Contributions to the epidemiology of *Campylobacter* infections

A review of clinical and microbiological studies

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CHAPTER 1. INTRODUCTION

Campylobacter was first described in 1886 by Theodor Escherich. He identified spiral form bacteria in stool specimens from diarrhoeal infections in neonates as well as in kittens. Attempts to grow these organisms on solid medium were unsuccessful. In 1909, McFaydean & Stockman obtained the first pure culture of a "vibrio", which we now know as *Campylobacter fetus*, from the uterus of a sheep. Smith & Taylor proposed in 1919 the name *Vibrio fetus* to organisms isolated from clinical cases of vibrionic abortion in cattle. Closely related organisms were later described as *V. jejuni* isolated from the jejunum of cattle, and *V. coli* from pigs (Jones, 1931; Doyle, 1944).

A milkborne outbreak of diarrhoea affecting 355 inmates of two adjacent state institutions in May 1938 in Illinois is now regarded as the first well-documented instance of human infection caused by *C. jejuni* (or *C. coli*). Faecal cultures from 73 tested patients were negative (microscopy positive in 31), but organisms resembling *V. jejuni* were grown in broth cultures of blood samples from 13 patients (Levy, 1946). A milestone in the history of *Campylobacter* was the work of Elisabeth King who made a systematic study of several *Vibrio* isolates and discriminated between *V. fetus* and the thermo-tolerant *V. jejuni* and *V. coli*, though she kept the provisional names "related vibrios" for the two latter (King, 1957; King, 1962).

Still, it lasted until 1972 before *Campylobacter* was isolated from human stools by Dekeyser & Butzler in Brussel, Belgium (Dekeyser et al., 1972; Butzler et al., 1973). Their initial papers inexplicably elicited no response until several years later they were picked up by Skirrow (1977). The development of Skirrow's selective medium enabled routine diagnostic microbiology laboratories to isolate campylobacters and to evaluate their clinical role. This brought to light the true dimension of *Campylobacter* as the leading bacterial cause of human enteritis in the world (Friedman et al., 2000).

The taxonomic complexity of the genus *Campylobacter* has risen dramatically during the past two decades. The genus *Campylobacter* was first proposed in 1963 by Sebald & Véron, who transferred *V. fetus* and *V. bubulus* (now *C. sputorum*) into a new genus, *Campylobacter*. Ten years later, Véron & Chatelain published a more comprehensive study on the taxonomy of the microaerophilic *Vibrio*-like organisms and considered four distinct species in the genus *Campylobacter*: *C. fetus*, *C. coli*, *C. jejuni* and *C. sputorum*. The availability of adequate isolation procedures led to an increased interest in *Campylobacter* research during the early 1980s. As a consequence, a manifold of *Campylobacter*-like organisms (CLOs) were isolated from a variety of human, animal and environmental sources. Gradually, these CLO groups were identified as novel *Campylobacter* species or biochemical variants of established species. From 1974 to 1988, 12 new species or subspecies were discovered although the taxonomic status of many of these taxa was later changed owing to the wider application of advanced taxonomic methods in the late 1980s. The idea that bacterial classification should be based on natural evolution, which is imprinted in the DNA sequence of highly conserved macromolecules, offered a new approach. Indeed, the potential of the 16S rRNA gene for determining phylogenetic relationships attracted much interest. Numerical comparison of partial 16S rRNA gene sequences identified distinct clades within the genus, and the new genus *Helicobacter* were proposed by Goodwin et al. (1989). In 1991, a complete revison of the taxonomy and nomenclature of the genus *Campylobacter* and related bacteria was proposed by Vandamme et al. By use of DNA-rRNA hybridization and by cross-reference with other phenotypic and genetic data, the relative phylogenetic positions were determined. This study provided the basis of the taxonomic structure used at present, and delineated *Campylobacter* spp. as a diverse, yet phylogenetically distinct group, rRNA superfamily VI (also known as the ε-division of the *Proteobacteria*). This comprise rRNA homology group I (*Campylobacter* and *Bacteroides ureolyticus*), II (*Arcobacter*) and III (*Helicobacter* and *Wollinella succinogenes*). Based on the close relatedness of rRNA homology between group I and II, these constitute the family *Campylobacteraceae*. Present members of the family *Campylobacteraceae* and related organisms belonging to "*Campylobacteria*" are presented in the **Table 1**.

Table 1. Present members of the family *Campylobacteraceae* (A-D)[a] and related organisms (E).

	Taxon
A	*C. jejuni* subsp. *jejuni*
	C. jejuni subsp. *doylei*
	C. coli
	C. lari
	C. upsaliensis
	C. insulaenigrae
	C. helveticus
B[b]	*C. concisus*
	C. curvus
	C. rectus
	C. showae
	C. gracilis[c]
	C. hominis[c]
	C. sputorum bv. *sputorum*
	C. sputorum bv. *faecalis*
	C. sputorum bv. *paraureolytycus*
	[Bacteroides] ureolyticus
C	*C. fetus* subsp. *fetus*
	C. fetus subsp. *venerealis*
	C. hyointestinalis subsp. *hyointestinalis*[d]
	C. hyointestinalis subsp. *lawsonii*[d]
	C. mucosalis
	C. lanienae
D	*A. cryaerophilus*
	A. butzleri
	A. skirrowii
	A. nitrofigilis
	A. cibarius
	Sulfurospirillum spp.
E[e]	Enterohepatic *Helicobacter* spp.
	Sutterella wadsworthensis
	Anaerobiospirillum succiniproducens
	Anaerobiospirillum thomasii

a) Members of the family *Campylobacteraceae* that at present have been isolated from humans are underlined.
b) Hydrogen-requiring campylobacters.
c) Anaerobic growth only.
d) Some strains require hydrogen for growth.
e) For group E, only campylobacterial taxa that at present have been isolated from human faeces are included.

As shown, most of these organisms have been isolated from humans, including from faecal samples. However, the disease potential of a number of non-*jejuni/coli Campylobacter* species, including *C. concisus*, remains to be definitively determined.

The present work was initiated as a result of the first registered *C. jejuni* water-borne outbreak in Denmark, which started at almost the same day as the author began his work in the field of clinical microbiology. The work with the intriguing *Campylobacter* conumdrum was further encouraged by the increased number of reported *Campylobacter* infections in Denmark in mid 1990s and by the increasing international reports on the emergence on diarrhoeal illness possibly associated with non-*jejuni/coli Campylobacter* spp. The aims of the present investigations were:

1. To conduct an outbreak investigation in order to verify the cause and determine the burden of illness associated with the outbreak (Engberg et al., I).
2. To optimise culture-based diagnostic methods for *Campylobacter* and related organisms, including address the importance of non-*jejuni/coli Campylobacter* spp. in diarrhoeal infections (Engberg et al., II).
3. To compare different tests for antibiotic susceptibility testing of thermophilic *Campylobacter* spp., necessary to secure comparability of the data from the different participants in the Danish integrated antimicrobial resistance monitoring and research programme (DANMAP) (Engberg et al., III).
4. To study the antimicrobial susceptibility of *Campylobacter* isolated from humans including prevalence of macrolide and quinolone resistance, as well as sources and risk factors for quinolone-resistant *C. jejuni* infections (Engberg et al., IV).
5. To implement and evaluate molecular typing methods for the purposes of molecular epidemiological typing of *Campylobacter* infections, including outbreak investigations and determination of disease associations and manifestations (Engberg et al., I, Nielsen et al., V & VI, Engberg et al., VII & VIII).

CHAPTER 2. CLINICAL EPIDEMIOLOGY

2.1. EPIDEMIOLOGY OF *C. JEJUNI* AND *C. COLI* INFECTIONS IN DENMARK AND OTHER DEVELOPED COUNTRIES

In Denmark, laboratory based national surveillance for *C. jejuni/coli* has been carried out since 1980. The annual incidence of registered infections was relatively constant in the period from 1980 to 1992, but from 1992 to 2001, the number of *Campylobacter* infections quadrupled from 1.129 cases (21 per 100,000 inhabitants) to 4657 (86 per 100,000) (**Figure 1**).

After a drop in 2002 and 2003, the number of registered infections increased slightly again in 2004 to 3733 (70 per 100,000). The trend parallels the rising incidence registered in several other industrialized countries (Friedman et al., 2000).

In a detailed analysis of Danish surveillance data collected over the period from 1992 to 1999, the increase in *Campylobacter* infections was found to follow a log-linear trend with a yearly increase of 1.14 for indigenous (domestically acquired) cases whereas there was no apparent increase in the numbers of travel associated. The increase in infections with *Campylobacter* was significantly more pronounced in older children and adults, whereas the incidence remained more or less stable in infants infections (Mølbak, 2001).

2.1.1. Clinical manifestations

After an incubation of approximately three days, the onset of disease caused by *Campylobacter* is usually abrupt with cramping pain in the abdomen, followed by diarrhoea. The mechanisms by which *C. jejuni* and *C. coli* induce diarrhoea are not well understood, but it is clearly a complex and multifactorial process. Flagella mediated motility has been shown to be necessary for *Campylobacter* to colonize the intestinal tract, and data obtained from clinical infections, experimental infections in humans and animals, and in vitro analyses of adherence and invasion in cultured human cells have demonstrated that cell invasiveness is a necessary step in *Campylobacter*-induced inflammatory diarrhoea. Several bacterial components have been shown to have adhesive properties (lipopolysaccaride (LPS), flagella, fimbrial filaments, surface-exposed proteins), but the relative importance of these structures for adhesion in vivo as a requirement for colonization and invasion remains to be determined (Wassenaar et al., 1999). Likewise, a direct role of toxins including of a cytolethal distending toxin (CDT) in disease remains to be demonstrated (Wassenaar et al., 1999; Bang et al., 2003).

The diarrhoea is commonly profuse and may be watery or bloody. Further clinical features of *Campylobacter* enteritis include fever, headache, myalgia, nausea and vomiting (Skirrow et al., 2000). The diarrhoeal stage is commonly reported to be of a few days. However, in a Danish case-control study the median duration of illness was 10 days (interquartile range = 7-14 days) (Neimann, personal communication). Analysis of registered *C. jejuni/coli* episodes by age group shows a bimodal distribution with the highest number of infections in small children and with a second peak in young adults (II). The same incidence of *C. jejuni/coli* infections by age group has been found in other developed countries (Friedman et al., 2000).

Complications of *Campylobacter* infections are rare, but the infections may be followed by the development of reactive arthritis or Guillain-Barré syndrome (GBS). Schiellerup et al. (2003), surveyed 1.339 *Campylobacter* infections and 171 (19.9%) reported joint

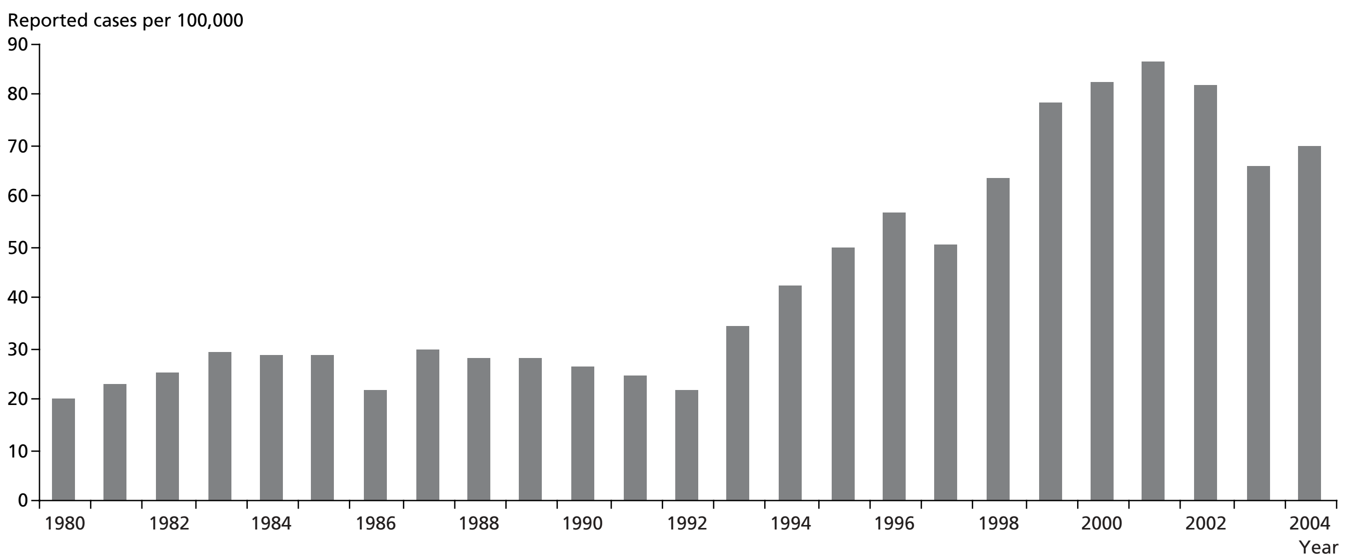

Figure 1. Incidence of *Campylobacter*, Denmark, 1980 to 2004.

pain. Interestingly, complains of joint pain was not associated with duration of diarrhoea and the prevalence of HLA-B27 tissue type was 11.6% in patients with joint pain compared to 6.5% in patients with gastroenteritis only. In a study from Finland, only 7% developed *Campylobacter*-triggered reactive arthritis (Hannu et al., 2002).

While relatively little is known about reactive arthritis following *Campylobacter* infection, the knowledge on the pathogenesis of *Campylobacter*-induced GBS is expanding rapidly. GBS is an autoimmune-mediated disorder of the peripheral nervous system. Affected persons may rapidly develop weakness of the limbs and of the respiratory muscles and areflexia. In most cases, most patients have uneventful recovery, but 15-20% may have severe neurologic deficits. Guillain, Barré, and Strohl first described the syndrome of flaccid paralysis, areflexia, and albuminocytological dissociation in the spinal fluid in 1916. The occurrence of an infectious illness preceding GBS, primary virus respiratory disease, has been known for long time, but Rhodes & Tattersfield reported the first case of *Campylobacter* infection preceding GBS in 1982. Molecular mimicry of *C. jejuni* lipooligosaccharides (LOS) with nerve gangliosides is thought to induce cross-reacting antibodies leading to GBS. The concept of pathogenesis is that antibodies recognising epitopes shared between *C. jejuni* LOS and nerve gangliosides are allowed entry into the peripheral nerve system compartment at locations where the blood-nerve barrier is incomplete (nerve roots) or absent (motor nerve terminals). Subsequently, binding of anti-ganglioside antibodies to reactive antigens at these accessible target sites causes peripheral nerve damage and loss of physiological nerve function (Schwerer, 2002; Ang et al., 2004). The link between *Campylobacter* and GBS has been reviewed by Engberg (2002) with the main finding being:

- *Campylobacter* gastroenteritis is the most frequent identified antecedent event for GBS and accounts for 30-40% of all cases of GBS.
- Certain *Campylobacter* strains are more often isolated from GBS patients than others.
- The structure of *C. jejuni*-LOS seems to determine the specificity of antiganglioside antibodies and hereby the clinical presentation of GBS.
- Host factors are of importance in the pathogenesis of GBS following a *Campylobacter* infection.
- Post-*Campylobacter*-GBS appears to be more severe than GBS triggered by other infections.
- Post-*Campylobacter*-GBS is a rare complication, but has a tendency to cause long-term sequelae and therefore is an important contributor to the total burden of illness of *Campylobacter*.

Godschalk et al. (2004) have recently demonstrated, for the fist time, that specific types of the LOS biosynthesis gene locus are associated with GBS and with the expression of ganglioside-mimicking structures, i.e., it is now clear that specific bacterial genes are crucial for the induction of anti-ganglioside antibodies. The association between certain *Campylobacter* sero- and genotypes and GBS is discussed in more detail in VII and in chapter 4 of this review.

2.1.2. Sporadic Campylobacter infections

In general, zoonotic agents, including *Campylobacter*, may cause sporadic infections or common source outbreaks. It is assumed that patients, who have not been associated with known outbreaks, are sporadic. Most *Campylobacter* infections are sporadic, making the search for the source of infection difficult. The epidemiology of *Campylobacter* infections is not entirely elucidated, but the major sources have long been identified. Descriptive epidemiological studies have identified *Campylobacter* in the intestinal tract of a wide variety of wild and domestic food animals and pets. Among food animals, *C. jejuni* predominates among cattle, broiler chickens and turkeys, whereas *C. coli* is most common among pigs. As a result of faecal contact during processing, the meat may become contaminated. The *Campylobacter* contamination rates at retail level varies between food item and country, but in general, beef and pork show low rates, whereas poultry, especially chicken, consistently show high rates (Pezzotti et al., 2003; Whyte et al., 2004; Anonymous, 2004).

Analytic epidemiological studies, i.e. case-control studies have provided important information on the sources of human infections. At least 19 out of 24 case-control studies in the US, Canada, New Zealand, Australia and Western European countries have identified poultry (especially the consumption of undercooked chicken) as risk factor for sporadic *Campylobacter* infections (Tenkate et al., 2001; Neimann et al., 2003 and references therein; Kapperud et al., 2003; Potter et al., 2003; Friedman et al., 2004; Michaud et al., 2004; Schönberg-Norio et al., 2004; Carrique-Mas et al., 2005; Wingstrand et al., 2005). The kind of poultry found to be a risk, where it was consumed (i.e. at home vs. at restaurants), and the relative importance of such consumption has varied. Additional identified risk factors included contact with pet animals, contaminated drinking water, milk, barbecuing, swimming in recreational waters, occupational exposure to animals, and traveling. Several of the case-control studies have also indicated that infection is more likely to occur as a result of cross-contamination from raw poultry products than because of poultry consumption per se. Cross-contamination in the home and in the restaurant kitchen is therefore probably a frequent route of transmission for *Campylobacter*, but also difficult to quantify (Frost et al., 2002; Kapperud et al., 2003). In addition, poultry is a commonly consumed food product and therefore frequently noted as a recent exposure among both cases and controls. Consequently, case-control studies of risk factors for sporadic *Campylobacter* infections will have relatively small population attributable risk (PAR) of exposures, i.e. tend to underestimate the importance of poultry products as source of *Campylobacter* infections (Neimann et al., 2003). Person-to-person transmission of *C. jejuni/coli* is rare and probably of no epidemiologic importance (Endtz et al., 1991).

Other epidemiological data also support the assumption that poultry is an important source of human infections, e.g. the unintentional "intervention study" that occurred in Belgium during the dioxin crisis where withdrawal of domestically-produced poultry products from the market resulted in a 40% decrease in the *Campylobacter* infections (Vellinga et al., 2002). In addition, the link to poultry is supported by the marked seasonality of human *Campylobacter* infections; a seasonality that in part overlaps the seasonal increase in the prevalence of positive broiler and turkey flocks (Anonymous, 2002a).

In a number of countries, including Iceland, Norway, and Denmark, poultry consumption has increased steadily over the last 10-15 years, and raw refrigerated products have become increasingly popular (Hänninen et al., 2000; Kapperud et al., 2003). In the previous years, a majority of the products were frozen. While frozen storage has been shown to reduce the number of viable campylobacters, *Campylobacter* survives well throughout the shelf life of fresh poultry products stored at refrigeration temperature in modified and normal atmospheres. Increased consumption of fresh poultry may have contributed to the rising incidence of *Campylobacter* infections in a number of countries (Kapperud et al., 2003). In Iceland, the number of domestically acquired *Campylobacter* infections reached epidemic proportions in 1998-2000. Subsequently, a broad campaign was launched directed on reducing *Campylobacter* in poultry during production, processing and marketing, but simultaneously focusing on consumer education. In order to reduce the distribution of *Campylobacter* contaminated poultry, all positive flocks had to be frozen to reduce bacterial counts before going to retail. As a consequence, in 2000, the incidence of domestically acquired *Campylobacter* infections dropped from 116/100,000 in 1999 to 33/100,000 in 2000, a reduction of 72% in domestic infections. The

combination of public education, enhanced on-farm biological security measures, carcass freezing and other factors, such as variations in weather, contributed to the large reduction in poultry-borne campylobacteriosis. There was no immediate basis for assigning credit to any specific intervention (Stern et al., 2003). Based on the Icelandic experience, results from research projects in the primary production, and results from a Danish risk assessment of campylobacteriosis associated with *Campylobacter* in chickens (Rosenquist et al., 2003), comparable mitigation strategies have been adopted in Norway and Denmark (Anonymous, 2005; Anonymous, 2004).

The importance of the other well-known risk factors, including contaminated drinking water, for sporadic infections in Denmark remains to be answered. In the Danish case-control study by Neimann et al. (2003), drinking water with a bad taste or smell tended (but not significant at the 95% confidence level) to be associated with an elevated risk for the acquisition of *Campylobacter*. However, a subsequent Danish case-control study by Wingstrand et al. (2005) did not identify drinking water as a risk factor for campylobacterosis. Notably, a recent preliminary assessment of environmental risk factors for *Campylobacter* infections in Sweden found associations between *Campylobacter* incidence and average water-pipe length per person, ruminant density, and a negative association with the percentage of the population receiving water from a public water supply (Nygard et al., 2004).

2.1.3. Campylobacter outbreaks

A food-borne *Campylobacter* outbreak is as other food-borne disease outbreaks, defined as the occurrence of two or more cases of illness resulting from the ingestion of a common food source. According to guidelines for confirmation of food-borne-disease outbreaks from the Centers for Disease Control and Prevention (CDC), a *C. jejuni/coli* food-borne outbreak is confirmed by isolation of the organism from clinical specimens from two or more ill persons OR isolation of the organism from epidemiologically implicated food (Olsen et al., 2000). The definition of a waterborne outbreak, including one caused by *Campylobacter* is that ≥2 persons must have experienced a similar illness, either after ingestion of drinking water or exposure to water encountered in recreational or occupational settings and secondly, epidemiological evidence must implicate water as the probable source of the illness (Blackburn et al., 2004).

With these definitions, food- and water-borne outbreaks account for a very small fraction of registered *Campylobacter* infections. In Denmark, outbreaks of food- and water-borne infections caused by zoonotic agents are reported in three different systems. First, general practitioners and hospitals are obligated to notify all infections suspected to be food-borne, without awaiting microbial analyses, to the public health authorities. Secondly, gastrointestinal pathogens identified at clinical microbiology laboratories are reported to the Unit of Gastrointestinal Infections at Statens Serum Institut. Thirdly, individuals who experience food poisoning may report these incidents to the Regional Veterinary and Food Authorities. There is at present no systematic evaluation of the overlap between the three parallel systems, nor has the completeness of these systems been formally evaluated. However, with this reporting system, only 139 minor general and family *Campylobacter* outbreaks were reported in the period from 1997 to 2003, and among these, only one was waterborne (a small family outbreak) (Anonymous, 1998a; Anonymous, 1999; Anonymous, 2000; Anonymous, 2001; Anonymous, 2002a; Anonymous, 2003; Anonymous, 2004). In comparison, 27125 laboratory-confirmed *Campylobacter* infections were reported in the same period. In the United States, *Campylobacter* outbreaks represent approximately 1% of reported food-borne and waterborne outbreaks, and most of them are food-borne especially due to raw milk. From 1978 to 2002, only 17 waterborne outbreaks of *Campylobacter*, affecting a total of approximately 6625 people were reported in the United States (Friedman et al., 2000; Barwick et al., 2000; Lee et al., 2002; Blackburn et al., 2004). In the UK, *Campylobacter* accounted for only 2% of registered food- and waterborne outbreaks between 1995 and 1999 (Frost et al., 2002).

A Danish register study on household outbreaks among 26,479 *Campylobacter* infections from 1991-2001 found 3.2% of the *Campylobacter* infections to be part of household outbreaks. The applied definition of a *Campylobacter* outbreak in this study was ≥2 cases who shared address and became infected within three weeks of one another (Ethelberg et al., 2004). The study shows that family outbreaks are much more frequent than indicated by the reported number of outbreaks. In addition, recent Danish studies combining serotyping and molecular typing of sporadic human infections suggest that almost one third of apparently sporadic *Campylobacter* infections may be epidemiologically connected (Fussing et al., 2003). These studies are described in more detail in chapter 4.

2.1.4. Own investigations

Large waterborne *Campylobacter* outbreaks have been described in a number of countries including from Denmark. Engberg et al. (I) investigated the first registered waterborne outbreak with *C. jejuni* in Denmark in 1995-96 in the town of Klarup. It occurred as a result of an unintended leak of sewage to the groundwater reservoir.

Epidemiological data was provided by a retrospective follow-up study for culture confirmed cases and for residents without a bacteriological diagnosis. Stored clinical and environmental isolates were analysed by serotyping and genotyping with Restriction Endonuclease Analysis (REA), Pulsed Field Gel Electrophoresis (PFGE), and Ribotyping. The validity of these typing methods in the investigation of *Campylobacter* outbreaks is discussed in chapter 4.

C. jejuni was isolated from 110 residents and visitors to the area. However, an estimate based on a telephone survey indicated that some 2400 people out of a population of 3925 (61%) were affected by the outbreak. Water samples obtained from the community waterworks contained *C. jejuni* serotype HS:2, the same serotype as in all but one of the thirty stored isolates from the outbreak. The water and clinical isolates also showed the same DNA profile except for the single strain with the different serotype. The contamination of the water supply was traced back to contamination of ground water due to a break on a sewage pipe.

The Communicable Disease Surveillance Centre in the UK has developed a categorizing system for levels of evidence of waterborne disease outbreaks (Tillett et al., 1998). The categories take into account the epidemiology, microbiology and water quality information. Thus, outbreaks are classified as being associated with water either "*strongly*", "*probably*" or "*possibly*". According to this system, the waterborne disease outbreak investigation by Engberg et al. was "*strongly*" associated with water, as the pathogen identified in clinical cases was also found in water and descriptive epidemiology suggested that the outbreak was water-related and obvious alternative explanations were excluded. In contrast, in the United States, a CDC waterborne-disease outbreak surveillance system is used (Blackburn et al., 2004). This classification scheme with classes of evidence I-IV is also based on the provided epidemiological and water-quality data. A classification of I indicates that adequate epidemiological and water-quality data are reported. By adequate epidemiological data is meant data provided regarding exposed and unexposed individuals. According to this classification system, a drawback of the outbreak investigation by Engberg et al. is that the epidemiological data was not obtained by a case-control study. A case-control study among individuals who did and did not develop illness in Klarup could possibly have shown a dose-response relationship between amounts of drinking water and risk of illness. However, a likely dose-response effect was actually demonstrated in the Klarup study, with less severe disease among patients from the southern area than among patients from the heavily exposed northern area of the town. The frequency of bloody diarrhoea were approximately one third of confirmed infections compared with only 3% and 0% of not culture

confirmed infections from the northern and southern areas of a town, and reflects the severity bias in surveillance activity exclusively based on passive case detection from samples received by the health care system. In addition, the epidemiologic link between sewage and illness among residents in Klarup was compensated by the strong molecular epidemiological typing data. The cause was confirmed by the finding of indistinguishable *C. jejuni* isolates in the drinking water, and in the clinical samples by multiple typing methods. Additional flagellin typing of outbreak isolates has later confirmed initial typing results. (Bang, personal communication). The approach with a case-control design has been used in three more recent, reported waterborne outbreak investigations from Finland, France and Sweden (McCarthy et al., 1998; Kuusi et al., 2004; Gallay et al., 2005).

In Denmark, the drinking water supply is mainly based on untreated groundwater and groundwater rarely requires disinfection because of its high microbiological quality. However, in case of distribution system deficiency (e.g. cross-connection, contamination of water mains during construction or repair, or contamination of storage facility due to flooding and surface runoff related to heavy rain), ground water may be a vehicle for transmission of campylobacters to humans and farm animals. In a review of 14 waterborne epidemics in Finland during 1998-1999, all except one of the waterborne epidemics were associated with undisinfected groundwaters and only one was due to insufficient disinfection of surface water. *Campylobacter* caused three of the outbreaks, all associated with groundwater (Miettinen et al., 2001). In contrast, in a review of 19 waterborne *Campylobacter* outbreaks in Norway from 1988-2002, all outbreaks with more than 10 cases were associated with surface water (Nygard et al., 2003).

Although groundwater works usually serve small communities compared to surface water works it is unlikely that waterborne outbreaks in Denmark will often remain undetected and unreported to public health authorities. This is due to the capacities for *Campylobacter* to survive and persist in the aqueous microcosm (absence of molecular oxygen, low temperature, and protection for the effects of UV and desiccation (Jones, 2001)) and with subsequent high attack rates, high disease burden, household clustering and individuals experiencing more than one episode within a short timeframe until corrective measures are implemented.

2.1.5 Conclusions and future directions

The current leading hypothesis for the recent increase in registered *Campylobacter* infections in a number of countries is primarily an increased transmission from the poultry reservoir, in part due to the increased consumption of fresh chilled poultry products. However, the importance of the other well-known risk factor for sporadic *Campylobacter* infections remains to be fully determined. Therefore, additional studies on the relative importance of each of these to the epidemiology of *Campylobacter* infections are clearly needed.

2.2. DIAGNOSIS AND PREVALENCE OF *CAMPYLOBACTERACEAE* AND RELATED ORGANISMS IN FAECAL SAMPLES FROM HUMAN DIARRHOEAL INFECTIONS

2.2.1. Culture and isolation

Since the early 1970s, several isolation methodologies have been developed for *Campylobacter*. These range from the original centrifugation filtration methods developed by the pioneering workers in Belgium to subsequent development of selective agar media and enrichment broth formulations. The basic media need supplements for optimal growth of *Campylobacter* species. Complex substrates such as blood, serum and charcoal have been used. For the effective isolation of *Campylobacter* species from the faecal flora, the culture media must be selective. Selection for *Campylobacter* is usually achieved by the addition to the medium of antimicrobial agents like cephalosporins, trimethoprim, polymyxins, novobiocin, vancomycin, teicoplanin, bacitracin, rifampicin, and sodium deoxycholate to which most campylobacters are resistant. Some antimicrobial agents present in selective media, however, may be inhibitory to *Campylobacter* species, too. Cephalothin, colistin, and polymyxin B which are present in some selective media formulations may be inhibitory to some strains of *C. jejuni* and *C. coli*, and are inhibitory to *C. fetus* subsp. *fetus*, *C. jejuni* subsp. *doylei*, *C. upsaliensis* and *A. butzleri* (Goossens et al., 1986; Ng et al., 1988). For this reason the incidence of infection by different *Campylobacter* spp. may be understated. Finally, some selective agars also contain amphotericin to inhibit molds and yeasts (Nachamkin et al., 2000).

Selective media in use worldwide include blood containing media such as Skirrow medium and Campy-CVA medium, and blood-free media such as modified charcoal cefoperazone deoxycholate agar (mCCDA), cefoperazone, amphotericin and teichoplanin medium (CAT medium), charcoal-based selective medium (CSM) and semisolid blood-free motility medium (SSM) (Nachamkin et al., 2000).

Since some species of *Campylobacter* and *Arcobacter* may be susceptible to various antibiotics present in selective media, a passive filtration technique was developed by Steele & McDermott in 1984. This method works on the principle that *Campylobacter* bacteria are selected by their ability to migrate through membranes of pore sizes 0.65 or 0.45 μm. Drops of faecal suspension are placed on top of a filter membrane which is located on the surface of a blood-agar medium. This is left at either room temperature or at 37°C for 45 minutes and *Campylobacter* bacteria, if present, may migrate through the membrane and onto the surface of the blood agar medium. The membrane is removed with sterile forceps and the culture plates are incubated microaerobically at 37°C. The method has been used to isolate these organisms from faecal samples (II; Goossens et al., 1986; Bolton et al., 1988; Goossens et al., 1990; Kiehlbauch et al., 1991). Filtration using non-selective media is most often reported as less sensitive than selective media for primary culture of thermophilic campylobacters (Goossens et al., 1992; Lopez et al., 1998; Vandenberg et al., 2004). Thus, filtration should not be used as a replacement but as a supplement to selective plating media for detection of *Campylobacter* spp. that do not grow well on these media.

In the acute phase of infections, campylobacters can readily be isolated from faecal specimens. Enrichment cultures may be useful for looking for low numbers of organisms, e.g. in delayed specimens, family contacts (asymptomatic carriers) or when looking for triggering infectious agents in patients with clinical presentations suggesting postinfectious sequelae such as reactive arthritis and GBS (Nachamkin, 1997). Several enrichment media, such as Preston enrichment broth, Campythio, *Campylobacter* enrichment broth and Bolton enrichment broth have been used regularly (Nachamkin et al., 2000; Bolton, 2000).

Campylobacter species are microaerophilic bacteria and need an microaerobic atmosphere containing approximately 5-10% O_2 and 5-10% CO_2 for recovery (Bolton et al., 1997). An atmosphere containing an increased concentration of hydrogen is required to isolate the hydrogen requiring *Campylobacter* species (Table 1, group B). The optimal concentration of hydrogen has not yet been established, but the flammable limits of hydrogen in air are from 4% hydrogen and higher concentrations may form explosive mixtures and caution should be exercised (Cox, 1997). Engberg et al. (II; Engberg et al., 2000b) have proven that a gas mixture of 6% O_2, 6% CO_2, 3% H_2 and 85% N_2 is sufficient for isolating hydrogen requiring species. A hydrogen-enriched atmosphere is not a necessity for the isolation of *C. jejuni* and *C. coli*, however hydrogen strongly enhances the growth of these species.

Campylobacter and *Arcobacter* species have different optimal growth temperatures, and the choice of temperature used for routine laboratory use will determine the spectrum of species that will be isolated. Many laboratories use 42°C as the primary incubation temperature and this will allow growth of *C. jejuni* and *C. coli* on selective media. Studies comparing the effect of incubation temperature on the isolation of thermophilic species of *Campylobacter* from

faeces show conflicting results. A study by Bolton et al. (1988) demonstrated an increased isolation rate of *C. jejuni* and *C. coli* if mC-CDA plates were incubated at 37°C rather than at 42°C, whereas Gee et al. (2002) isolated more thermophilic *Campylobacter* species at 42°C compared to 37°C. In a small study of 600 faecal samples, Engberg et al. (2000c) found comparable isolation rates with incubation at 37°C compared to 42°C. *C. upsaliensis* grows well at 42°C, but it is usually not recovered on selective media, with the CAT medium as an exception. *Campylobacter fetus* may be missed in stool samples plated on media incubated at 42°C and non-thermophilic campylobacteria including *Arcobacter* spp. will generally not be recovered at 42°C. Most *Campylobacter* and *Arcobacter* species grow well at 37°C. However, several of the selective media, such as Skirrow medium and SSM, were devised for use at 42°C and have poor selective properties at 37°C, whereas mCCDA and CSM show good selective properties at 37°C.

2.2.2. Identification methods

The most widely adopted approach for the identification of *Campylobacter* spp. is based on classical phenotypic characteristics, including: colony morphology, motility, catalase, oxidase, hippuricate hydrolysis test, indoxyl acetate hydrolysis, production of H_2S, and antibiotic sensitivity to cephalothin and nalidixic acid (Nachamkin, 2003). A drawback of this classical phenotypic approach is that the discrimination amongst species often relies on one or two differential characters such as presence of hippuricase and urease activity. The hippuricase activity test differentiates most *C. jejuni* strains from other *Campylobacter* species. However, an estimated 5-8% of *C. jejuni* does not express hippuricase activity and are therefore false negative by the hippuricate hydrolysis test. In addition, increasing resistance of *C. jejuni* and *C. coli* to quinolones is emerging in many countries (Engberg et al., 2001; Engberg et al., 2005). This will cause problems with species identification when this is solely performed by phenotypic tests, the most frequently used way of differentiating *Campylobacter* spp. in routine laboratories. For definitive identification of atypical *C. jejuni* and non-jejuni *Campylobacter* spp., additional biochemical tests and/or molecular methods are needed (On, 1996).

The usefulness of a number of commercially available identification systems for *Campylobacter* spp. has been described in detail in a review by On (1996). In brief, commercial systems for identification of *Campylobacter* species have not been found to be more accurate than conventional tests. For example, in evaluations of the API Campy (API Biomériux Ltd., Marcy l'Etoile, France), commercial identification kit for campylobacteria, misidentifications of *C. concisus* as *C. mucosalis*, and of *A. butzleri* as *A. cryaerophilus* or *H. cinaedi* occurred, and with reported additional problems in identifying certain *C. coli* and *C. lari* strains suggest that caution should be exercised when using the API Campy system (On, 1994; Huysmans et al., 1995; Reina et al., 1995; On, 1996). Also serological tests using latex particles coated with immunoglobulins raised against several *Campylobacter* spp. are available, but evaluations of these tests suggest that they should only be used to assist in preliminary diagnosis of campylobacterial infection (On, 1996).

During recent years, a large number of molecular methods, including DNA probe or PCR based identification assays, have been published and to some extent evaluated. In these assays, many different gene targets have been used including: GTPase-based PCR-reverse hybridization assay (van Doorn et al., 1999), the *ceuE* gene (Gonzalez et al., 1997), the 16S rRNA gene (Linton et al., 1996; Cardarelli-Leite et al., 1996; Marshall et al., 1999), the 23S rRNA gene (Eyers et al., 1994; Bastyns et al., 1995; Hurtado et al., 1997), the *glyA* gene (al Rashid et al., 2000), the flagellin gene (*flaA*) (Comi et al., 1996), the lipid A gene *lpxA* (Klena et al., 2004), or random (Vandamme et al., 1997) or a multiplex of the 16S rRNA, hippuricase and aspartokinase genes (Linton et al., 1997). The sensitivity and specificity of each of the tests were examined in each study, but there are large differences in the number and choices of strains used to evaluate each test. On & Jordan (2003) evaluated the sensitivity and specificity of 11 PCR assays described in the literature for the species identification of *C. jejuni* and *C. coli.* The study examined boiled lysate and purified DNA templates of well-characterized type, reference, and field strains of *C. jejuni* (n = 62), *C. coli* (n = 34), and *C. lari* (n = 15). The tests varied considerably in their sensitivity and specificity for their respective target species. Noteworthy, no assay was found to be 100% sensitive and/or specific for all *C. jejuni* strains tested, but four assays for *C. coli* gave appropriate responses for all strains examined. The study endorses the use of multiple strains that reflects the diversity and taxonomy of *Campylobacter* spp. to evaluate PCR-based identification methods.

Species-specific identification of *Campylobacter* spp. by 16S rRNA gene sequencing have been shown to be unable to discriminate between *C. jejuni* and *C. coli*, the two dominant *Campylobacter* spp. in clinical stool samples, and is therefore not an appropriate method when one of these taxa is suspected (Gorkiewicz et al., 2003). However, it was a useful supplementary method for the identification of non-*C. jejuni/coli Campylobacter* spp. in the studies by Engberg et al. (II) and by Gorkiewicz et al. (2003). There are some concerns with the method. There is still incomplete knowledge on the strain-to-strain variation within a single species and occurrence of identical 16S rRNA sequences in strains belonging to different *Campylobacter* spp. For example, Harrington & On (1999), found that strains of *C. hyointestinalis* differed up to 4.5%. Very recently, PCR-Restriction Fragment Length Polymorphism (RFLP) identification of *Campylobacter* spp. based on partial *groEL* gene sequences were reported to provide better resolution than for the 16S rRNA gene (Kärenlampi et al., 2004). Finally, highly discriminatory molecular typing methods, such as Amplified Fragment Length Polymorphism (AFLP) have also proven useful for *Campylobacter* strain identification to the species level (On et al., 2000; Duim et al., 2001).

The review of the above studies illustrates that, even with molecular methods, accurate discrimination of closely related campylobacterial taxa may be insufficient when only a single test is used. It also supports the strategy of a polyphasic approach that uses both phenotypic and genotypic methods for identification of *Campylobacter*, as suggested by On (1996), and applied in Engberg et al., II.

2.2.3. Non-culture methods

Traditional microscopic methods, such as Gram stain response and cell motility, have been used for examining fresh acute-phase clinical specimens with sensitivity reported to range from 66 to 94% and the specificity is very high (Sazie et al., 1982; Park et al., 1983). Recently, a commercially available system for rapid detection of *C. jejuni* and *C. coli* antigens in stool samples has been made available (ProSpecT *Campylobacter* Microplate Assay (Alexon-Trend, Minneapolis, MN, USA)). When compared with culture, the immunoassay had sensitivity of 89 to 96% and specificity of 98 to 99% in three independent studies (Hindiyeh et al., 2000; Tolcin et al., 2000; Dediste et al., 2003).

Molecular methods based on PCR have also been developed for direct detection of *Campylobacter* in stool specimens (Oyofo et al., 1992; Waegel et al., 1996; Lawson et al., 1999; Kulkarni et al., 2002; Maher et al., 2003; Iijima et al., 2004; Amar et al., 2004; Persson, personal communication). When compared with culture, the PCR assays had lower or comparable sensitivity for detection of *C. jejuni* and *C. coli* (Oyofo et al., 1992; Waegel et al., 1996; Lawson et al., 1999; Kulkarni et al., 2002; Iijima et al., 2004; Amar et al., 2004). However, in a study by Maher et al. (2003), additional 2.6% specimens were positive for *C. jejuni* using the PCR method. For detection of non-*C. jejuni/coli Campylobacter* spp., the approach with a PCR-based method may result in additional *Campylobacter* spp. being detected, as shown by Kulkarni et al. (2002), but the method need further automatisation for routine use in the diagnostic laboratory.

The value of serodiagnosis of *Campylobacter* enteritis is limited. However, serologic assays have been valuable tools in understanding the role of *Campylobacter* in post-infectious sequelae, such as GBS (Engberg, 2002). Isolation of *Campylobacter* in stool samples from patients with GBS is difficult, since the median period of excretion of *Campylobacter* in stools of infected patients is short; in a Swedish study it was only 16 days (Svedhem et al., 1980). Thus, GBS patients frequently have negative stool cultures because of the 1- to 3-week time lag between onset of diarrhoeal illness and the onset of GBS. Numerous serologic assays for the measurement of serum antibodies to *Campylobacter* have been developed, but there are no standards for serological testing, either with regard to the antigens used or the end points for positivity (Nachamkin et al., 2000; Strid et al., 2001). Therefore, measurement of serum antibodies to *C. jejuni* as the sole marker of prior infection may be an unreliable method of determining the association between *Campylobacter* and GBS (Taylor et al., 2004). The combination of serological testing, recovery of isolates by enrichment culture prior to stool culture combined with antigen-based or DNA-based detection methods and clinical history is useful in identifying GBS cases, which are likely to have had an antecedent *Campylobacter* infection (Nachamkin, 1997; Sinha et al., 2004).

2.2.4. Own investigations

Within the genus *Campylobacter*, *C. jejuni* and *C. coli* are the most common species associated with diarrhoeal illness and are clinically indistinguishable. Although most laboratories do not routinely distinguish between these species, 85 to 95% of *Campylobacter* infections in industrialised countries are due to *C. jejuni* and 5 to 15% are due to *C. coli*, when the diagnosis is performed solely on selective media (Sopwith et al., 2003; Vandenberg et al., 2004). Of the 975 *Campylobacter* isolates recovered by Engberg et al. (IV), 926 isolates were identified as *C. jejuni* (95.0%), 42 as *C. coli* (4.3%), and 1 as *C. lari* (0.1%). Six of the 975 isolates were not speciated due to logistic problems.

The distribution of species may be different in other parts of the world and if a non-selective isolation technique, such as the filter technique, is applied in conjunction with a selective medium (II; Lindblom et al., 1995; Van Etterijck et al., 1996; le Roux et al., 1998; Labarca et al., 2002; Lastovica et al., 2003; Vandenberg et al., 2004). Engberg et al. (II) re-evaluated three selective media, (mCCDA, Skirrow medium, and CAT medium) and the filtration method for the efficacies to isolate *Campylobacter* spp. with well appreciated disease potential and to estimate the prevalence of new and emerging campylobacterial pathogens. The study population consisted of both clinical samples and samples from healthy individuals and the study pointed out several important issues. First, mCCDA proved to be the most effective selective medium for the isolation of *C. jejuni* and *C. coli*. Second, another six taxa could be isolated, mostly after an extended incubation period of 5-6 days, but with the filtration method as the essential method. Third, a polyphasic approach in order to identify all of the eight isolated taxa was needed: conventional phenotypic tests, *C. concisus* species-specific PCR, extended phenotypic characterization, whole-cell protein profiling and 16S rRNA gene sequence analysis. Fourth, the study provided evidence for the existence of *Sutterella wadsworthensis* in human faeces from clinical cases of gastrointestinal disorders and in faeces from a healthy individual. Fifth, *C. concisus* was isolated from a large number of diarrhoeal cases, particularly from those at the extremes of age, but was additionally isolated from the faeces of healthy people at a similar rate. *C. concisus* isolates from this study were subsequently evaluated for a number of phenotypic and genotypic characteristics (VIII).

Three *C. curvus*-like strains were isolated using the filter method. They formed a discrete group by SDS-PAGE protein analysis, and the strains were identified as *C. curvus* by means of 16S rDNA gene sequencing. However, subsequent whole-cell protein analysis showed that these strains resembled, but were not identical to the type strain of *C. curvus*. The frequency of *C. curvus* in the gastrointestinal tract of symptomatic individuals has previously been reported exceedingly low (Lastovica et al., 2000; Maher et al., 2003). However, in a 2005 reported study from California 20 strains of *C. curvus* and *C. curvus*-like organisms were isolated by the filter method and prolonged incubation during the course of two investigations: one involving a search for possible bacterial agents causing bloody diarrhoea and a second concerning a small outbreak of persistent (= 4 weeks) diarrhoea (Abbott et al., 2005).

Surprisingly, *C. upsaliensis* was not recovered by Engberg et al. (II), even though a variety of media and methods principally designed for the isolation of this species were applied. In a study from Sweden (Lindblom et al., 1995), *C. upsaliensis* was the most common species next to *C. jejuni* among diarrhoeal children. However, the finding by Engberg et al. is supported by two large studies. Wareing et al. (1998) compared the CAT agar with mCCDA for the isolation of *Campylobacter* spp. from 7000 human clinical samples in the UK and only five *C. upsaliensis* isolates were recovered. In a very large 8-year study of 67,599 stool samples from 40,995 patients in Belgium by Vandenberg et al. (2004), only 0.2% of the patients were infected with *C. upsaliensis*. Even though the study included two selective media and the filter method with the latter incubated at 37°C in a hydrogen-enriched atmosphere for up to 10 days, only 27 *C. concisus* isolates were recovered. In contrast, Lastovica & le Roux (le Roux et al., 1998; Lastovica et al., 2000), consistently identify an impressive number of non-*C. jejuni/coli Campylobacter* spp. from paediatric patients in Cape Town. The possible explanations for discrepancies in the reported isolation rates between diagnostic centers are multiple, but may include differences in applied diagnostic techniques, the number of faecal samples tested and study populations, but may also reflect true geographical differences in the prevalence of various campylobacteria, differing sources, and routes of transmission of campylobacterial species in these countries (II; Engberg et al., 2000b).

2.2.5. Conclusions and future directions

A range of campylobacteria may cause diarrhoeal infections in Denmark. The study by the author and colleagues for comprehensive diagnosis of *Campylobacter* spp. in human faeces emphasizes that at present, no single method will succesfully isolate all campylobacteria. This is in line with generally accepted recommendations for a comprehensive isolation strategy, where filtration should be used to complement culturing on selective plating media and not as a replacement. Further investigations are needed to establish the role of a number of the emerging campylobacteria, including *C. concisus* and *S. wadsworthensis* in enteric disease.

More sensitive and less work-intensive molecular techniques will have to be developed for direct detection of thermophilic and nonthermophilic campylobacters in faeces, if they are going to be applied in the routine clinical microbiology laboratory. Such methods might be optimized rapid multiplex PCR assays.

CHAPTER 3. ANTIMICROBIAL SUSCEPTIBILITY OF *C. JEJUNI*, *C. COLI* AND *C. CONCISUS*

Development of resistance in pathogenic bacteria is the largest threat against the use of antimicrobial agents for therapy. This chapter reviews the literature and own studies on antimicrobial susceptibility testing methodology in *Campylobacter* and what is known about resistance profiles and trends in antimicrobial resistance in clinical *Campylobacter* isolates in Denmark and in different parts of the world. Also, risk factors for quinolone-resistant *Campylobacter* infections will be reviewed and discussed in relation to current recommendations for the clinical management of infection.

3.1. TREATMENT: GENERAL MEASURES AND ANTIMICROBIAL THERAPY

Infection with thermophilic *Campylobacter* spp. usually leads to an episode of acute gastroenteritis, which resolves within a few days to

a few weeks. Fluid and electrolyte replacement constitutes the cornerstone of treatment of diarrhoeal diseases. Current practices of optimal fluid replacement for the mildly to moderately dehydrated cases are oral re-hydration therapy (ORT) and appropriate early feeding. The severely ill patient may be admitted to hospital for observation, re-hydration and antimicrobial treatment. This group of patients often need parenteral fluid replacement in addition to ORT (Skirrow et al., 1995; Snyder, 1995).

Antibiotic treatment of enteric infections other than typhoid fever, paratyphoid infections and shigellosis is still controversial, due to the risk of emergence of resistance and the lack of clear clinical effect (Wistöm et al., 1995; Sjögren et al., 1997). Most cases of *Campylobacter* enteritis do not require antimicrobial treatment, as they are self-limiting. However, antimicrobial treatment is needed for systemic *Campylobacter* infections and for severe or long-lasting cases of *Campylobacter* enteritis. Erythromycin has been the agent of choice, but therapy with extended-spectrum macrolides, such as clarithromycin or azithromycin, is probably equally effective (Hardy et al., 1988; Taylor et al., 1991; Skirrow et al., 1995; Blaser, 2000). It is seldom possible to establish the causative agent of an acute case of diarrhoea in a patient before treatment is begun. The decision of which antimicrobial-drug to use has to be taken on empirical basis in most cases in the clinical setting. Fluoroquinolones are the drug of choice in this situation. However, *Campylobacter* isolates often develop resistance during therapy with this class of drugs (Adler Mosca et al., 1991; Ellis Pegler et al., 1995; Wistöm et al., 1995; Tee et al., 1998). Intravenous aminoglycosides should be included for the treatment of *C. jejuni* and *C. coli* bacteraemias in patients who appear very ill (Blaser, 2000). In case of resistance, other antimicrobial agents such as selected third generation cephalosporins, tetracycline or meropenem may be used for treatment. The mechanisms of antimicrobial resistance as well as antimicrobial resistance in *Campylobacter* spp. other than *C. jejuni*, *C. coli* and *C. concisus* have been thoroughly reviewed elsewhere and will not be discussed in this review (Nachamkin et al., 2000; Aarestrup & Engberg, 2001; Engberg et al., 2005).

3.2. SUSCEPTIBILITY TESTING OF *CAMPYLOBACTER*

Speciation of campylobacters in relation to susceptibility testing is important for surveillance purposes, but less important in the routine clinical laboratory, where timely susceptibility testing and reporting is more important to facilitate and ensure appropriate treatment of the patient (Nachamkin et al., 2000). In vitro susceptibility testing of a microorganism to an antimicrobial agent is determined to predict the effect of treatment and to surveillance trend of resistance. The classification of bacterial strains into susceptible, intermediate and resistant categories with regard to an antimicrobial agent is based on critical values determined for minimal inhibitory concentrations (MIC) or for inhibition zone diameters. MIC is defined as the lowest concentration of an antimicrobial agent required for the inhibition of growth of a particular bacterial isolate. With regard to the relationship between the MIC determined in vitro and the antibiotic concentrations at the site of infections, a strain is considered susceptible if its MIC is lower than achievable at the main pathologic centre of infection following usual doses. The strain is considered resistant if the MIC is higher than the highest concentration achievable in vivo (Sirot et al., 1996). At present, there are only internationally accepted clinical breakpoints for resistance for *Campylobacter* for a few antimicrobial agents (EUCAST, 2005). The only way to define provisional breakpoints is based on population distributions. In the histogram analysis, the epidemiological cut-off value separates microorganisms without (wild type) and with acquired resistance mechanism (non-wild type) to the drug in question. A bacterial strain is regarded as resistant (non-wild type) to an agent when the zone of inhibition is smaller and the MIC is considerably higher than the normal population of zones/MICs formed by wild type bacteria of the same species. The MICs for the susceptible population must also be lower than the concentration obtained at the site of infection. Several different methods for susceptibility testing are available, but until very recently no international standards for *Campylobacter* susceptibility testing have been described. Due to fastidious growth requirement for *Campylobacter*, such as enriched medium, microaerobic atmosphere and sometimes incubation for a prolonged period of up to 48 hours, they cannot be tested accurately using the methods described by for example The Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS) for testing of rapid growing organisms, such as *Enterobacteriaceae*, or other bacterial families and genera. The special growth conditions may significantly influence the results of susceptibility testing. An atmosphere enriched with H_2 promotes the growth of *Campylobacter*, but the potential influence on MIC or zone diameters have not been established. In a pilot study (data not presented) of a study by Engberg et al. (III), the use of hydrogen-enriched atmosphere resulted in difficulties of measurement of zone diameters due to excessive swarm into areas of inhibited growth.

Antimicrobial susceptibility testing can be performed using either dilution or diffusion methods. The choice of method depends on several factors, including preference, ease of performance, and availability of methods in individual laboratories.

3.2.1. Dilution methods

The MIC is classically determined by dilution methods. MICs are determined either by using inoculation of bacterial isolates onto agar plates or into liquid media with different concentrations of the antimicrobial agent to be tested. Several different variations of the agar dilution method have been used for *Campylobacter*, varying from the type of agar to inoculum, atmosphere, incubation time and temperature. Mueller-Hinton agar supplemented with 5% blood (bovine, horse or sheep) was preferred in most studies (Baker, 1992; Aarestrup et al., 1997; Gee et al., 2002; Rautelin et al., 2003; Unicomb et al., 2003; Gaudreau et al., 2003). However, Iso-sensitest agar, Colombia agar, Danish blood agar, *Haemophilus* test medium, gonococcus agar and Schaedler agar have also been used (Karmali et al., 1981; Andreasen, 1987; Sjögren et al., 1997; Thwaites et al., 1999; Lucey et al., 2002; Rautelin et al., 2003; Luber et al., 2003; McDermott et al., 2004). The inoculum has varied from 10^3 to 10^6 CFU per spot, but the effect on MIC of different inocula has not been compared systematically. In most studies, an incubation temperature of 35 to 37°C has been used. However, temperatures of 42-43°C have also been used. The atmosphere chosen has not been reported in all studies, but has also varied considerably – from 5-15% CO_2, 5-7% O_2, and 0-7% H_2 (Aarestrup et al., 1997; Sjögren et al., 1997; McDermott et al., 2004). Incubation in CO_2 lowers the pH of the medium and may have significant influence on results for some antimicrobial agents, especially macrolides (Andreasen, 1987).

MIC-determinations in broth dilution has also been performed for *Campylobacter* using Mueller-Hinton broth supplemented with 5% blood (Huang et al., 1992; Baker, 1992; Luber et al., 2003).

For interpretation of the results, cut-off values recommended by CLSI (NCCLS) for bacterial isolates grown aerobically have been chosen in most cases (Gaudreau et al., 1997; Hoge et al., 1998). However, national cut-off values or cut-off values established through population distribution have also been used (Huysmans et al., 1997; Gaudreau et al., 1997; Thwaites et al., 1999). As mentioned above, the interpretation of MIC-values in relation to clinical outcome of infections has not been established for most antimicrobial agents and until this has been done, classification of MIC-results into groups of susceptibilities has to be empirically based.

In 2004, an international working group standardized an agar dilution susceptibility test for *Campylobacter* (McDermott et al., 2004). For *C. jejuni* and *C. coli*, the group determined quality control ranges for ciprofloxacin, doxycycline, erythromycin, gentamicin and meropenem for incubation temperatures at both 36°C and 42°C in a multi-laboratory study based on CLSI protocols. The

quality control ranges, testing conditions, testing method as well as the *C. jejuni* quality control strain has been accepted by the CLSI (NCCLS, 2004).

3.2.2. Diffusion methods

For *Campylobacter*, a number of different diffusion methods i.e. disks, tablets (e.g. Rosco Neosensitabs) and the epsilometer testing method (E-test) have been used, and in some cases compared to results obtained using MIC-determinations assessed by dilution methods (Huang et al., 1992; Baker, 1992; Huysmans et al., 1997; Gaudreau et al., 1997; Luber et al., 2003).

The results of a susceptibility test using diffusion methods are influenced by the composition and thickness of the agar medium, pH and electrolytes, interaction between antimicrobial agents and the agar or substances in the agar medium such as serum proteins or blood, inoculum density and temperature and incubation conditions and time (Acar et al., 1996). Thus, susceptibility testing using the agar diffusion test requires good standardisation and continuous quality assurance. The interpretation of the results from the agar diffusion tests is normally based on inter-calibration to agar dilution testing (III; Huysmans et al., 1997). Different zones of inhibitions are defined as the concentration where a bacterium can be defined as fully susceptible, intermediately resistant or resistant. With the E-test, it has become possible to read the MIC values directly on the strips applied to the dishes. This method involves a strip coated with an antimicrobial agent that is placed on a seeded agar plate.

Most diffusion tests have been performed using Mueller-Hinton agar supplemented with either horse blood or sheep blood, but other agars have been used. As is the case for dilution methods, the range of inocula and incubation conditions have varied, but have not been evaluated (Gaudreau et al., 1998; Saenz et al., 2000; Lucey et al., 2002; Janosi et al., 2003; Chu et al., 2004). The international working group, which standardized an agar dilution susceptibility test for *Campylobacter*, tested the disk diffusion test in a multi-laboratory format, but found lack of intra- and inter-laboratory reproducibility, which was greater for certain antimicrobial agents (McDermott et al., 2004). However, researchers have reported consistent results for certain drugs obtained by disk diffusion within a single laboratory (Gaudreau et al., 1997).

3.2.3. Own investigations

In 1995, the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) was initiated as a collaborative programme for the surveillance and research of antimicrobial resistance. The objective of the programme is to monitor the occurrence of antimicrobial resistance in bacteria isolated from food animals, food of animal origin and from humans; to monitor the consumption of antimicrobials for humans and animals; to detect and quantify the spread of resistant bacteria and resistance genes from animals to man; to provide guidelines for medical and veterinary antimicrobial chemotherapy to ensure that they continue to be used prudently (Anonymous, 1998b). The system was the first of its kind in the world and included animal pathogens, indicator bacteria and zoonotic bacteria, including *C. jejuni* and *C. coli*. The zoonotic bacteria including *Campylobacter* were included because they can develop resistance in the animal reservoir, which may compromise therapy when causing human disease. In order to survey the antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. isolated from food animals, food of animal origin and humans in different laboratories, comparative studies on the performance of testing procedures are required. MIC-determinations are normally considered the golden standard for susceptibility testing. However, a variety of different methods including diffusion tests were routinely used in Danish laboratories involved in DANMAP. Thus, comparative studies on the performance and the comparability of testing procedures were needed to achieve meaningful surveillance.

Engberg et al. (III) compared the results obtained by the methods used in four clinical, veterinary and food microbiology reference laboratories in Denmark in a study involving 98 *Campylobacter* strains. This intra- and inter-laboratory base-line study included four antimicrobial agents: nalidixic acid, erythromycin, streptomycin and tetracycline. Nalidixic acid was chosen as nalidixic acid susceptibility is a marker for flouroquinolone susceptibility in *C. jejuni* and *C. coli*. Nalidixic acid susceptible strains are susceptible to fluoroquinolones while most of the resistant ones are resistant to fluoroquinolones.

Interpretive criteria for breakpoints were made by comparison of the distribution of the population of MICs for the dilution methods and zones of inhibition for the diffusion methods.

The study showed complete agreement between the three MIC-methods to separate isolates into a susceptible and a resistant population when tested for all four antimicrobial agents. However, for nalidixic acid, two isolates were resistant according to MIC-methods (MIC ranges 32 to 64 μg/ml), but had zones of inhibition between 26 and 31 mm with the two tablet diffusion methods.

For nalidixic acid and erythromycin, the E-test tended to produce lower values compared to the two agar dilution methods. The discrepancies in MICs were clearly separated from the cut-off values and did not cause problems in aspect to interpretation of susceptibility of the individual agent or to investigation of the agreement between methods to separate isolates in susceptible and resistant groups, which was the overall purpose of the study.

The general tendency of the E-test to produce lower values than the agar dilution methods were more pronounced for erythromycin than for nalidixic acid, and was mainly registered in the very low end of MICs. Lower values produced by the E-test have also been identified by others (Huang et al., 1992; Baker, 1992; Gee et al., 2002). Huang et al. (1992) reported very low comparability of the E-test to agar dilution (38.7%) for clindamycin and the E-test should therefore probably not be used to test *C. jejuni* for susceptibility to this antibiotic. However, the results for erythromycin and ciprofloxacin also correlated well with agar dilution in their study. In contrast, in the study by Gee et al. (2002), the correlation between the E-test and agar dilution MICs varied greatly depending on the antimicrobial agent tested, and with only 61.0% overall agreement of the MICs between the two methods. For nalidixic acid and erythromycin, the correlations were 21.4% and 65.6%, respectively.

In another study by Engberg et al. (IV), the E-test was re-evaluated for susceptibility testing nalidixic acid. The correlation between the E-test and tablet diffusion was 100% in this study (data not shown).

In the initial susceptibility study (III), the interpretive criteria were suggested to be considered tentative because of the low level of resistance to some antibiotics among a moderate number of isolates at study. By histogram analyses of susceptibility populations of additional isolates in the subsequent year in the DANMAP surveillance programme, the tentative cut-off values were subject to minor adjustments: for the tablet method D, *C. jejuni* and *C. coli* isolates are considered susceptible to nalidixic acid when zones of inhibition are larger than or equal 27 mm; for the E-test, MICs larger than or equal to 64 μl/mL are considered resistant, whereas strains with MIC less than or equal to 32 μl/mL are considered in vitro susceptible. For erythromycin, the same cut-off values as for nalidixic acid are now applied.

3.2.4. Conclusions and future directions

It is recommended that routine clinical laboratories as a minimum screen all *Campylobacter* isolates for susceptibility to erythromycin and a quinolone and – in case of resistance to the former – timely report it to the clinicians.

Even though monitoring of the resistance for surveillance purpurses is performed in different laboratories and with different techniques, the two studies by Engberg et al. (III-IV) confirmed that reliable results can be achieved for intra- and inter-laboratory com-

parison. However, the agar diffusion test requires good standardisation and continuous quality assurance. The recent availability of a standardized testing method will improve the intra- and inter-laboratory comparability of *Campylobacter* susceptibility testing results. The recommendation is a major achievement for the surveillance of resistance in *Campylobacter*. However, the standardisation is confined to the agar dilution method, a method that is unsuitable for the daily routine use in the average clinical microbiology laboratory. For routine diagnostics, the procedures for susceptibility testing must still be managed locally. Diffusion methods using disks, tablets or E-test strips will probably in most cases give reliable results. However, it is recommended that quality control strains are included and the values of those are continuously recorded to ensure reproducibility over time. Furthermore, with the lack of accepted cut-off values it is recommended that raw data (mm inhibition zones, MIC values) are stored and that histograms for the population distributions are examined continuously for the detection of any shifts in the population.

In future, direct determination of the actual genes encoding resistance using molecular methods may become important as a supplement to the conventional phenotypic tests. Recently it has become clear that mutations in adenine residues in all three copies of the 23S rRNA gene (*rrnB* operon) are responsible for the majority of erythromycin resistance in *Campylobacter*, although in a few cases, mutations in only two 23S rRNA genes are all that is necessary for expression of macrolide resistance (Trieber & Taylor, 1999; Jensen & Aarestrup, 2001; Gibreel et al., 2004). The mutations are base substitutions at positions 2074 and 2075 (corresponding to positions 2058 and 2059 in the nomenclature for *E. coli* numbering) in the 23S rRNA genes of erythromycin-resistant *C. jejuni* and *C. coli* (Trieber et al., 1999; Jensen et al., 2001; Niwa et al., 2003; Vacher et al., 2003). Jensen & Aarestrup (2001) have sequenced a 699 base pair (bp) amplicon of the domain V of the 23S ribosomal DNA (rDNA) in phenotypic macrolide resistant and susceptible *C. coli* strains and identified adenin to guanin point mutation at position 2058 (using *E. coli* numbering) in phenotypic macrolide resistant strains, indicating that this is the mechanism of resistance. The mutation led to the appearance of an additional target for the restriction enzyme *Bsm*AI. Using this restriction enzyme, the presence of this specific point mutation could be visualised by the appearance of an additional fragment in the digest of the 699-bp PCR amplicon using *Bsm*AI, i.e. Jensen & Aarestrup developed a simple PCR-RFLP applicable for testing a large number strains.

Campylobacter quinolone resistance is primarily mediated by single point mutations in *gyrA* in the presence of a constitutively expressed multidrug efflux pump, CmeABC (Zhang et al., 2003). Mutations at Thr-86, Asp-90 and Ala-70 in the genes encoding DNA gyrase (*gyrA*) result in quinolone resistance in *C. jejuni* (Wang et al., 1993), with mutations at Thr-86 being the most common (Hakanen et al., 2002; McIver et al., 2004). High-level resistance to nalidixic acid (64-128 mg/mL) and ciprofloxacin (16-64 mg/mL) is associated with mutations at Thr-86-Ile (Wang et al., 1993; Ruiz et al., 1998; Beckmann et al., 2004). Even higher resistance to ciprofloxacin (125 mg/mL) occurs when mutation at Arg-139 in the *parC* gene encoding topoisomerase IV occurs together with a mutation at Thr-86 in *gyrA* (Gibreel et al., 2004). Other double mutations, such Thr-86 with Asp-85 or Pro-104, may also occur in ciprofloxacin-resistant *C. jejuni* (Piddock et al., 2003; McIver et al., 2004).

Thus, simple and rapid determinations of the genetic mechanism determining resistance to drugs of choice may have great potential in the future clinical microbiology laboratory. However, the major drawback of these methods is that they will not detect resistance if a new unexpected resistance mechanism is present.

3.3. ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF *C. JEJUNI, C. COLI* AND *C. CONCISUS* AND TRENDS OF ANTIMICROBIAL RESISTANCE IN *C. JEJUNI* AND *C. COLI*

Antimicrobial therapy of *Campylobacter* infections is based on in vitro antimicrobial susceptibility studies in order to predict the efficacy when similar drugs are used in vivo. However, in reports on in vitro susceptibilities, major differences in sampling strategy, level of bacterial identification procedures, resistance testing and interpretative criteria are used, making it necessary to interpret results between different reports with caution. Some reports cover data from a single centre with a modest number of tested isolates, while others are based on a high number of isolates from a whole country. The reports are typically based on susceptibility testing on clinical faecal isolates from both in- and out-patients, and often no differentiation has been made on whether isolates originated from children or other age groups. Likewise, distinctions have seldom been made between domestic cases and cases associated with travelling.

Bearing this in mind reported macrolide and quinolone resistance

Table 2. Data on macrolide resistance (%) among *Campylobacter jejuni, C. coli* and *C. jejuni/coli* combined since 1997.

Country	*C. jejuni*	*C. coli*	*C. jejuni/coli*	Reference
Argentina	3[a]	6[a]		(Fernandez, 2001)
Australia	3			(Sharma et al., 2003)
Austria			<1-2	(Feierl et al., 2001, 2003 & 2004)
Belgium	4	6		(Vandenberg et al., 2003)
Bosnia & Herzegovina	20	25	22	(Uzunovic-Kamberovic, 2003)
Canada	0-12			(Gaudreau et al., 2003; Gibreel et al., 2004)
Chile	6	-		(Fernandez, 2001)
Denmark	0-7[b]/0-7[c]	4-21		(IV; Engberg, unpublished data; DANMAP 2003, 2004)
Egypt	0[a]	0[a]		(Putnam et al., 2003)
Finland			0[b]/3[c]	(Rautelin et al., 2003)
France	3	11		(Megraud et al., 2004)
Germany	0-4	0-29		(Steinbrueckner et al., 2001; Luber et al., 2003; Wagner et al., 2003)
India			6	(Jain et al., 2005)
Indonesia	0	-		(Tjaniadi et al., 2003)
Ireland			2	(Lucey et al., 2002)
Italy	1	24		(Pezzotti et al., 2003)
The Netherlands	4[b]/3[c]	6[b]/11[c]		(MARAN-2002, 2003)
New Zealand	3			(Goodchild et al., 2001)
Norway	0-2[b]/<1-3[c]			(Afset et al., 2001; NORM/NORM-VET 2003, 2004)
Mexico	14			(Tuz-Dzib et al., 1999)
Spain	2-5[a]	35		(Saenz et al., 2000; Campos et al., 2001)
Sweden	3		0[b]/5[c]	(Österlund et al., 2003; Rönner et al., 2004)
Thailand	1[a]-2	17[a]-26		(Bodhidatta et al., 2002; Isenbarger et al., 2002)
United Kingdom	1-3	25	2-11	(Moore et al., 2001; Wickins et al., 2001; Anon., 2002; Rao et al., 2005)
United States	1-5	4-9		(Nachamkin et al., 2002; Gupta et al., 2004; NARMS 2002, 2004)
Vietnam	0	0		(Isenbarger et al., 2002)

a) Isolates exclusively from children. b) Isolates acquired domestically. c) Isolates acquired abroad.

profiles and trends over time in *Campylobacter* isolates from humans will in short be reviewed in the following sections and compared with own surveillance data (IV).

3.3.1. Macrolide resistance

Since the recognition of *Campylobacter* enteritis in the 1970s, erythromycin has been the most commonly used agent with which to treat a patient with uncomplicated enteritis.

Table 2 shows data on macrolide resistance in percentage among *C. jejuni*, *C. coli* and *C. jejuni/coli* combined, isolated from human sources around the world since 1997.

There are notable differences between countries and species. Almost all studies report a higher frequency of erythromycin resistance in *C. coli* than in *C. jejuni* with rates reported in proportions ranging from 0% to 20% in *C. jejuni* and 0% to 29% in *C. coli*. In a number of industrialized countries, a higher proportion of *C. coli*, including macrolide-resistant *C. coli*, have been reported among travel-related patients than among domestically acquired infections. Trend over time for macrolide resistance shows stable low rates in most countries, which is comforting as erythromycin or, alternatively, one of the newer macrolides, such as azithromycin, is the drug of choice for treating *C. jejuni/coli* enteritis.

As described above the macrolide resistance mechanism in *Campylobacter* is likely to be chromosomal mutations in the drug-sensitive target. Thus, resistance to macrolides in *Campylobacter* will spread with the bacteria and not be transferable to other bacteria. Development of resistance to macrolides in *Campylobacter* during therapy has not been documented in humans. The origin of resistant strains has been linked to the veterinary use of antibiotics of the macrolide-lincosamide group (Aarestrup et al., 1997). This group of antibiotics has been used worldwide for treatment of food animals for several decades. The most commonly used antimicrobial agents have been lincomycin and tylosin for the control of dysentery and *Mycoplasma* infections in swine and spiramycin for treatment of mastitis in cattle. In addition, for the past 20 years, tylosin has been the most commonly used antimicrobial agent for growth promotion in swine production worldwide, whereas spiramycin has been commonly used for poultry. The use of macrolides for growth promotion were banned in all EU-countries as of July 1999, but are still used in a number of countries out of Europe.

Engberg et al. (VIII), tested 43 *C. concisus* strains and found all the strains to be susceptible to 11 antimicrobial agents, including erythromycin. Macrolide resistance data on this species have only, own data apart, been identified from South Africa. Greg et al. (1993), tested the MIC values of eight isolates and found all but one to be resistant to erythromycin. The Danish study suggests that erythromycin (or a newer macrolide) may be considered if treatment with antimicrobial agents is needed for *C. concisus* infections in this country, as it is for thermophilic *Campylobacter* spp. infections. However, the role of *C. concisus* as a gastrointestinal pathogen first has to be established more firmly.

In conclusion, macrolides are the treatment of choice for most cases of *C. jejuni/coli* enteritis. The data stress the need for additional studies of antimicrobial use in the human and animal populations and that empiric antimicrobial therapy of *Campylobacter* enteritis should be based on locally assessed susceptibility profiles.

3.3.2. Quinolone resistance

The introduction in the 1980s of the fluoroquinolones provided a suitable therapeutic alternative to erythromycin for adults with gastroenteritis because of activity against most enteric pathogens. Fluoroquinolones had good in vitro activity for all *Campylobacter* species as well as for members of the family of *Enterobacteriaceae* and due to an advantageous pharmacokinetic profile with few serious, adverse effects, it looked as though there was finally a class of agents that could be used as the drug of choice for acute bacterial diarrhoea, including *Campylobacter* enteritis.

As reviewed by Wegener & Engberg (2003), the fluoroquinolones were introduced in veterinary medicine in the late 1980s and the early 1990s. Several types of fluoroquinolones are available for animals, however the usage of fluoroquinolones differs greatly as regard animal species, label indications, and geographic spread. In broilers, a principal *Campylobacter* reservoir for human infections oral formulations (water medication) are used to treat respiratory and enteric infections caused by *E. coli*, *Mycoplasma* spp., *Pasteurella* spp. and *Salmonella* spp. A Dutch study (Endtz et al., 1991) was the first one to document a link between veterinary use of fluoroquinolones and occurrence of resistant *Campylobacter* among both food animals and humans. In 1987, fluoroquinolones were introduced for use in veterinary medicine in the Netherlands. No fluoroquinolone-resistant *Campylobacter* isolates were found in poultry products or in humans before 1987. The percentage of fluoroquinolone resistant

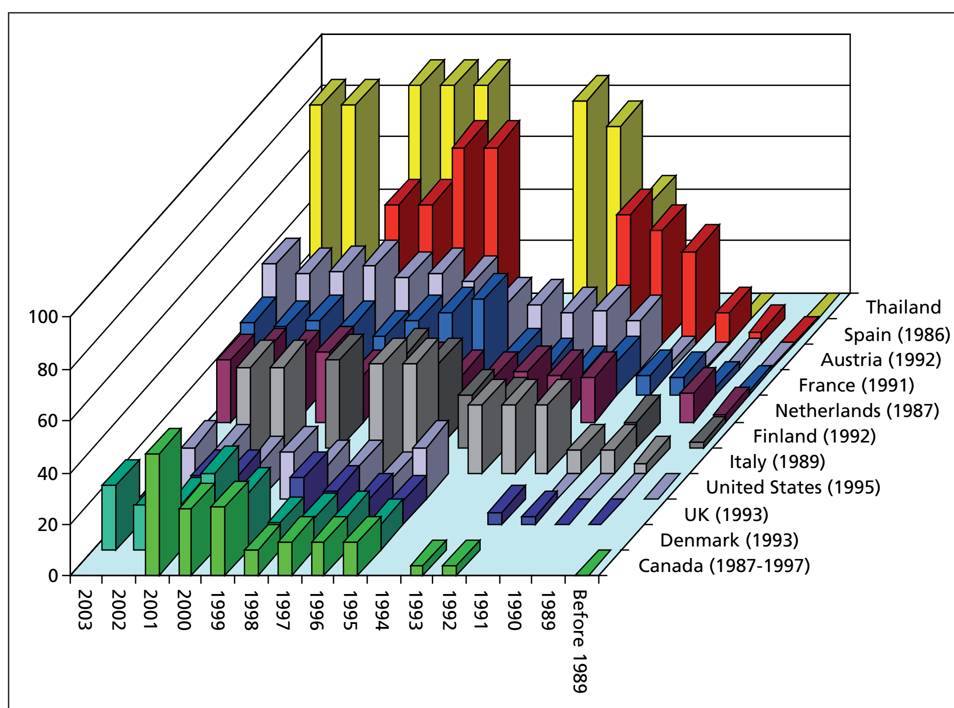

Figure 2. Trends in quinolone resistance (in percentage) among *Campylobacter* from humans. Updated and modified from Engberg et al., 2001. References therein plus (IV; DANMAP 2003, 2004; Boonmar et al., 2005; Sanders et al., 2002; MARAN-2002, 2003; MARAN-2003, 2004; Megraud et al., 2004; Feierl et al., 2001, 2003 & 2004; Pezzotti et al., 2003; Rautelin et al., 2003; Bodhidatta et al., 2002; NARMS 2001, 2003; NARMS 2002, 2004; Wickins et al., 2001}, plus pers. comm. from Prouzet-Mauléon, and unpub. data from Engberg).

isolates in poultry products increased to 8.4% in 1987 and 14% in 1989 (Endtz et al., 1991). During 1992 and 1993, the percentage of resistant isolates from broilers was 29% (Jacobs-Reitsma et al., 1994). This emergence of resistance among poultry products and broilers has been closely followed by an emergence and subsequent increase in resistance among isolates causing infections in humans. The percentage of resistance was 8% during 1988 and 1989, 11% in 1989 and 29% in 1997 (Endtz et al., 1991; Talsma et al., 1999).

Figure 2 shows trends for quinolone resistance rates (in percentage) of among *C. coli* and *C. jejuni* combined from humans from 11 different countries covering the period 1989 to 2003. The bars represent both nalidixic acid and fluoroquinolone resistance and are based on mean values of resistance from numerous reports. Year in parenthesis is the year of licensure for use in veterinary medicine in each country. Canada banded veterinary use of fluoroquinolones in 1997.

The use of fluoroquinolones (mainly enrofloxacin) in veterinary medicine is correlated with an increase in quinolone resistance in food animals, in retail food of animal origin, especially in poultry products and, most importantly, in human *Campylobacter* infections. Before 1989, fluoroquinolones were mainly used in human medicine and resistance was rare, but with the introduction of fluoroquinolones in veterinary medicine, a rapid emergence of quinolone resistance in *Campylobacter* isolates from patients was reported from a number of countries. Similar trends have been observed in other countries where fluoroquinolones are approved in veterinary medicine. In some countries, the rise in resistance has been remarkably rapid and considerable, while the resistance rates have increased steadily in other countries. For instance, a recent report of quinolone resistance of human *C. jejuni* isolates found 86% resistance in Hong Kong (Chu et al., 2004). In the high-endemic quinolone resistance areas, fluoroquinolones cannot be recommended for community-acquired bacterial diarrhoea, as the predominant causes are often *Campylobacter* spp. Although lower frequencies are reported from other regions, recent trends over time show a clear and worrying tendency of emerging quionolone-resistance in many countries.

3.3.3. Own investigations

Veterinary use of fluoroquinolones is not the only selection pressure that acts upon *Campylobacter* to select for quinolone-resistance. Resistance occurs naturally, but the selection and dissemination of resistance is an inevitable result of any antibiotic use. Fluoroquinolone use in humans can in itself lead to the emergence of quinolone-resistant *Campylobacter* in treated infections.

By a systematic approach integrating standardized epidemiologic, antimicrobial susceptibility, and typing data, Engberg et al. (IV), conducted a 1-year prospective study to address the prevalence of macrolide and quinolone resistance in human *Campylobacter* isolates. Quinolone resistance was found to be significantly associated with the origin of infection: 76 (50.0%) of 152 infections among travelers returning to Denmark were quinolone-resistant whereas 52 (9.9%) of 526 domestically infected patients were infected with a quinolone-resistant strain ($p < 0.001$).

A case-comparison study to identify risk factors associated with acquiring quinolone-resistant *C. jejuni* infections was also conducted (IV). From December 1, 2001, to June 10, 2002, 42 patients were infected with quinolone-resistant *C. jejuni* isolates, and these patients were matched with 84 patients with quinolone-sensitive isolates. According to the multiple logistic regression analysis, the exposures independently associated with an increased risk for quinolone-resistant *C. jejuni* infection were foreign travel (OR = 16.81), eating fresh poultry other than chicken and turkey (OR = 19.10), and swimming in pools, oceans, lakes, or other places (OR = 5.01). Eating fresh chicken (of presumably Danish origin) was associated with a decreased risk (OR = 0.04). Age group did not affect the findings (younger or older than 15 years of age) neither in the univariate nor the multiple logistic regression analysis. At least three other case-control studies have specifically addressed risk factors for quinolone *Campylobacter* infections in the U.S., U.K. and Denmark (**Table 3**).

Three of the four studies evaluated current or recent treatment with antimicrobials. An association between treatment with a fluoroquinolone before stool-specimen collection and having a quinolone-resistant *Campylobacter* infection was only observed in the

Table 3. Studies evaluating risk factors for quinolone-resistant *Campylobacter* infections[a].

Reference	Potential risk factor	Patients with resistant isolates (%)	Patients with sensitive isolates (%)	Multivariate analysis	
				mOR (95% CI)	P-value
Smith et al., 1999	Foreign travel to				
	Mexico	47 (36)	30 (12)	26.0 (8.6-78.6)	<0.001
	Caribbean countries, South America, Central America (not Mexico)	14 (11)	7 (3)	45.5 (9.7-214)	<0.001
	Asia	23 (18)	8 (3)	40.7 (10.2-163.0)	<0.001
	Spain	7 (5)	1 (<1)	48.6 (4.1-570.0)	0.002
	Use of a quinolone before the collection of stool specimens	26 (20)	7 (3)	7.5 (2.6-21.3)	<0.001
Anon., 2002b	*Travel-related infections*				
	Portugal	8 (2)	3 (2)	22.4 (4.4-115.0)	<0.001
	Cyprus	5 (1)	1 (<1)	11.7 (1.3-108.0)	0.03
	Spain	48 14)	16 (11)	6.9 (3.5-13.4)	<0.001
	Chicken	92 (27)	82 (55)	5.0 (2.1-11.6)	<0.001
	Domestically acquired infections				
	Cold meats (pre-cooked)	80 (27)	71 (4)	2.1 (1.4-3.1)	<0.001
Engberg et al., IV	Foreign travel	30 (71)	12 (14)	16.8 (3.4-82.2)	0.001
	Fresh poultry other than chicken and turkey	14 (33)	58 (69.6)	19.1 (2.2-167.3)	0.008
	Swimming (pool, ocean, lake, or other places)	20 (48)	16 (19)	5.0 (1.14-22.0)	0.033
Kassenborg et al., 2004[b]	Eating chicken or turkey cooked at a commercial establishment	18 (55)	7 (21)	10.0 (1.3-78.0)	0.03

a) Only risk factors associated with increased risk of infection are presented.
b) Analysis of potential risk factors specifically on domestic acquired infections. Travel outside the U.S. were reported by 27 (42%) of 64 patients with fluoroquinolone-resistant *Campylobacter* and by 51 (9%) of 582 patients with fluoroquinolone-susceptible *Campylobacter* infection (odds ratio [OR] 7.6; CI 4.3-13.4).
Reprinted from Engberg J, Keelan M, Gerner-Smidt P, Taylor DE. Antimicrobial resistance in *Campylobacter*. In Aarestrup FM, editor, Antimicrobial resistance in bacteria of animal origin. Veterinary and public health aspects. Washington, D.C.: ASM Press, in prep. 2005, with permission from ASM Press.

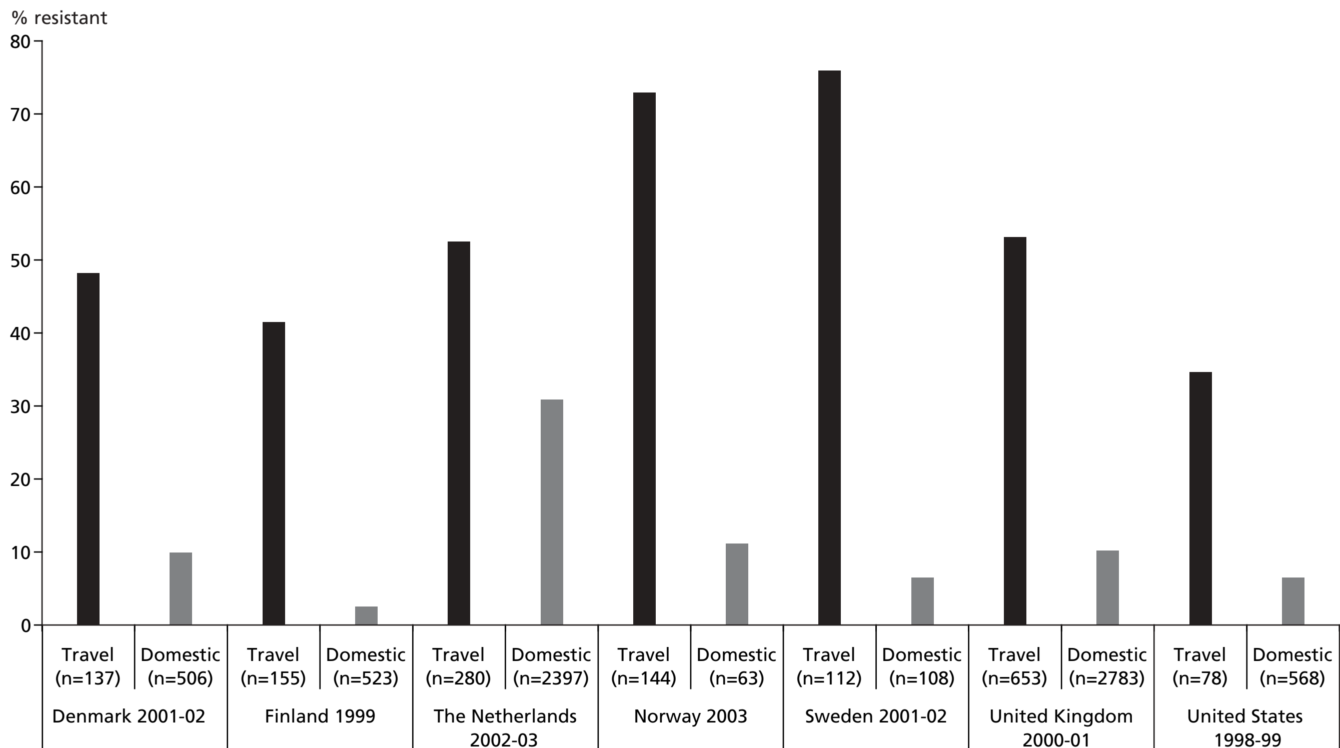

Figure 3. Quinolone-resistance of *Campylobacter* by origin. Quinolone resistance rates (in percentages) among *C. jejuni* (Denmark, The Netherlands and Norway) and *C. jejuni/coli* (Finland, Sweden, United Kingdom and United States) by history of travel (IV; Rautelin et al., 2003; MARAN-2002, 2003; Anon., 2002b; NORM/NORM-VET, 2003; Rönner et al., 2004; Kassenborg et al., 2004). Reprinted from Engberg J, Keelan M, Gerner-Smidt P, Taylor DE. Antimicrobial resistance in *Campylobacter*. In Aarestrup FM, editor, Antimicrobial resistance in bacteria of animal origin. Veterinary and public health aspects. Washington, D.C.: ASM Press, in prep. 2005, with permission from ASM Press.

study by Smith et al. (1999), but their study also showed that treatment with a fluoroquinolone before stool culture accounted for a maximum of 15% of resistant isolates in Minnesota during 1996 and 1998. The studies suggest that quinolone use in humans is not the major selective force for quinolone resistance among *Campylobacter* spp. causing human infection.

Foreign travel was identified as a risk factor in all four studies, and this is in agreement with recent surveillance data from a number of countries, which show a significant difference in quinolone resistance rates between travel-related infections and domestically acquired infections, and document the importance of stratifying susceptibility data by travel status (**Figure 3**).

Travel-related infections to destinations with recognized high quinolone-resistance in *Campylobacter* in poultry, as well as established high risk of attracting quinolone-resistant human *Campylobacter* infections, is associated with significantly higher prevalence of quinolone resistance compared to infections acquired domestically. The significantly lower prevalence of quinolone resistance amongst domestically acquired *Campylobacter* probably reflects a more limited or lacking veterinary usage of fluoroquinolones in these countries. For instance, in Australia, where fluoroquinolones have not been licensed for use in food production animals, and only cooked chicken products may be imported, no fluoroquinolone resistance has been found in domestically acquired human infections (Unicomb et al., 2003). In contrast, while foreign travel is also associated with quinolone-resistent infections in the United States, the majority of quinolone-resistant infections are nevertheless domestically acquired in this country (Gupta et al., 2004; Kassenborg et al., 2004).

In Engberg et al. (IV), *C. jejuni* infections and *C. coli* infections did not differ in severity, when assessed by frequency of diarrhoea, blood in stool, abdominal pain, fever, vomiting, mean duration of illness, or admission to hospitals. However, the mean duration of illness was longer for the 86 patients with quinolone-resistant *C. jejuni* infections and a known duration of illness (median 13.2 days) than for the 381 patients with quinolone-sensitive *C. jejuni* infections and a known duration of illness (median 10.3 days, p = 0.001). **Table 4** summarizes information from case-comparison studies evaluating the duration of illness in patients infected with quinolone-resistant *Campylobacter* strains versus quinolone-sensitive *Campylobacter* strains (IV; Smith et al., 1999; Anonymous, 2002b; Kassenborg et al., 2004).

The recent study by Nelson et al. (2004) evaluated duration of illness across a variety of analytical models, including a multivariable analysis-of-variance model, and identified a consistent correlation between quinolone resistance and prolonged duration of diarrhoea. Although the results from these studies are not all statistically significant, the estimates all point in the same direction, and taken together suggest that patients infected with quinolone-resistant strains have a longer duration of illness. Whether patients with resistant infections may experience a longer duration of illness because the antibiotic provided to them simply does not work against resistant *Campylobacter* and/or it may be due to a possible co-selection of virulence traits in resistant strains remains to be fully determined.

Additionally, Helms et al. (2005) have very recently determined that there is an excess risk of death or invasive illness following infection with resistant *Campylobacter* compared with susceptible strains. In 3471 Danish patients with *Campylobacter*, a total of 22 (0.63%) patients had an adverse event defined as invasive illness or death within 90 days of date of receipt of faecal sample. Patients with quinolone-resistant *Campylobacter* had an increased risk of an adverse event within 30 days compared with quinolone and erythromycin susceptible *Campylobacter* infection (adjusted OR 6.17, 95% CI 1.62-23.47). Compared with quinolone- and erythromycin-susceptible *Campylobacter* infection, infection with erythromycin-resistant strains was associated with a more than five-fold risk of adverse event at 90 days of receipt of sample (adjusted OR 5.51, 95% CI 1.19-25.50).

3.3.4. Conclusions and future directions

Campylobacter has become the leading cause of zoonotic enteric infections in developed and developing countries worldwide. Epide-

Table 4. Studies evaluating the duration of illness in patients infected with quinolone-resistant *Campylobacter* strains versus with quinolone-susceptible *Campylobacter* strains.

Reference	Resistant		Sensitive		P-value
	Number of patients	Duration of diarrhoea, days	Number of patients	Duration of diarrhoea, days	
Smith et al., 1999	69	10	115	7	0.03
(Neimann et al., 2001)[a]	5	14	31	9	0.13
Anon., 2002b[b]		12.7[d] 11.8[e]		13.5[d] 11.2[e]	0.56[d] 0.66[e]
Engberg et al., IV[b]	86	13.2	381	10.3	0.001
Nelson et al., 2004[c]					
Model A	26	9	264	7	0.04
Model B	7	12	56	6	0.04
Model C	9	8	76	6	0.2

a) Stratified by treatment, but not on antimicrobial agent used for treatment.
b) Analysis not stratified by treatment.
c) Model A: analysis of 290 persons who did not take anti-diarrhoeal medications; Model B: analysis of 63 persons who did not take antimicrobial agents or anti-diarrhoeal medications; Model C: analysis of 85 persons who took only fluoroquinolone antimicrobial agents.
d) Domestically acquired infections.
e) Travel-related infections.

Reprinted from Engberg J, Keelan M, Gerner-Smidt P, Taylor DE. Antimicrobial resistance in *Campylobacter*. In Aarestrup FM, editor, Antimicrobial resistance in bacteria of animal origin.' Veterinary and public health aspects. Washington, D.C.: ASM Press, in prep. 2005, with permission from ASM Press.

miological and microbiological studies show that poultry is the most important source for quinolone-susceptible and quinolone-resistant *Campylobacter* infections in humans. Trends over time for macrolide resistance show stable low rates in most countries and macrolides remain the drugs of choice for *Campylobacter jejuni/coli* enteritis. However, macrolide resistance is emerging in some countries and needs to be monitored; the causes of this resistance should be identified and if possible controlled. In countries with widespread veterinary use of quinolones and among returning travelers from these destinations, fluoroquinolones are, at present, not safe drugs for the treatment of patients with *Campylobacter* enteritis.

There is growing evidence that antimicrobial-resistant *Campylobacter* in the food chain has significant public health consequences. Quinolone resistance is emerging in *Campylobacter* and resistant *Campylobacter* infections may be associated with excess morbidity and mortality compared with infections with sensitive strains. As a consequence, The Center for Veterinary Medicine (CVM), U.S. Food and Drug Administration (FDA) proposed to withdraw the approval of enrofloxacin (or Baytril™) in poultry in the United States in late 2000. Following the initial decision of a hearing, the approval of Baytril™ was withdrawn from the U.S. market in March 2004. However, the manufacturer has appealed the decision. Mitigation of antimicrobial resistance in food-borne bacteria such as *Campylobacter* will likely benefit human health.

CHAPTER 4. TYPING OF *C. JEJUNI, C. COLI* AND *C. CONCISUS*

Phenotypic and genotypic characters used for determination of bacterial relatedness in an epidemiological context are termed epidemiological markers. Characterization of isolates for a given marker is called typing. Typing is used to characterize and identity strains. A strain is the term used for epidemiologically related isolates with common pheno- and genotypic characteristics. However, no typing method can prove the identity of two isolates; only the non-identity of isolates may be proved. Only by selection of multiple typing methods may assumptions be made about the strain relationship of two isolates. Epidemiological typing may be used to study bacterial population genetics, the study of pathogenesis of infections, epidemiological surveillance of infectious diseases and outbreak detection/investigation (Struelens et al., 1996). The method must therefore be able to discriminate between epidemiologically unrelated isolates of the same microbial species and assign isolates derived from the same outbreak or from a chain of transmission to one type or a closely related group of types to confirm that these are derivatives of the same ancestor. Hence, the interpretation of results must be the same, no matter who interprets them, or where and when the method is applied.

4.1.1. Criteria for performance of typing methods

It is important to clarify whether a typing method is definitive or comparative by nature. A *definitive* system produces results that are expressed in a simple, meaningful, and reproducible manner, making it possible to compare results from studies done at different times and or in different places. The *comparative* methods yield results that can only be compared with results obtained in the same experiment. Many genotyping methods such as ribotyping and Pulsed Field Gel Electrophoresis (PFGE) are only comparative by nature, but by standardisation of the experimental conditions definitive comparisons may be made.

In addition, the European study group on epidemiological markers (ESGEM) has proposed that several performance criteria need to be addressed, when a typing method is to be set up in a laboratory. These include 1) typability, 2) reproducibility, 3) stability, and 4) discriminatory power. The *typability* is the percentage of strains that could be typed by the method. The *reproducibility* is the ability of a typing method to assign the same type to a strain tested on independent, separate assays. When the reproducibility is tested, all steps in the technical procedure should be addressed. The reproducibility should be equal to or higher than 0.95 for a reliable definitive typing. The *stability* is the term used for the ability of a method to recognise the clonal relatedness of strains derived in vivo or in vitro from a common ancestor strain, despite the phenotypic or genotypic variation that may occur during clonal dissemination by nature, especially over prolonged periods or in large-scale epidemics, or during laboratory storage and replication. The *discriminatory power* is the ability of a typing method to discriminate between epidemiologically unrelated isolates. Hunter & Gaston (1988) have devised a discriminatory index (DI) based on Simpson's index of diversity to describe the discriminatory power of a typing system. The DI is an expression of the average probability that the typing method will distinguish two unrelated strains randomly sampled in a microbial population. The formula reads:

$$DI = 1 - \sum_{j=1}^{N} a_j / N(N-1)$$

where DI is the discriminatory index, N is the number of strains in the study population, a_j is the number of strains with a type that cannot be differentiated from that of the *j*'th strain. By the use of this formula, it is possible reliably to compare the discrimination of different typing systems.

Table 5. Phenotypic and genotypic methods used for typing *Campylobacter* in the literature and by the author and colleagues in the thesis.

Typing system	Paper	Author	Colleague
Phenotypic methods			
Biotyping			
Antibiogram typing			
Serotyping (heatstable, "Penner")	I, IV, V, VI, VII		x
Phagetyping			
MLEE (Multilocus enzyme electrophoresis)	VII		x
SDS-PAGE (Whole cell protein profiling)	VIII		x
Genotypic methods			
Restriction Fragment Length Polymorphism (RFLP)-based methods			
REA (Restriction Endonuclease Analysis)	I	x	
PFGE (Pulsed Field Gel Electrophoresis)	I, V, VI, VII	x	x
Ribotyping (incl. automated ribotyping, RiboPrinting)	I, IV, V, VI, VII, VIII	x	
PCR amplification-based methods			
PCR amplification of 23S rDNA	VIII	x	
RAPD (Random Amplified Polymorphic DNA analysis)	V, VIII	x	
DGGE (Denaturing Gradient Gel Electrophoresis)	V		x
Combined RFLP and PCR methods			
PCR-RFLP (Restriction digests of PCR products, e.g. of the *flaA* gene	V, VII		x
AFLP (amplified fragment length polymorphisms)			
Sequence-based methods			
MLST (Multilocus Sequence Typing)			
"Genomotyping"			
Whole-genome DNA microarrays			

All these performance criteria were evaluated in this thesis (V & VI) and will be discussed further below together with additional factors, such as cost, speed and relevance, for consideration when implementing a typing method.

Many laboratories routinely use various phenotypic and genotypic techniques to classify or identify organisms including of *Campylobacter* spp. There is, therefore, a need to properly store these data in a suitable integral database in order to meaningful group or identify the organisms based on all available biological data (phenotypic test results, all kinds of fingerprints and perhaps nucleic acid sequences). In other words, there is a need for *computer-assisted data analysis*. Various bioinformatics software programmes are commercially available, such as the GelCompar program used in Nielsen et al., V, and the BioNumerics program used in Engberg et al., VII & VIII. Both programmes have been developed by Applied Maths, Sint-Martens-Latem, Belgium, (http://www.applied-maths.com/home.html). Electrophoresis data from finger printing methods can be exported to a BioNumerics database as either 2D TIFF images of gels or densitometric curves produced by capillary electrophoresis in sequencers. In brief, electrophoresis data is then processed in a four-step manner by defining lanes, calculating curves, normalisation by interpolation to the nearest standard lanes, and band searching. The normalised profiles can be saved and be used for future analyses. New patterns may be compared with patterns already in the database. This is imperative for tracking specific subtypes of bacteria in prospective long-term studies (Fussing et al., 2003). The software packages offer a range of strain grouping features based on different similarity coefficients and clustering methods. In the present thesis, the Dice coefficient, which is generally recognised as the coefficient of choice for band based typing methods, has been used for such methods, e.g. PFGE and ribotyping. Pearson's product-moment similarity coefficient, which is not band based, is most commonly used for densitometric curves and has therefore been used for methods with a densitometric output, e.g. RAPD. A clustering dendrogram can subsequently be produced, e.g., by using the unweighted pair group method with arithmetic averages (UPGMA) to visualize the relationships between the profiles.

However, if instructed to, the software will generate a dendrogram whether or not the relationships in the data may be described this way and the calculations performed by the software is dependent on the quality of the data put into the system and user input on the position tolerance of the electrophoretic data. For these reasons, all results generated by the software needs to be checked visually.

4.1.2. Typing methods used for typing Campylobacter

A list of phenotypic and genotypic methods used to investigate the epidemiology of *Campylobacter* is presented in **Table 5**.

Phenotypic methods are based on expressed properties and for *C. jejuni* and *C. coli* the most important phenotypic test has been serotyping. Two serotyping schemes were developed in the 1980s in Canada based on heat-stable (capsular) antigens (Penner et al., 1980) and on heat-labile (flagellar) antigens (Lior et al., 1982), respectively. More recently, a modified Penner serotyping system was developed in the UK (Frost et al., 1998). The Penner serotyping system has been implemented in Denmark by Nielsen (1997) and have been extensively used to study the epidemiology of *C. jejuni* and *C. coli* inclusive in this thesis (Table 5). Biotyping and phagetyping can be complementary to other typing methods, but are only in use in a few centers due to their poor resolution. The advantages of phenotypic tests are the ability to screen large number of isolates with low cost and that the methods are definitive, whereas their disadvantages are the moderate discriminatory power, the need for specialist laboratories, the lack of commercially available quality controlled reagents and that they depend on gene expression.

Genotypic methods are based on genomic properties and a wealth of methods has been used to investigate the epidemiology of *Campylobacter* (Table 5).

The development of molecular methods has expanded the resolution of typing methods considerably and has provided additional evidence for epidemiological links.

However, many of the methods are comparative by nature and the discriminatory power is method-dependent: *low discriminatory:* plasmid profiling; *intermediate discriminatory:* PCR-RFLPs, Ribotyping, DDGE; *high discriminatory:* RAPD, PFGE, AFLP, MLST, and DNA microarrays.

Ribotyping (manual or automated (RiboPrinting)), RAPD and PFGE have been used extensively by the author in this thesis (Table 5).

Ribotyping was originally described by Grimont & Grimont in 1986. It detects RFLPs of chromosomal DNA containing the genes for ribosomal RNA (rRNA restriction patterns). The genome of any bacterial species contains genes encoding ribosomal DNA, usually in

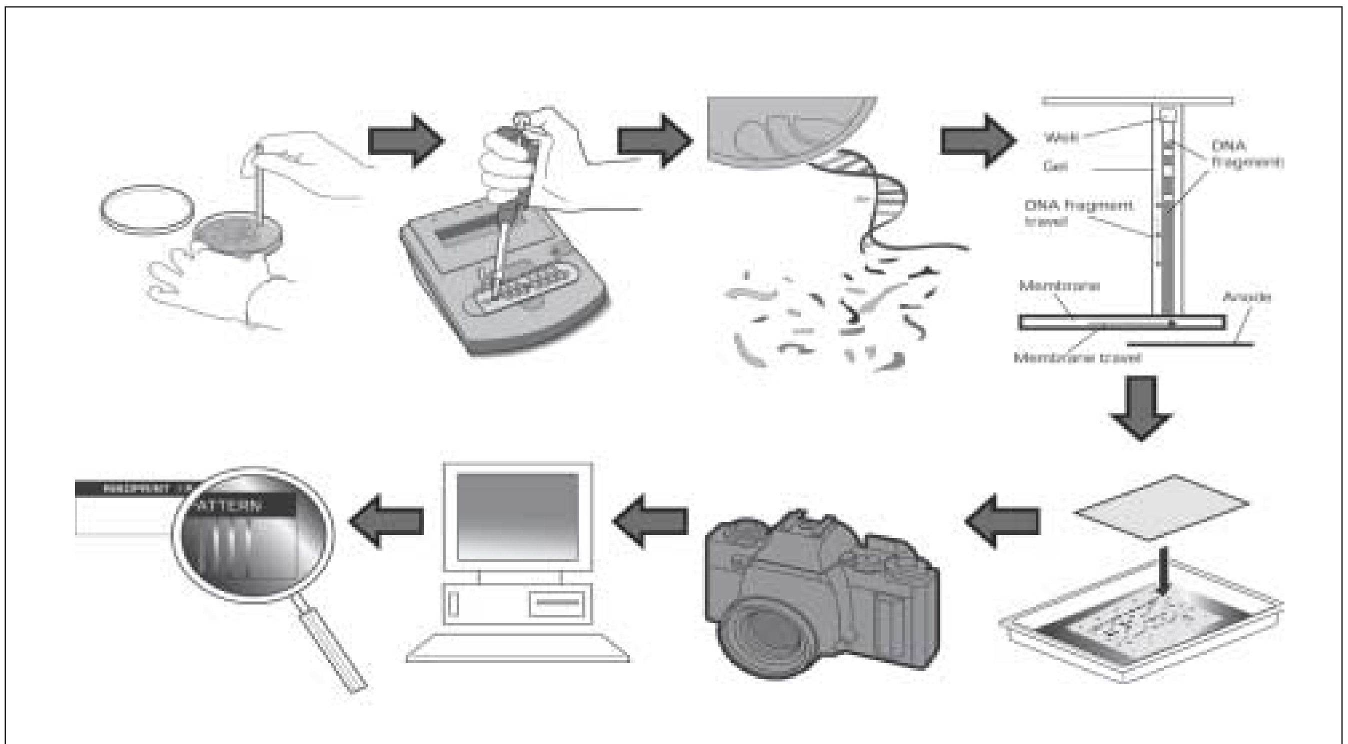

Figure 4. Schematic representation of automated ribotyping using the RiboPrinter® System from DuPont Qualicon. The process progresses from manual collection of the bacterial sample, through heat treatment in a heating block, into the automated instrument for DNA preparation, separation, and transfer, membrane processing, pattern detection, and data processing, to the production of a unique RiboPrint™ pattern for each sample. Used with permission from DuPont Qualicon.

several copies. Because the nucleic acid sequence of ribosomal DNA is highly conserved in large regions, a probe made for ribosomal DNA of one species may hybridize with genes encoding ribosomal DNA in almost any other species. In short, the principle of the methods is as follows: the genomic DNA is cut with a restriction enzyme that recognizes a short DNA-base sequence and cuts the DNA in relation to it; the DNA is, thereby, cut into a well-defined number of pieces (restriction fragments), usually >100 in number, which are then separated according to their size by electrophoresis in an agarose gel (Restriction Endonuclease Analysis (REA)); the fragments are transferred to a nylon membrane by Southern blotting and hybridized with the ribosomal DNA probe; thereby only the fragments (usually 4-15 in number) containing ribosomal genes are visualised. This manual ribotyping method was used in I. In the subsequent ribotyping analyses in this thesis, a commercially available automated system, RiboPrinter® (Qualicon, Wilmington, Del., USA), was used. The manual and automated steps incompassed by the RiboPrinter are principally the same as described above, but the process from DNA preparation, separation, and transfer, membrane processing, pattern detection, to data processing and the production of a unique pattern (RiboPrint(TM)) of each strain is automated (**Figure 4**).

In the instrument, the bacterial cells are lysed and the released DNA is digested with the *Eco*RI restriction enzyme as standard. However, this enzyme is not capable of digesting *Campylobacter* chromosomal DNA, and after a pilot screening (data not presented) of different restriction enzymes, the *Hae*III enzyme was found to provide most discrimination and most ease of interpretation and was therefore used in this thesis for *C. jejuni* and *C. coli*. The enzyme generates 6-8 bands over a size range of 1-15 kb (V-VII). A subsequently inter-laboratory (CAMPYNET) evaluation of *Pst*I, *Pvu*II and *Hae*III restriction enzymes for ribotyping *C. jejuni* and *C. coli* using the RiboPrinter, confirmed that *Hae*III was the optimal enzyme (Harrington et al., 2001). In contrast, in a screening study (data not shown) of a number of restriction enzymes prior to a typing study of *C. concisus* by Engberg et al. (VIII), only the *Pvu*II enzyme was able to properly digest DNA of this species and was therefore used.

Automated ribotyping has the advantage of automation, reproducible results and a fairly good throughput as the run time for a single batch of eight strains is 8 h, and a new batch may be placed in the instrument every 2 h. Furthermore, profiles are portable and due to standardization and automation, RiboPrinting can be regarded as a definitive typing system permitting data to be exchanged between laboratories. A disadvantage of the method is the cost. The RiboPrinter equipment is expensive and the consumables are costly as well. There are only three ribosomal gene copies in *Campylobacter* and therefore the method has only an intermediate discriminatory power (V).

Random Amplified Polymorphic DNA analysis (RAPD) was first described in 1990 by Williams et al., and Welsh & McClelland. The method is based on the use of a single short primer of arbitrary nucleotide sequence (typically 10 bases in length) to amplify multiple random fragments of the bacterial DNA under low stringency conditions (i.e. at a low annealing temperature, such as 35°C or lower). The primers anneal to multiple regions of the genome simultaneously, but amplification occurs only when the 3' end of the annealed primers face one another on opposite strands of DNA not more than several kilobases apart. Once a primer or more are selected, the method of RAPD analysis is relatively straightforward and consists of the following steps: preparation of DNA (break of cells and DNA extraction), PCR amplification, electrophoresis, visualization of bands, and data analysis. A detailed description of the procedure used in this thesis is presented in V.

The method has a number of advantages: it is simple, cheap and rapid and does not require complex equipment like RiboPrinting and PFGE. Random multiple fragments of the whole genome can be amplified with a single short primer of arbitrary nucleotide sequence and RAPD can provide a level of discrimination, which is higher than ribotyping, *fla*-RFLP, *fla*-DGGE and equal to or greater than that of PFGE (V) (Endtz et al., 2000; Scates et al., 2003).

Due to the low stringency PCR conditions, this method is extremely sensitive to slight changes within the different PCR parameters such as type of polymerase, buffer ingredients, DNA concentration and thermocycler, and the major drawback of RAPD is there-

fore its reproducibility (see below and for a review see Meunier et al., 1993, and Power, 1996). In this thesis, the author applied Ready-To-Go Analysis Beads® (Pharmacia Biotech, Freiburg, Germany), containing premixed, predispensed AmpliTaq DNA polymerase, buffer ingredients and nucleotides followed by fragment analysis on a DNA sequencer, to reduce the number of susceptible steps and hereby increase reproducibility.

Pulsed Field Gel Electrophoresis (PFGE). Since its initial description by Schwartz et al. in 1983, PFGE has emerged as the present "gold standard" molecular approach to the epidemiological analysis of many bacterial pathogens, including *Campylobacter*. The PFGE procedure requires the digestion of the bacterial DNA with rare cutting restriction enzymes and thus generates a small number (10-30, sometimes less) of restriction fragments. These fragments are usually too large to separate by conventional agarose gel electrophoresis. However, they can be effectively resolved by differential migration through agarose gels by constantly changing the direction of the electrical field during electrophoresis. Many configurations of PFGE have been devised, but contour-clamped homogeneous electric field (CHEF) gel electrophoresis has evolved to be the method of choice for resolving DNA macrorestriction fragments of bacterial genomic DNA. The principle of PFGE is lysis of bacterial cells in situ in agarose plugs to release intact chromosomal DNA, removal of impurities by extensive washing, restriction of genomic DNA with an appropriate restriction enzyme, PFGE of restricted DNA, and staining in ethidium bromide to visualize the separated restriction fragments. This basic format can be applied as a universal generic method for subtyping of bacteria. Only the choice of the restriction enzyme and conditions for electrophoresis need to be optimized for each species. For *Campylobacter*, the following enzymes *Sma*I, *Sal*I, *Kpn*I, *Sac*II and *Bam*HI are commonly used, either alone or in combination as they yield an optimum number and size range of chromosomal fragments for analysis (V; VI; VII; On et al., 1998; Endtz et al., 2000; Lehner et al., 2000; Nachamkin et al., 2001; Hänninen et al., 2001; Ono et al., 2003). A detailed description of the procedure used in this thesis is presented in VI.

The method has a number of advantages. First, the sensitivity of the technique lies in the fact that whole-genome restriction site polymorphisms are detected and strain differences are far easier to ascertain compared with the highly complex patterns obtained by REA. Second, comparisons of the discrimination for typing of a number of bacterial genera, including *Campylobacter*, have repeatedly shown high discriminatory indices (V; Endtz et al., 2000; Ono et al., 2003; Lindmark et al., 2004). Third, although comparative in principle, use of standard protocols, reference strains on every gel, and storage of profiles in a database permits comparison of data from one laboratory with those from another using the same protocol. Standardized protocols are imperative in networks like the American molecular subtyping-based surveillance system for food-borne bacterial disease, PulseNet (http://www.cdc.goc/pulsenet). In PulseNet, standardized PFGE protocols for subtyping of clinical and food isolates of *Escherichia coli* O157:H7, *Salmonella, Listeria monocytogens* and *Shigella* has been developed. A rapid standardized PFGE method for *C. jejuni/coli* has most recently been added to this program (Ribot et al., 2001), but routine subtyping of all *Campylobacter* isolates is not feasible due to the large degree of diversity in PFGE patterns among isolates that limits the usefulness of PFGE for outbreak detection. Instead, in the PulseNet PFGE is presently used to delineate outbreaks detected by other means, e.g. simple clustering in time and place of cases of *Campylobacter* infection (Hedberg et al., 2001).

A disadvantage of PFGE for subtyping campylobacters is its sensitivity to genetic instability (see below and for a review see Wassenaar et al., 2000).

Other disadvantages of PFGE profiling are the production of DNase of some *Campylobacter* strains which must be deactivated (e.g. by the toxic chemical formaldehyde (Gibson et al., 1994)) to ensure that DNA samples do not degrade before electrophoresis. Finally, the enzymes commonly used to produce PFGE profiles do not digest the DNA of some strains (Newell et al., 2000).

4.1.3. Own investigations

Engberg et al. (I) applied a multidisciplinary approach to epidemiologic, environmental and microbiological investigations, using a combination of serotyping, REA, manual ribotyping and PFGE to full understand an outbreak in the Danish town Klarup (Desenclos, 1998). In spite of the six week ongoing outbreak with continued transmission and with sampling over a period of more than two months from human diarrhoeal cases, *C. jejuni* isolates recovered from the community water system had the same serotype and the same DNA profile in all but one of the thirty stored outbreak isolates. The DNA profile of the outbreak isolates was not found in control isolates obtained from cases unrelated to the outbreak. Serotyping alone was not a sufficient epidemiologic marker, as serotype HS:2 is the most common human serotype in Denmark and was also identified in control isolates. The combination of multiple typing methods, to verify similarity or dissimilarity of *Campylobacter* isolates have been found useful in a number of outbreak investigations. Most often serotyping together with PFGE typing of patient and water isolates have been used, but also other combinations have been applied (Bopp et al., 2003; Clark et al., 2003; Hänninen et al., 2003; Kuusi et al., 2004; Gallay et al., 2005). The nearly ideal situation with lack of genetic diversity among *Campylobacter* isolates from patients and the environment in the Klarup outbreak is uncommon for reported waterborne outbreaks where multiple pathogens or multiple *Campylobacter* strains are often demonstrated (Bopp et al., 2003; Clark et al., 2003; Hänninen et al., 2003; Gallay et al., 2005). This emphasizes that laboratory findings alone are not sufficient to prove or disprove the source of an infection. Typing, epidemiologic and environmental findings need to be evaluated together.

Campylobacter is generally considered to have an almost panmictic population structure rather than a clonal structure due to considerable rearragements (Dingle et al., 2001; Suerbaum et al., 2001; de Boer, 2002; Dingle et al., 2005). Some *Campylobacter* strains, however, might be exceptional with respect to their genomic stability. For example, several studies indicate that HS:19 and HS:41 have a clonal population structure (Fujimoto et al., 1997; Misawa et al., 1998; Wassenaar et al., 2000; Nachamkin et al., 2001). In addition, HS:19 and HS:41 have been reported to be over-represented among isolates from GBS cases compared to enteritis cases in Japan, South Africa, China, and Mexico suggesting that these serotypes might have unique virulence properties linked to relationship between infection and GBS (Kuroki et al., 1993; Yuki et al., 1997; Lastovica et al., 1997; Nachamkin et al., 1999).

Nachamkin et al. (2001) and Engberg et al. (VII) evaluated in two parallel studies if a particular HS:19 clone is associated with GBS and whether GBS-associated HS:19 strains differ from strains isolated from patients with *Campylobacter* gastroenteritis and likewise for non-HS:19 strains. Using MLEE, three major phylogenetic clusters were identified among 83 *C. jejuni* strains including 64 HS:19 and 19 non-HS:19 strains. Cluster I contained all HS:19 strains and a single electropherotype (ET) ET4, accounted for the majority of HS:19 strains. HS:19 strains did not occur in any of the other clusters. ET4 contained isolates from different geographic locations, indicating global spread of this clone. Futhermore, ET4 contained isolates from patients with uncomplicated enteritis, GBS, as well as isolates from animal sources. Additional analysis with three genotyping methods, including RiboPrinting confirmed the findings of the MLEE analysis. The results of this study showed that HS:19 strains comprise a clonal, although not monomorphic population distinct from non-HS:19 strains within *C. jejuni*, but a unique clone associated with GBS was not identified. The findings of this study were subsequently confirmed in a DNA microarray study using a subset of the same strains (Leonard et al., 2004).

In the non-HS:19 study (VII), Engberg et al. examined whether *C. jejuni* non-HS:19 serotypes associated with GBS have a clonal structure and differ from strains isolated from patients with *Campylobacter* gastroenteritis. The study population consisted of 11 non-HS:19 GBS-related *C. jejuni* strains isolated from patients in six geographically distinct locations and 47 control strains. Twelve HS:19 strains were included for comparative purposes. Genetic diversity across the nine loci examined by MLEE was high indicating the nonclonal nature of these serotypes. All typing methods (Penner serotyping, MLEE, RiboPrinting, PFGE with *Sma*I, *Sal*I, and *Kpn*I restriction enzymes and *fla*A-RFLP) showed a high diversity in the studied population of strains; however, group definition of strains varied considerably between metods. The 11 GBS-related strains of different serotypes did not represent separate genetic lineages distinct from enteritis-associated strains or strains from animals. A specific epidemiological marker, i.e., a specific band(s) or protein allelic profile associated with GBS strains only could not be identified. This finding was confirmed by a subsequent MLST study which showed that *C. jejuni* strains associated with GBS and Miller-Fisher syndromes are of diverse genetic lineage, serotype, and flagella type (Dingle et al., 2001).

In Engberg et al. (VII), the discriminatory potential of the three enzymes used for PFGE typing differed from each other. The 58 *C. jejuni* strains were divided into 39, 28, and 47 PFGE types by means of *Sma*I, *Sal*I, and *Kpn*I, respectively. A number of strains could be identified as genetically identical by two enzymes, but complete agreement among all three enzymes was seen for only eight strains of serotypes HS:1.44; HS:6.7; HS:19; and HS:41. The need of multiple restriction enzymes to determine genetically identical *Campylobacter* strains is in line with other studies (On et al., 1998; Lehner et al., 2000; Hänninen et al., 2001; Ono et al., 2003; Lindmark et al., 2004). On et al. (1998) studied the validity of *Sma*I genotypes of 34 *C. jejuni* isolates by *Sal*I, *Kpn*I, and *Bam*HI polymorphisms and recommended that strains with identical *Sma*I profiles are subjected to further analysis with additional enzymes such as *Kpn*I that demonstrate high supplementary discriminatory potential.

In Nielsen et al. (V), strains of serotype HS:1.44 and HS:2 were found to be more homologous than were strains of the HS:4 complex, i.e., within serotypes HS:1.44 and HS:2, several large clonal groups of isolates were identified with the genotypic methods, whereas none were found in the HS:4. In a study by Petersen et al. (2001) 95 combinations of sero- and genotypes (*fla* and PFGE types) were seen among 120 examined isolates of various serotypes. Finally, serotyping and RiboPrinting of 975 isolates, which were obtained from clinical cases, revealed 309 combinations of sero-ribotypes (Fussing et al., 2003).

In conclusion, *C. jejuni* serotypes may vary greatly in genetic diversity. While certain HS serotypes, such as HS:19 and HS:41, appear to be highly genetically homogeneous, it appears that there is no such clonality in other serotypes (V; VII; Gibson et al., 1995; Owen et al., 1995; Fujimoto et al., 1997; Endtz et al., 2000).

Nielsen et al. (VI) studied the stability of four typing methods. The sero- and genotypic stability of three *C. jejuni* strains were evaluated after sub-culturing 50 times in triplicate and after colonising mice for up to 26 days. The employed methods were serotyping, RiboPrinting, PFGE, and RAPD using the three primers 1254, 1281 and HLWL85. Diversity of isolates using RAPD is often evident when using one primer, but reliable similarity data may require the use of multiple primers. In this thesis, a large number of primers available from the literature were evaluated in a pilot study (data not shown) to obtain an acceptable pattern of fragments of variable size. The combination of primers 1254, 1281, and HLWL85 was found to cover a range of discriminatory indices for typing of *C. jejuni* and *C. coli*. HLWL85 and 1254 most often produced more informative patterns than did 1281, and these initial findings were re-affirmed in V.

No changes in any of the DNA profiles or in the reactions to heat-stable antigens were identified among these strains after the in vitro and in vivo passages. However, one isolate became untypeable (no profile could be produced) with RAPD after passage in one of the mice. The results indicate that the applied typing methods are reliable and applicable for typing of *Campylobacter* isolates from different sources over time, and that many *C. jejuni* strains are genetically stable as tested by these methods. This is supported by a Japanese study that tested the genotypic stability of eight *C. jejuni* strains before and after subculturing 50 times. Both RAPD (using 1 primer) and PFGE (using *Sac*II) genotypes of all strains remained stable during the in vitro passages (Ono et al., 2003). Laturnus et al. (2005), identified considerable degrees of genomic conservation and the occurrence of long-term O:2 serotype-associated clonal lineages in *C. jejuni* in different geographical regions and hosts. Moreover, Manning et al. (2001), have found evidence for longer-term genetic stability of a *C. jejuni* strain for almost 20 years, despite having been sub-cultured on many occasions in the laboratory. The genetic background of strain differences is beginning to emerge. de Boer et al. (2002), concluded that the clonality of distinct *Campylobacter* lineages is caused by a defect in the natural transformation machinery that is most likely located at the level of DNA uptake.

de Boer et al. (2002) also investigated the molecular basis of genetic diversity of *C. jejuni* and its influence of genetic variation on genotyping. Exchange of antibiotic resistance markers between two *C. jejuni* strains was examined both by co-cultivation in vitro and in vivo in the intestine of chicken. Bidirectional transfer of resistance genes was demonstrated and could be detected by PFGE, *flaA*-RFLP, AFLP and MLST.

With the wide range of phenotypic and genotypic typing systems used for epidemiological typing of *Campylobacter* spp., issues of comparability of patterns and interpretation of data become essential. In V, six methods (Penner serotyping, *fla*-DGGE, RiboPrinting, *fla*-RFLP, PFGE and RAPD) were used for subtyping a collection of 90 *C. jejuni* isolates from animal sources, sporadic human cases, and the waterborne outbreak in Klarup. The methods were evaluated and compared on the basis of their abilities to identify outbreak isolates and discriminate between unrelated isolates and the agreement between methods in identifying probable clones. The discriminatory power differed among the six marker systems with D indices in the range of 0.868 to 0.984. PFGE and RAPD were the most discriminatory methods followed by RiboPrinting and *fla*-RFLP. Serotyping and *fla*-DGGE typing were the least discriminatory methods. The findings are comparable with findings from other evaluation studies using the same methods for subtyping *Campylobacter* (Madden et al., 1996; de Boer et al., 2000; Ono et al., 2003).

In Nielsen et al. (V), all typing methods had a typability of 100%. The 11 isolates related to the waterborne outbreak were clearly identified by all six typing methods. The typing methods are thus sufficiently stable to correctly group isolates of clonal origin. Serotyping was the least discriminatory method, but the best primary method in the sense that the other methods could form the best hierarchic structure based on the serotyping, e.g., only one of the RAPD groups was subdivided by serotyping. *fla*-RFLP and RiboPrinting were not as discriminatory as PFGE and RAPD, but both methods grouped the isolates in generally good accordance with the other methods. However, several RiboGroups and *fla*-RFLP types were subdivided by all other methods, e.g., the 15 isolates of RiboGroup 23, the most common group, were of three different serotypes (HS:1.44, HS:2, and HS:4 complex) and eight different *fla*-RFLP types. In general, typing based on the conserved ribosomal genes is considered a stable typing method. This could be the reason why other typing methods further divide some RiboGroups, e.g., RiboGroup 23. Denaturing Gradient Gel Electrophoresis (DGGE) allows the discrimination of PCR-amplified DNA fragments of similar lengths but different sequences. Separation is based on the differential motility of partially denatured double-stranded DNA fragments in a gel with a gradient of denaturants. In V, most of the groups formed by DGGE of the *flaA* gene were subdivided by all other

methods, including the least discriminatory one, serotyping, and the method needs to be further developed and evaluated.

The more typing systems showing the same pattern, the better the predictability of relationships between isolates. The most discriminatory methods, PFGE and RAPD, showed some level of agreement in terms of strain differentiation and grouping, but for about 40% of the isolates, the two methods disagreed. Both methods subdivided groups formed by the other method. Although both methods detect whole-genome polymorphisms, the principles underlying each method are quite different and different genetic variations may be detected. When the grouping of isolates formed by at least four typing systems was used for evaluation of concordance of methods, the highly discriminatory PFGE most often disagreed with the other methods.

Many other *Campylobacter* species than *C. jejuni* and *C. coli* exhibit genetic diversity; these species include *C. lari*, *C. upsaliensis*, *C. helveticus* and *C. concisus* (VIII; Duim et al., 2001; Aabenhus et al., 2002; Aabenhus et al., 2003; Duim et al., 2004). Engberg et al. (VIII) compared phenotypic and genotypic characteristics of 39 *C. concisus* isolates from Danish patients with diarrhoea, three strains from healthy individuals and the type strain, and found a large degree of variability among the strains. Protein profile analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and PCR amplification of 23S rDNA assigned the strains into two distinct, but disconcordant groups. PCR amplification of 23S rDNA was performed by the method described by Bastyns et al. (1995) with modifications (Istivan et al., 2004). The method was modified by using the two reverse primers independently rather than as a mixture, and was used to group the isolates. Analysis of combined RAPD DNA profiles based on each of the three primers identified 37 unique reproducible profiles. Interestingly, an almost complete agreement between the RAPD method (PCR amplification of amplicons throughout the genome) and PCR amplification of 23S ribosomal genes was noted, with only two strains (CCUG 13144 and strain 74321) branching in the "wrong" clusters. However, as the two typing methods have different targets of amplification, complete concordance between the two methods cannot be expected. In fact, a similar overall good, but incomplete agreement between the PCR typing method and the RiboPrinting method was also found. Although both methods are based on conserved ribosomal genes, genetic changes in the *Pvu*II restriction enzyme digesting sites (RiboPrinting) or annealing sites (PCR typing method) may explain the divergent clustering of some strains. Although the sequences of the two reverse primers used for the PCR typing were quite different, a very weak binding with the "wrong" reverse primer was sometimes noticed for some *C. concisus* strains. This weak reaction at low annealing temperatures has also been experienced by others using the same PCR assay for typing *C. concisus* (*Istivan*, personal communication). Engberg et al. (VIII), applied an annealing temperature of 60°C, and the weak bands could possibly have been eliminated by using higher annealing temperatures (60-65°C), but it illustrates a potential weakness of the specificity of the method: one or more mutations in the 23S rRNA gene locus for the CON1 (or CON2) could theoretically lead to annealing and amplification with the "wrong" reverse primer resulting in a shift of PCR type. In conclusion, although reproducible profiles were obtained, minor genetic changes may explain the disagreement between typing methods for some strains.

Six strains were not tested with the RAPD method due to sudden unexplained loss of reproducibility of the method. In this study, we examined the strains within a short time frame and applied a PCR set-up with a commercially available RAPD analysis kit to ensure reproducibility. However, even with this set-up and unchanged PCR running conditions, thermocycler, fragment separation apparatus and with the same technicians to reduce the number of susceptible steps and factors hereby increasing reproducibility, the profiles suddenly changed for primers HLWL85 and 1254 and the pending six strains could not be tested with this method. This stress the importance of including control strains in each batch, and confirm that RAPD is a method that is comparative by nature.

The identified diversity is in line with other typing studies of *C. concisus*. Van Etterijck et al. (1996) found 49 unique RAPD fingerprints among 51 clinical strains. Matsheka et al. (2002) identified 51/53 strains to have unique PFGE patterns using a single restriction enzyme (*Not*I).

The intestinal pathogenicity of *C. concisus*, or of a subgroup of this species, remains to be proven. The study by the author and colleagues did not find any clear phenotypic or genotypic differences between strains from patients with diarrhoea and from healthy carriers. However, as only a limited number of strains from healthy carriers were available, it is not possible to draw firm conclusions about phenotypic or genotypic differences between the two groups of individuals. Future studies should include additional strains from diverse sources including from healthy individuals, and preferably from a case-control study in order to include analytic epidemiologic data.

4.1.4. Limitations of typing to study the epidemiology of Campylobacter

Epidemiological typing of *Campylobacter* is hampered by the plasticity of the genome. Phenotypic and genotypic diversity of *Campylobacter* has been known for a number of years and has been shown by serotyping, ribotyping, PFGE, and several PCR-based techniques including RAPD and *fla*-RFLP. Moreover, diversity within *C. jejuni* has also been observed for characteristics implicated in pathogenecity, such as GBS (Nachamkin et al., 2001), toxin production (Bang et al., 2003), sialylation of LOS (Linton et al., 2000), and ability to colonize chickens (Korolik et al., 1998). Unfortunately, these methods are unable to further characterize the genetic basis for this observed variability. In general, genetic diversity originates from horizontal gene transfer (natural transformation, conjugative DNA transfer and transposable elements) and mutations, and genomic rearrangements. Phenotypic diversity may also due to polymorphisms within homonucleotide stretches throughout the genome that may rapidly alter the phenotype of the organism through variation in gene expression or posttranslational modification (Manning et al., 2003). At present, horizontal gene transfer is recognised as a major cause of diversity. Multilocus sequence typing has in recent years been valuable to study the genetic diversity of *C. jejuni* and *C. coli* at the sequence level. MLST determines the partial nucleotide sequence of a small number of housekeeping genes that are expected to be subject to neutral spontaneous sequence variation and minimal recombination. Based on the number, type and position of observed mutations, it is possible to estimate the evolution of genes and relationships of strains and to distinguish strains that only differ in a few point mutations. MLST is similar to MLEE in that it measures variation in housekeeping genes located around the genome, but MLST has the advantage that the variation is determined at level of DNA sequence, thus making the technique both highly reproducible and portable (Manning et al., 2003). MLST of *C. jejuni* has shown that the overall population structure is largely non-clonal with some clonal lineages. This means that there is evidence of frequent recombination within a clonal framework (Dingle et al., 2001; Suerbaum et al., 2001; Dingle et al., 2002).

Theoretically any change in the DNA may influence the result of genotyping. Point mutations can affect the outcome of all genotyping methods making use of restriction enzymes, due to the introduction or deletion of restriction site. Furthermore, PCR based methods can be affected when point mutations occur in the primer binding sites (de Boer, 2002). However, the influence of point mutations on the generation of genetic diversity seems to be limited compared to recombination events. Schouls et al. (2003) estimated the role of recombination in sequence variation is 50 times greater than that of mutation. The potential effects of genetic recombination on geno-

typing depend on the method employed. DNA microarray data have revealed both divergent and highly conserved gene classes. The majority of the divergent genes are related to surface modifications (LOS, capsule, and flagellar loci) (Dorrell et al., 2001; Leonard et al., 2003). When a method targets just one or two loci, as has become evident in *fla*-RFLP, any recombination affecting these loci will have effect on the results (Harrington et al., 1997; de Boer, 2002). Recently, Dingle et al. (2005) used a combination of MLST and sequencing of the short variable region (SVR) of the *flaA* flagellin gene and found that *C. coli* and *C. jejuni* share approximately 86.5% identity at the nucleotide sequence level within the MLST loci. There was evidence of genetic exchange of the housekeeping genes between the two species, but at a very low rate; only one sequence type from each species showed evidence of imported DNA. The *flaA* gene was more variable and has been exchanged many times between the two species.

Genetic alterations can also have significant effect on the result of PFGE. Insertion or deletion of DNA sequences or rearrangements, which alter band sizes and do not directly involve the restriction sites targeted in PFGE, may result in different PFGE profiles (Wassenaar et al., 1998; Wassenaar et al., 2000; Steinbrueckner et al., 2001; de Boer, 2002).

Another potential limitation of typing in epidemiological investigations is the possibility of co-infection with multiple *Campylobacter* strains or species. In nearly all epidemiological studies, only a single or a few colonies from a *Campylobacter* isolate are typed, while it is known that the various reservoirs may carry multiple strains at the same time. There are conflicting results in literature of whether this is an epidemiological problem.

In a study of the effect of incubation temperature on isolation of *C. jejuni* genotypes from foodstuffs (poultry and lamb) enriched in Preston Broth, it was shown that the incubation temperature had no significant effect on the number of positive samples or on the species isolated (Scates et al., 2003). However, genotyping of the *C. jejuni* isolates (two colonies per treatment were characterized) revealed profound differences in the types obtained. The use of a single incubation temperature, 37°C, gave 56% of the total number of RAPD *C. jejuni* genotypes, and, hence, 44% remained undetected. The paper suggested that to detect the widest range, food samples should be incubated at both 37 and 42°C. Richardson et al. (2001) investigated 10 single *Campylobacter* colonies cultured from each of 53 positive human faecal samples. The majority of patients were infected with a single strain of *Campylobacter*, but from each of four samples, 7.5%, two strains of *C. jejuni*, were identified, as confirmed by molecular typing. In contrast, Steinbrueckner et al. (2001) determined the rate of human intestinal infections with more than a single *Campylobacter* strain and the genetic variabilities of *Campylobacter* strains throughout an infection episode. The applied typing methods in this study were PFGE and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR). For 48 and 49 of 50 patients, all isolates from one sample showed identical patterns by PFGE and ERIC-PCR, respectively. Throughout an infection episode in 47 of 52 patients, the PFGE fingerprints of the isolates remained stable, while in one patient two different species were observed and in four patients different patterns were observed. The authors concluded that human infection with more than one *Campylobacter* strain is rare and should not significantly impair epidemiologic analyses. However, changes in the genetic fingerprint throughout an infection should be considered in the assessment of epidemiologic studies of *Campylobacter* spp.

Taken together, the above-mentioned studies show that co-infection, although relatively rare, may occur in sporadic cases of campylobacteriosis and may have impact on epidemiological analyses, as a lack of capture of the full diversity of strains present may underestimate epidemiologic relations as mentioned in VI.

4.1.5. Applications

Conventional phenotypic methods have been and still are of great importance in studies of bacterial epidemiology including for risk assessment purposes, e.g. serotyping and phagetyping of *Salmonella*, and phagetyping of *Staphylococcus aureus*. For *Campylobacter* spp., as already discussed, a number of phenotypic and genotypic methods are at present needed to investigate their epidemiology.

Most molecular typing performed in public health is to determine if a bacterial isolate is part of an outbreak. In this case, the phylogenetic relatedness of one isolate to another is virtually irrelevant. The investigator is asking a seemingly simple question: "Did these two people get their infection from the same source?" Unfortunately, molecular typing alone cannot answer that question. As reviewed by Barrett et al. (2004), a number of questions should be considered in the interpretation of molecular typing data including:

1. Which method was used? (Is it discriminating for this organism?).
2. How much genetic diversity does this organism demonstrate? (Similarities in typing result are more important as diversity increases).
3. How common is the particular molecular type observed? (The importance of matching types decreases with frequency of occurrence).
4. What is the nature of the outbreak being investigated? (Ongoing outbreaks with continued transmission are more likely to show genetic diversity than are point-source outbreaks).
5. What is the big picture? (How do molecular typing data fit with epidemiologic and environmental investigations?).

These questions are indeed relevant for typing of *Campylobacter* and should therefore be considered when choosing a typing method for a defined purpose and when evaluating typing studies of this genus such as I & V-VIII.

In Nielsen et al. (V) it was concluded that the typing methods can be recommended for different uses. Penner serotyping proved to be useful for typing of large numbers of isolates to obtain a rough grouping of isolates and comparing the type distribution in other sources, other time periods, other countries and regions. *FlaA*-RFLP and RiboPrinting are both fairly discriminative and can be used for screening high numbers of isolates. However, the inherent potential for instability in *fla* typing probably makes this method unsuitable for global or long-term time-related epidemiological studies (Wassenaar et al., 2000).

PFGE and RAPD are highly discriminatory methods and these methods are therefore useful for ensuring genotypic similarity in cases of outbreaks, investigations of possible secondary spread of infection within institutions or family clusters and treatment failure by comparing pre- and post-treatment isolates like in IV and in Llovo et al., 2003. Also when the question is whether successive chicken flocks are colonised by the same *C. jejuni* strain or by different strains, a highly discriminatory method, or even better, a combination of methods should be applied. This was illustrated in a study of in vivo recombination of *C. jejuni* by de Boer (2002). In these experiments, extensive PFGE analysis indicated changes in bacterial genotype, while AFLP, *flaA*-RFLP and MLST suggested stable genotype for the same specimen.

As discussed in chapter 1, *Campylobacter* infections are characterized as either sporadic cases or outbreaks. However, *Campylobacter* infections are also characterized by a high number of infections acquired by cross-contamination at the different levels from "stable to table", where it may be difficult to recognise that the persons who fell ill shared a common food source. The rigorous distinction between "sporadic cases" and outbreaks, with the assumption that patients who have not been associated with known outbreaks are sporadic cases, may therefore have limitations to understand the complex epidemiology of campylobacteriosis, to determine the burden of disease caused by *Campylobacter* and to guide on prevention. An alternative approach to achieve more information on campylobacte-

riosis has been launched in at least Denmark, the UK and Canada (Sopwith et al., 2003; Fussing et al., 2003; Michaud et al., 2005). The approach is based on the linkage of typing data with epidemiological data.

To monitor the distribution of subtypes of *Campylobacter* and to identify clusters, 975 isolates of *Campylobacter* spp. was obtained from two Danish counties during a one-year period (Fussing et al., 2003). The evaluation was based on timely serotyping and RiboPrinting, and confirmation of selected clusters by PFGE profiling. Two cluster criteria were defined according to either a rare or frequent presence of combined serotype and ribotype subtypes. Using these cluster criteria, a total of 43 clusters of subtypes were found during the study period, representing 29% (273) of the isolates. The observed clustering of *Campylobacter* isolates with identical type in time and place may indicate that common source outbreaks of campylobacteriosis are more common than usually recognized. This is in agreement with Swedish results; that although a large number of genotypes may be found among *C. jejuni* strains infecting humans, many may be genetically related (Hook et al., 2004).

Subsequently, the subtypes of *Campylobacter* isolates from human infections in the two Danish counties were compared to isolates from retail food samples and faecal samples from chickens, pigs and cattle (Nielsen et al., 2003). During the one year period, a total of 1285 *Campylobacter* isolates from these sources were typed and a large subtype overlap was found between human isolates and isolates from food (66%), chickens (59%) and cattle (83%). This was further substantiated by additional PFGE typing of selected strains. All frequent (n>3) subtypes found in food were also isolated from humans and 61% of the domestically acquired human isolates had a subtype that was also found in food, whereas this was only the case for 31% of the patients who had traveled abroad within the last week before disease onset. The diversity of these travel-associated isolates was considerably higher than isolates from domestic cases. The results show that most *C. jejuni* subtypes found in poultry food samples, broiler chickens, and cattle were represented in the domestically acquired human cases, indicating that *C. jejuni* from these reservoirs are likely/important sources of human infections in Denmark (Nielsen et al., 2003). In a study from Finland, 34% of the sporadic *C. jejuni* infections during the seasonal peak in 1999 were caused by indistinguishable sero- and genotype combinations found in chicken flocks at slaughter, suggesting that chickens may be a source of human infections, either directly or by increasing the environmental load of *C. jejuni*. However, human strains with overlapping sero- and genotype combinations with a chicken strain were also isolated prior to the slaughter of the chicken flock, suggesting common environmental sources for both human infection and flock contamination during the seasonal peak (Kärenlampi et al., 2003). Finally, in a Austrian report of a multi-state *C. jejuni* outbreak caused by chicken showed that without the proper epidemiological investigation, the cases would have been registered as "sporadic" by the local health authorities (Allerberger et al., 2003). This report is also remarkable, as the investigational team succeeded in performing trace-back to the incriminated chicken farm, finding outbreak isolates genetically indistinguishable by *Sma*I PFGE patterns from meat isolates obtained from slaughtered animals from the farm.

A major limitation of all present typing methods is that none of them are suited for attribution analysis of *Campylobacter* cases. The reason is the lack of host-specificity and the weak clonal structure of *Campylobacter*. At present, the use of layered multiple method strategy to suit application seems to be the approach when typing is used to study the epidemiology of *Campylobacter* spp.

4.1.6. Conclusions and future directions

What have we learned about *C. jejuni*, *C. coli* and *C. concisus* epidemiology from typing studies? Typing methods should be selected according to the epidemiological problem addressed, e.g. local outbreak assessment or long-term surveillance. Typing data are best interpreted in the complete context of epidemiologic, environmental, and laboratory investigations. The genetic diversity of *Campylobacter* together with the occurrence and distribution of common types makes it difficult to establish universally applicable definitive interpretive criteria for molecular subtyping methods. In the absence of epidemiologic information, molecular typing is best used to identify cases that are most likely linked (detect possible outbreaks, such as in Fussing et al. (2003)), and to help separate an outbreak from sporadic cases (facilitate outbreak investigations, such as in I) rather than drawing independent conclusions regarding strain relatedness (risk assessment of sources). Genetic instability may be a problem, in particular when only one genotyping method is applied. Because MLST produces defined sequence data, it is possible to develop a large database accessible from all over the world, in which MLST results may be directly compared. This makes MLST a promising typing method for *Campylobacter* spp. For the non-sequence-based methods, the development of standardized typing methods will facilitate international comparison, and institutions should be encouraged to co-operate on their development. In particular, there is a critical need for a typing method that relates *Campylobacter* types to pathogenic potential.

CHAPTER 5. SUMMARY

In this chapter, the major results and conclusions from each objective listed in chapter 1 will be presented.

1. Conducting an outbreak investigation in order to verify the cause and determine the burden of illness associated with the outbreak

Engberg et al. (I) investigated the first registered waterborne outbreak with *C. jejuni* in Denmark in 1995-96 in the town of Klarup. It occurred as a result of an unintended leak of sewage into the groundwater reservoir. Epidemiological data were provided by a retrospective follow-up study for culture confirmed cases; for residents without a bacteriological diagnosis a telephone-based survey was conducted. The cause was confirmed by the finding of indistinguishable *C. jejuni* isolates in the drinking water, and in the clinical samples by multiple typing methods. The epidemiological investigation estimated that some 2400 people out of a population of 3925 (61%) were affected by the outbreak. A likely dose-response relationship between amounts of drinking water and risk of illness was demonstrated, with less severe disease among patients from the southern area than among patients from the heavily exposed northern area of the town.

The author caught an interest in *Campylobacter* as a result of this outbreak, subsequently leading to the work performed in this thesis.

2. Optimisation of culture-based diagnostic methods for Campylobacter and related organisms, including addressing the importance of non-jejuni/coli Campylobacter spp. in diarrhoeal infections

Re-evaluation of three selective media, (mCCDA, Skirrow medium, and CAT medium) and the filtration method for the efficacies to isolate *Campylobacter* spp. with well appreciated disease potential and estimation of the prevalence of new and emerging campylobacterial pathogens pointed out several important issues (II). First, mCCDA proved to be the most effective selective medium for the isolation of *C. jejuni* and *C. coli*. Second, another six taxa could be isolated, mostly after an extended incubation period of 5-6 days, but with the filtration method as the essential method. Surprisingly, *C. upsaliensis* were not recovered in this study. Third, a polyphasic approach in order to identify all of the eight isolated taxa was needed: conventional phenotypic tests, *C. concisus* species-specific PCR, extended phenotypic characterization, whole-cell protein profiling and 16S rRNA gene sequence analysis. Fourth, the study provided evidence for the existence of *Sutterella wadsworthensis* in human faeces from clinical cases of gastrointestinal disorders and in faeces from a healthy individual. Fifth, *C. concisus* was isolated from a large

number of diarrhoeal cases, particularly from those at the extremes of age, but was additionally isolated from the faeces of healthy people at a similar rate. Further investigations are needed to establish the role of a number of the emerging campylobacteria, including *C. concisus* and *S. wadsworthensis* in enteric disease.

3. To compare different tests for antibiotic susceptibility testing of thermophilic Campylobacter spp., necessary to secure comparability of the data from the different participants in the Danish integrated antimicrobial resistance monitoring and research programme (DANMAP)

MIC-determinations are normally considered the golden standard for susceptibility testing. However, a variety of different methods including diffusion tests were routinely used in Danish laboratories involved in DANMAP (III). The study included four antimicrobial agents: nalidixic acid, erythromycin, streptomycin and tetracycline. Epidemiological cut-off values were made by comparison of the distribution of the population of MICs for the dilution methods and zones of inhibition for the diffusion methods. The study showed complete agreement between the three MIC-methods to separate isolates into a susceptible and a resistant population when tested for all four antimicrobial agents. However, for nalidixic acid, two isolates were resistant according to MIC-methods (MIC ranges 32 to 64 μg/ml), but had zones of inhibition between 26 and 31 mm with the two tablet diffusion methods. The interpretive criteria were suggested to be considered tentative because of the low level of resistance to some antibiotics among a moderate number of isolates at study. By histogram analyses of susceptibility populations of additional isolates in the subsequent year in the DANMAP surveillance programme, the cut-off values were subject to minor adjustments: for the tablet method D, *C. jejuni* and *C. coli* isolates are considered susceptible to nalidixic acid when zones of inhibition are larger than or equal 27 mm; for the E-test, MICs larger than or equal to 64 μl/mL are considered resistant, whereas strains with MIC less than or equal to 32 μl/mL are considered in vitro susceptible. For erythromycin, the same cut-off values as for nalidixic acid are now applied. For nalidixic acid and erythromycin, the E-test tended to produce lower values compared to the two agar dilution methods. The discrepancies in MICs were clearly separated from the cut-off values and did not cause problems in aspect to interpretation of susceptibility of the individual agent or to investigation of the agreement between methods to separate isolates in susceptible and resistant groups, which was the overall purpose of the study.

4. To study the antimicrobial susceptibility of Campylobacter isolated from humans including prevalence of macrolide and quinolone resistance, as well as sources and risk factors for quinolone-resistant C. jejuni infections

Quinolone resistance was found to be significantly associated with the origin of infection: 76 (50.0%) of 152 infections among travelers returning to Denmark were quinolone-resistant whereas 52 (9.9%) of 526 domestically infected patients were infected with a quinolone-resistant strain ($p < 0.001$). Only 3 (0.3%) isolates (all *C. coli*) were erythromycin-resistant (IV). Exposures independently associated with an increased risk for quinolone-resistant *C. jejuni* infection were foreign travel (OR = 16.81), eating fresh poultry other than chicken and turkey (OR = 19.10), and swimming in pools, oceans, lakes, or other places (OR = 5.01). Eating fresh chicken (of presumably Danish origin) was associated with a decreased risk (OR = 0.04). Typing data showed an association between strains from retail food products and broilers and quinolone-sensitive domestically acquired *C. jejuni* infections. An association between treatment with a fluoroquinolone before stool-specimen collection and having a quinolone-resistant *Campylobacter* infection was not observed. *C. jejuni* infections and *C. coli* infections did not differ in severity, when assessed by frequency of diarrhoea, blood in stool, abdominal pain, fever, vomiting, mean duration of illness, or admission to hospitals. However, the mean duration of illness was longer for the 86 patients with quinolone-resistant *C. jejuni* infections and a known duration of illness (median 13.2 days) than for the 381 patients with quinolone-sensitive *C. jejuni* infections and a known duration of illness (median 10.3 days, $p = 0.001$).

Engberg et al. (VIII), tested 43 *C. concisus* strains and found all the strains to be susceptible to 11 antimicrobial agents, including erythromycin.

5. Implementation and evaluation of molecular typing methods for the purposes of molecular epidemiological typing of Campylobacter infections, including outbreak investigations and determination of disease associations and manifestations

With the wide range of phenotypic and genotypic typing systems used for epidemiological typing of *Campylobacter* spp., issues of comparability of patterns and interpretation of data become essential. In Nielsen et al. (V) six methods (Penner serotyping, *fla*-DGGE, RiboPrinting, *fla*-RFLP, PFGE and RAPD) were used for subtyping a collection of 90 *C. jejuni* isolates. The discriminatory power differed among the six-marker systems with D indices in the range of 0.868 to 0.984. PFGE and RAPD were the most discriminatory methods followed by RiboPrinting and *fla*-RFLP. Serotyping and *fla*-DGGE typing were the least discriminatory methods. Penner serotyping proved to be useful for typing large numbers of isolates to obtain a rough grouping of isolates and comparing the type distribution in other sources, other time periods, other countries and regions. *FlaA*-RFLP and RiboPrinting are both fairly discriminative and can be used for screening high numbers of isolates. However, the inherent potential for instability in *fla* typing probably makes this method unsuitable for global or long-term time-related epidemiological studies (Wassenaar et al., 2000). PFGE and RAPD are highly discriminatory methods, and these methods are therefore useful for ensuring genotypic similarity in cases of outbreaks, investigations of possible secondary spread of infection within institutions or family clusters and treatment failure by comparing pre- and post-treatment isolates like in IV. In Engberg et al. (VII), we examined whether *C. jejuni* non-HS:19 serotypes associated with GBS have a clonal structure and differ from strains isolated from patients with *Campylobacter* gastroenteritis. All typing methods (Penner serotyping, MLEE, RiboPrinting, PFGE with *Sma*I, *Sal*I, and *Kpn*I restriction enzymes and *flaA*-RFLP) showed a high diversity in the studied population of strains. However, group definition of strains varied considerably between methods. The 11 GBS-related strains of different serotypes did not represent separate genetic lineages distinct from enteritis-associated strains or strains from animals. A specific epidemiological marker associated with GBS strains only could, therefore, not be identified.

Epidemiological typing of *Campylobacter* is hampered by the plasticity of the genome. In Nielsen et al. (VI) the sero- and genotypic stability of three *C. jejuni* strains were evaluated after sub-culturing 50 times in triplicate and after colonising mice for up to 26 days. No changes in any of the DNA profiles or in the reactions to heat-stable antigens were identified among these strains after the in vitro and in vivo passages. However, one isolate became untypeable (no profile could be produced) with RAPD after passage in one of the mice. The results indicate that the applied typing methods are reliable and applicable for typing of *Campylobacter* isolates from different sources over time, and that many *C. jejuni* strains are genetically stable as tested by these methods. However, phenotypic and genotypic diversity of *Campylobacter* has been known for a number of years and has been shown by serotyping, ribotyping, PFGE, and several PCR-based techniques including RAPD and *fla*-RFLP. Genetic diversity originates from horizontal gene transfer, mutations, and genomic re-arrangements (Manning et al., 2003). At present, horizontal gene transfer is recognised as a major cause of diversity. When a method targets just one or two loci, as has become evident in *fla*-RFLP, any recombination affecting these loci will have effect on the results. Bidirectional transfer of resistance genes has been

demonstrated in *C. jejuni* strains and could be detected by PFGE, *flaA*-RFLP, AFLP and MLST (de Boer, 2002).

Engberg et al. (VIII) compared phenotypic and genotypic characteristics of 39 *C. concisus* isolates from Danish patients with diarrhoea, three strains from healthy individuals and the type strain, and found a large degree of variability among the strains.

In conclusion, typing methods for typing *Campylobacter* should be selected according to the epidemiological problem addressed, e.g. local outbreak assessment or long-term surveillance. Typing data are best interpreted in the complete context of epidemiologic, environmental, and laboratory investigations. The genetic diversity of *Campylobacter* together with the occurrence and distribution of common types makes it difficult to establish universally applicable definitive interpretive criteria for molecular subtyping methods. In the absence of epidemiologic information, molecular typing is best used to identify cases that are most likely linked (detect possible outbreaks, such as in Fussing et al. (2003)), and to help separate an outbreak from sporadic cases (facilitate outbreak investigations, such as in I) rather than drawing independent conclusions regarding strain relatedness (risk assessment of sources)). It is a major limitation of all present typing methods that none of them are suited for attribution analysis of *Campylobacter* cases. The reason is the lack of host-specificity and the weak clonal structure of *Campylobacter*. At present, the use of layered multiple method strategy to suit application seems to be the approach when typing is used to study the epidemiology of *Campylobacter* spp. The more typing systems showing the same pattern, the better the predictability of relationships between isolates.

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