juhkam. Magistritöö "Tootmishügieeni hindamine lihatööstusettevõttes". Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut.

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Juhendamisel kaitstud Diplomitööd:

Jane Mägi. 2002. Juhendajad: Prof. Meili Rei ja assistent Mati Roasto. Lihatööstuse puhastamise program, seadmed ja vahendid. Eesti Põllumajandusülikool. Loomaarstiteaduskond.

Silja Sillakivi. 2006. Juhendajad: Prof. Meili Rei ja lektor Mati Roasto. Sanitatsioon lihatööstuse näitel. Eesti Maaülikool. Veterinaarmeditsiini ja loomakasvatuse instituut.

Juhendamisel kaitstud Bakalaureuse tööd:

Helina Volmer. 2006. Juhendaja lektor Mati Roasto. "Sanitaarkorraldus Eesti piimatööstuses ja selle kontrolli võimalused kasutades Hygiene Management System™-i". Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut.

Teadustöö põhisuunad: Toiduhügieen ja veterinaarne rahvatervishoid

Osalemine uurimisprojektides:

Grant 4979 "Toiduainete saastatus termofiilsete kampülobakteritega, probleemi tõsidus ja tõrje võimalused", 2002-2004, põhitäitja.
Baasfinantseerimise projekt: "Bioloogilised ja keemilised ohud toidu tootmise ahelas ja nende minimeerimise võimalused", 2005 – 2008, põhitäitja.

Pilootprojekt: "Toiduainete tehnoloogia uurimissuuna väljaarendamine", 2004-2007, alateema nr. 4 vastutav täitja..

Teaduspublikatsioonid:

1.1 artiklid ajakirjades, mida katab *ISI Web of Science*

Roasto, M., Juhkam, K., Tamme, T., Hörman, A., Häkkinen, L., Reinik, M., Karus, A., Hänninen, M.-L. (2007). High level of antimicrobial resistance in *Campylobacter jejuni* isolated from broiler chickens in Estonia in 2005-2006. *Journal of Food Protection*, **70(8)**, 1940-1944;

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Karus, A., Saprõkina, Z., Tikk, A., Järv, P., Soidla, R., Lember, A., Kuusik, S., Karus, V, Kaldmäe, H., **Roasto, M**., Rei, M. (2007). Effect of dietary linseed on insulin-like growth factor-1 and tissue fat composition in quails. *Archiv für Geflügelkunde/European Poultry Science*, **71**(2), 81-87;

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1.5 artiklid muudes teadusajakirjades

Lillenberg, M., **Roasto, M**., Püssa, T. (2003). Ravimijäägid keskkonnas. Fluorokinoloonide määramine mullas ja toidutaimedes. *Agraarteadus*, **14(1)**, 13-26;

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2.5 õpikud või õpikute peatükid

Reinik, M., Tamme, T., **Roasto, M.** Naturally occuring nitrates and nitrites in food, In: J. Gilbert and H. Senyuva (Eds.), Bioactive compounds in foods – Natural toxicants and heat processing contaminants. Blackwell Publishers, London, UK. In press;

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3.3 peatükid/artiklid kohalike (Eesti või välisriikide) kirjastuste kogumikes

Roasto, M., Juhkam, K., Tamme, T., Praakle, K., Hörman, A., Hänninen, M.-L. (2007). Antimicrobial resistance in *Campylobacter jejuni* isolated from broiler chickens in Estonia during periods 2002-2003 and 2005-2006. XIII International Congress in Animal Hygiene ISAH-2007, Proceedings Volume 2, 785-789;

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K. Praakle, **M. Roasto**, M.-L. Hänninen and H. Korkeala. 2004. Thermophilic *Campylobacter* spp. in raw poultry products at the retail level in Estonia. Conference on Farm to Fork Food Safety: A Call for Common Sense. Conference proceedings. Athens, Greece;

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Tamme, T., Reinik, M., Kiis, A., Juhkam, K., **Roasto, M**. (2003). Nitraadid ja nitritid köögiviljades ja toormahlades. *Veterinaarmeditsiin*, 91-100;

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Õppetöö VL.0644 Toiduhügieeni üldkursus (6,0 AP);
VL.0700 Hügieen ja veterinaarkontroll lihatööstuses (2,5 AP);
VL.0434 Lihainspektsioon (3,0 AP);
VL.0325 Toiduhügieen ja veterinaarne rahvatervishoid (2,0 AP).

Erialane enesetäiendus:
NOVABA (Nordic-Baltic) research course – Hygiene of fish and fish products, Tartu, December 7-11, 1998;
NOVABA research course – Novel Food, Tartu, October 25-29, 1999;
NOVABA research course – Welfare of Slaughter Animals and Meat Quality, Tartu, February 26 – March 2, 2001;
Food Microbiology and Principles of PCR- diagnostics, Helsinki University, March 3 – March 7, 2003;
Toiduhügieeni alane täiendkoolitus – Berliin, Freie Universitet, 24.08 – 07.09.2003;
Molecular typing methods in food microbiology and evaluation of the microbiological quality of water, Helsinki University, January 19 - January 23, 2004;
Microbial ecology of meat and microbiological hazards in meat industry, Latvia University of Agriculture, May 5 - May 6, 2004.
Detection of pathogens using the Roche LightCycler, Potsdam, Biotecon Diagnostics GmbH, Germany August 4 – August 5, 2004;
Õpetamine kõrgkoolis, Tartu, 13.05 – 11.06.2005, maht 2,0 AP (3,0 ECTS);
Course „Production Animals Medicine", Tartu, 15.07.2006 – 17.07.2006;

Microbial Contaminants and Contamination Routes in Food Industry, Espoo, Finland, January 22-23, 2007;
Täiendkoolitusprogramm „Kaugkoolituskursuse loomine WebCT vahenditega, Tartu, ajavahemikul 09.02.2007 – 02.03.2007, maht 1 AP (1,5 ECTS);
Täiendkoolitusprogramm „Statistikapakett STATISTICA kasutamine“, Tartu Ülikool, Tartu, ajavahemikul 01.03.2007 – 09.03.2007, maht 12 tundi;
Risk Assessment of Microbial Problems and Preventive Actions in Food Industry, Istanbul, Turkey, October 22-23, 2007;
SAFOODNET – 1st workshop of the SAFOODNET project "Detection and Identification, of Pathogenic Microorganisms", Veterinary Research Institute Brno, Czech Republic, December 10-12, 2007;
Koolitus: Eneseväljenduskunsti alused, Tartu, 06.02.2008 – 08.02.2008;
Rahvusvaheline kursus "Methodology of research", Harjumaa, Saku, 11.02.2008 – 12.02.2008.

Annex 1. Procedure for serotyping of *C. jejuni* by heat-stable antigen (Denka Seiken, Tokyo, Japan)

Preparation of sensitized bacterial antigen solution:
Place 0.25 ml of saline into a 1.5 ml volume centrifuge tube.
Suspend bacteria into saline using a bacteriological loop. (i.e. an amount equivalent to the size of a matchhead).
Add 0.25 ml each of extract reagent 1 and 2 into the bacterial suspension.
After mixing the suspension using a tube mixer, incubate for 10 minutes at room temperature.
Add 0.25 ml extract reagent 3 and mix well.
Centrifuge for 5 minutes at 7000 rpm and use the supernatant as antigen solution to sensitize chick red blood cells.

Preparation/washing of fixed chick red blood cells:
As each test requires 0.5 ml of the fixed red blood cells, dispense an appropriate volume into a test tube (number of tests x 0.5ml) and then add an equivalent volume of phosphate-buffered saline (PBS).
Centrifuge at 3,000 rpm for 10 minutes.
Discard the supernatant and resuspend the red blood cells in an equivalent volume of PBS (number of tests x 0.5 ml)

Preparation of sensitized cells:
Add 0.5 ml of fixed chick red blood cells into a 1.5 ml centrifugation tube which contains 0.5 ml of the bacterial antigen solution for sensitizing.
Incubate at 37 °C for 30 minutes and mix often.
Centrifuge at 6000 rpm for 30 seconds and discard the supernatant.
Add 1.0 ml buffer and resuspend the pellet using a test tube mixer. Use as the sensitized cell suspension.

Passive haemaglutination (PHA) test:
Place one drop of each antiserum individually into microplate wells.
Set aside a single well as a spontaneous aglutination control by placing one drop of control serum into it.
Place 25µl of the sensitized cell suspension into each well.
Mix well using a microplate mixer and place in a moisture box and incubate for 30 minutes.
Check for aglutination.

Interpretation

Results	Determination
Red blood cells (RBCs) sediment to form a tight button at the center of the well	-
RBCs aglutinate slightly, but do not cover the complete surface of the well	+
RBCs aglutinate to cover the complete surface of the well, but RBCs still sediment to form button at the center of the wall	++
RBCs completely aglutinate	+++

Regards aglutination stronger than + as a positive

Annex 2. The PFGE protocol. Application for the analysis of *Campylobacter jejuni* and *Campylobacter coli*

Two days before PFGE analysis
Subculture strains from frozen tubes onto Brucella agar media. Grow under appropriate conditions for one day.

One day before PFGE analysis
Subculturing from Brucella agar to Brucella agar.

First step: Washing the cells: Put into 8 ml centrifugation tubes 900 µl 0,95% NaCl + bacterial mass (with 10 µl loop) + 100 µl 40% formaldehyde (Formaldehyde is extremely toxic and must be added carefully in a fume cabinet). Vortex and keep about 30 min. on ice.

Second step: Put cooling centrifuge + 4 ºC per 20 minute/ 1000 X g
Centrifuge: 1250 X g, 10 min, 4 ºC
NB: After centrifugation should be visible sediment on a bottom of tube.
Pour supernatant away immediately after centrifugation
Resuspend in Pett IV buffer (PIV) 2-3 ml/tube and vortex
Centrifuge 10 min
Pour supernatant away again and resuspend in PIV 1-2 ml (depends on amount of sediment) and vortex
Keep on ice approximately 20 minutes.

Third step: Prepare the 2% agarose (within above mentioned 20 min.)
15 ml PIV + 0.3 g Low-melting-point (LMP)-agarose
LMP-agarose, a volume of 0.5 ml is needed for each strain + about 2 ml extra.
Add an aliquot of 0.5 ml of agar to each eppendorf tube (on waterbath, 56 ºC)
Check that you have all the following beside the water bath:

- 1 ml disposable syringes
- vortex
- ice with sloping surface
- the samples on ice
- garbage bag for the used pipettes and strain tubes

NB: For the next operation - don´t shake the bacterial cell suspension

before adding to eppendorf tube and don´t take the suspension from the bottom of suspension tube.

Add 0.5 ml bacterial cell suspension to an eppendorf, vortex immediately and suck into a 1 ml syringe. Put the syringe on the ice and allow cooling for at least 10 minutes. The eppendorf tube may be used as a cup.

Fourth step: take out from the freezer the lysozyme and RNAse (to melt up)
Enzymes are in freezer, in ready-to-use aliquots
Mark the milk tubes
Explanation: Lysozyme and RNAse are in ready-to-use aliquots:
Lysozyme 500 µl/ per 50 ml lysis-buffer
RNA-se 100 µl / per 50 ml lysis-buffer
Fifth step: Prepare the lysis-solution as following:
Lysis-buffer – 50 ml
Lysozyme – 500 µl
RNA-se – 100 µl

Pour 2.5 ml lysis-solution to each milk tube

Sixth step: Take the plug out of syringe and cut it into two pieces and put it into a milk tube (lysis-solution was added already before). Incubate in a shaker (80/min), 37 °C for 16 hours.

Wash the syringes with 2% hypochlorite (30 min) and rinse three times with distilled water.

Second day – Washing with EDTA (pH 8.0) + 10% sodium lavroyl sarcosine + proteinase K solution (ESP solution)

First step: Prepare the ES-buffer (0.5 M EDTA, sodium *N*-lauroylsarcosine)
For making 20 x ESP-solution: pronase 1 ml + ES-buffer 9 ml (1:9)
For making 1 x ESP-solution: 20 x ESP-solution 3.7 ml + ES-buffer 70 ml

Second step: Pour away the lysis-solution and add 1 x ESP-solution 2.5 – 3 ml – a plug should be covered totally by ESP-solution. Incubate at 50 °C for 6 hours. During those 6 hours change ESP-solution several times. Use the following scheme: 0 → 2 t → 2t → done

Third step: Transfere one half of the plug into an eppendorf tube containing fresh 1 x ESP (can be stored in a fridge (5 °C) for 1-2 years).

Fourth step:
Take the Pefabloc SC (for inactivating pronase from the ESP-solution) from freezer to melt up. The amount 0,5 ml (Pefabloc) is needed + TE 10:1 (pH around 7.5) 50 ml. Add 2.5 ml to each milk tube and incubate in a shaker 37 °C.
If your time schedule is allowing – change the solution after 1-2 hour.
Incubate overnight (16 hours).

Third day of PFGE analysis
First step: Change Pefabloc for TE 10:1 2.5 – 5 ml/tube

a) 37 °C 2 x 2 h. or
b) at 50 °C 1 x 2 h.

Number the eppendorfs.
For 2 pm. Make ready digestion-buffer.

Keep on ice!

Second step:
Digestion buffer needed:
If using *Sma*I: 500 µl buffer 4 (New England Biolabs) + 4500 µl dH_2O – (totally 5 ml).
If using *Kpn*I: 500 µl buffer 1 (New England Biolabs) + 50 µl BSA + 4450 µl dH2O

Keep on ice!

Third step:
Pipet 100 µl digestion-buffer/eppendorf. Keep on ice.

When buffer is ready cut a slim, about 0.5 mm thick slice from the plug and put it into the eppendorf with digestion buffer – one slice is going to buffer 4 (New England Biolabs)-buffer 4 and other one to buffer 1. Let cool on ice for 15-30 minutes. Put the rest of the plug into a tube with TE 2:5 and store in fridge.

Add the enzyme to the digestion-buffer, according to the instructions of the manufacture:

ON ICE!

*Sma*I: 20 Units per tube
In commercial original tube there is 20 000 Units
The concentration of *Sma*I is 20 Units per 1 µl/NEB
Take 25 µl enzyme + 2.5 ml digestion-buffer (for 25 sample)
Pipet the mere digestion-buffer out of the eppendorfs and replace with the enzyme digestion buffer (100 µl).
Incubate 16 h, 25 °C

*Kpn*I: 40 Units per tube
In commercial original tube there is 10 000 Units
The concentration of *Kpn*I is 10 Units per 1 µl/NEB
Take 100 µl enzyme + 2.5 ml digestion-buffer (for 25 sample)
Pipet the mere digestion-buffer out of the eppendorfs and replace with the enzyme digestion buffer (100 µl).
Incubate 16 h, 37 °C

Fourth day of PFGE analysis
Pipet away the enzyme digestion buffer, and add 250 µl 0.5 x TBE (Tris-Borate-EDTA solution) (the same buffer used in the electrophoresis). At the same time put markers into 0.5 x TBE, to be balanced. Store in a fridge. Plug can be stored in this stage for at least one week in a fridge.

Preparing the gel electrophoresis (1% agarose):

10 x TBE	5.5 ml
dH_2O	104.5 ml
agarose (SeaKem Gold®)	1.1 g

Weigh before melting!

Boil in a microwave oven for about 6-7 minutes, covered with clock-glass. Stir well after approximately 3 minutes of boiling. At the same time, boil water in a small container.
Weigh after melting and replace evaporated water, using the boiling water from the container.
Shake well and let cool to below 60 °C before casting.
Measure 2.5 ml of gel into a separate tube and keep 50-60 °C.
Cast the gel, at first fill the wholes. Apply "the comb" and let solidify for 15 minutes, put the gel into electrophoresis buffer for at least 2 hour before electrophoresis.

Preparing the electrophoresis buffer
0.5 x TBE buffer: 125 ml 10 x TBE + 2375 ml dH_2O
Put the gel to the electrophoresis buffer for 3 hour to cool down.

Loading the gel
Apply the markers at first, and then the samples from left to right. Finally cover plugs with the melted gel.

Electrophoresis:
Pour the 0.5 TBE-buffer to the PFGE application. Start up the pump for the water circulation system, and let cool to 8 °C.
Add the hexagonal electrode.
Put on the Gene Navigator and choose programme:
***Sma*I**: 1s→30 s; 19 h.
***Kpn*I**: 1s→25 s; 19 h.
200V
Check that electrodes bubble, before leaving the site.
80 – 100 mA (if you have two gels, the value is doubled)

Fifth day
Switch off the electricity of the device.
Take off the electrode.
Take off the gel with the plastic mould.
Staining solution: electrophoresis buffer 1 liter + 50 µl ethidiumbromide.
Cut off the posts from the gel with scalpel, and put gel into the staining solution for about 30 minutes. Save the rest of the electrophoresis-buffer for destaining. The gel is distained for at least two hours. Wash the application with distilled water.