MOLECULAR TYPING OF *CAMPYLOBACTER JEJUNI*

PhD thesis

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„ If it was the responsibility of laboratory scientists to determine
if *Campylobacter* spp. could become a foodborne pathogen,
it would be deemed the least likely candidate.
However, not for the first time bacteria
have proved the scientists wrong..."
(*Murphy et al., 2006*)

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CONFERENCE PRESENTATIONS

LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AIDP	acute inflammatory demyelinating polyradiculoneuropathy
AMAN	acute motor axonal neuropathy
AMSAN	acute motor and sensory axonal neuropathy
CDT	cytolethal distending toxin
DLV	double locus variant
DNA	deoxyribonucleic acid
GBS	Guillain-Barré syndrome
HL	heat-labile
HS	heat-stable
IL-8	interleukin 8
IS	insertion sequence
Kdo	keto-deoxyoctulonosic acid
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MAMA	mismatch amplification mutation assay
MLEE	multi locus enzyme electrophoresis
MLST	multi locus sequence typing
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
ND	not determined
NF-κB	nuclear factor κB
NJ	neighbour joining
NT	non typable
OS	oligosaccharide
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
QRDR	quinolone resistance determinant region
RFLP	restriction fragment length polymorphism
SLV	single locus variant
SVR	short variable region
UPGMA	unweighted pair group method with arithmetic mean
V	variable

PUBLICATIONS INCLUDED IN THE THESIS

This thesis is based on the experimental studies reported in the following papers being referred to in the text by their Roman numerals:

I. Suerbaum S, Lohrengel M, **Sonnevend A**, Ruberg F, Beuerle B, Kist M: Allelic diversity and recombination in *Campylobacter jejuni*. 2001. *J. Bacteriology* **183:**2553-2559 (**IF: 3,984**)

II. **Sonnevend A**, Pál T: Heterogeneity of non-serotypable *Campylobacter jejuni* isolates 2006 *Acta Microbiol. Immunol. Hung.* **53**:171-181

III. **Sonnevend A**, Rotimi VO, Kolodziejek J, Usmani A, Nowotny N, Pál T : High level of ciprofloxacin resistance and its molecular background among *Campylobacter jejuni* strains isolated in the United Arab Emirates. 2006 *J. Med. Microbiol.* **55**:1533-1538. (**IF: 2,18**)

NOTE:

A. *The format of this Thesis follows the requirements described under 19§(1)(b) of the PTE ÁOK Doktori Szabályzat, 2008.*

B. *Of the studies described in paper I. only those parts of the work are included and discussed in this Thesis I was directly and extensively involved with (i.e. development of an MLST system, sequence diversity and recombination frequency analysis). Results mostly generated by other members of the team (i.e. phylogenetic interpretations and linkage disequilibrium analysis) are left out intentionally.*

1. INTRODUCTION

1.1. MICROBIOLOGY OF THE GENUS *CAMPYLOBACTER*

Campylobacter cells are small, curved, Gram-negative, flagellated rods exhibiting rapid darting motion under the microscope. Campylobacters are strictly microaerophilic, requiring 5-10% ambient oxygen and do not grow in air. The genus *Campylobacter* includes 17 species. They can be distinguished by their optimal growth temperature and by the biochemical characteristics listed in **Table 1.** according to *Fitzgerald and Nachamkin, 2007* (next page). The species most frequently isolated from human diseases are *C. jejuni*, *C. coli* and *C. fetus*, with the first being way the most common one encountered in human specimens. Campylobacteriosis is commonly caused by the so called "thermophilic" campylobacters: *C. jejuni* and *C. coli*. Some laboratories do not routinely distinguish between these organisms, but when it is done the number of infections caused by *C. jejuni* exceeds the one's caused by *C. coli* (*Lin et al., 1998; Popović-Uroić, 1989*).

C. jejuni is the only species in the genus which hydrolyses hippuric acid, although some of the strains may phenotypically be negative. Consequently, when speciating *C. jejuni,* it is recommended to look for the hippuricase gene rather than to observe the phenotypic characteristics, only.

Table 1. Biochemical characteristics of different *Campylobacter* species

	Catalase	H_2 required	Urease	H_2S (TSI)	Hyppurate hydrolysis	Indoxyl acetate hydrolysis	Aryl sulfatase	Selenite reduction	Growth in 1% glycine
C. jejuni subsp. *jejuni*	+	-	-	-	+	+	V	V	+
C.jejuni subsp. *doylei*	V	-	-	-	V	+	-	-	+
C.coli	+	-	-	V	-	+	-	+	+
C.fetus subsp.*fetus*	+	-	-	-	-	-	-	V	+
C.fetus subsp. *venerealis*	V	-	-	-	-	-	-	V	V
C.lari	+	-	V	-	-	-	-	V	+
C.upsaliensis	-	-	-	-	-	+	-	+	+
C.hyointestinalis subsp. *hyointestinalis*	+	V	-	+	-	-	-	+	+
C.hyointestinalis subsp. *lawsonii*	+	V	+	+[a]	-	-	-	+	V
C.lanienae	+	-	-	-	-	-	ND	+	V
C. sputorum bv.sputorum	-	+	-	+[a]	-	-	+	V	+
C. sputorum bv. faecalis	+	+	-	+	-	-	+	V	+
C. sputorum bv. paraureolyticus	-	+	+	+	-	-	+	V	+
C. helveticus	-	-	-	-	-	+	ND	-	V
C. hominis	-	+[b]	ND	-	-	-	ND	-	+
C. mucosalis	-	+	-	+	-	-	-	V	V
C. concisus	-	+	-	V	-	-	+	V	V
C. curvus	-	+	-	V	V	V	+	-	+
C. rectus	V	+	-	-	-	+	+	-	+
C. showae	+	+	-	-	-	V	+	-	V
C. gracilis[b]	V	ND	-	-	-	V	ND	-	+

[a] Normally produces large amount of H_2S in triple sugar iron (TSI) agar [b] Anaerobic growth only.

1.2 THE HISTORY OF CAMPYLOBACTERS

A member of the genus *Campylobacter* as a causative agent of diarrhoea was most likely first recognised by *Theodore Escherich* at the end of the ninetieth century. He described spiral bacteria in the colon content of children who had died of what he called 'cholera infantum' (*cit. in Butzler, 2004*). From the beginning of the twentieth century these "vibrio-like bacteria" were considered by many as veterinary pathogens although occasionally systemic infections in humans had been reported, as well. In 1963, based on the DNA content, the organism was renamed by *Sebald* and *Veron* as *Campylobacter*, *i.e.* the Greek expression for „curved rod" (*cit. in Butzler, 2004*) (**Figure 1.**).

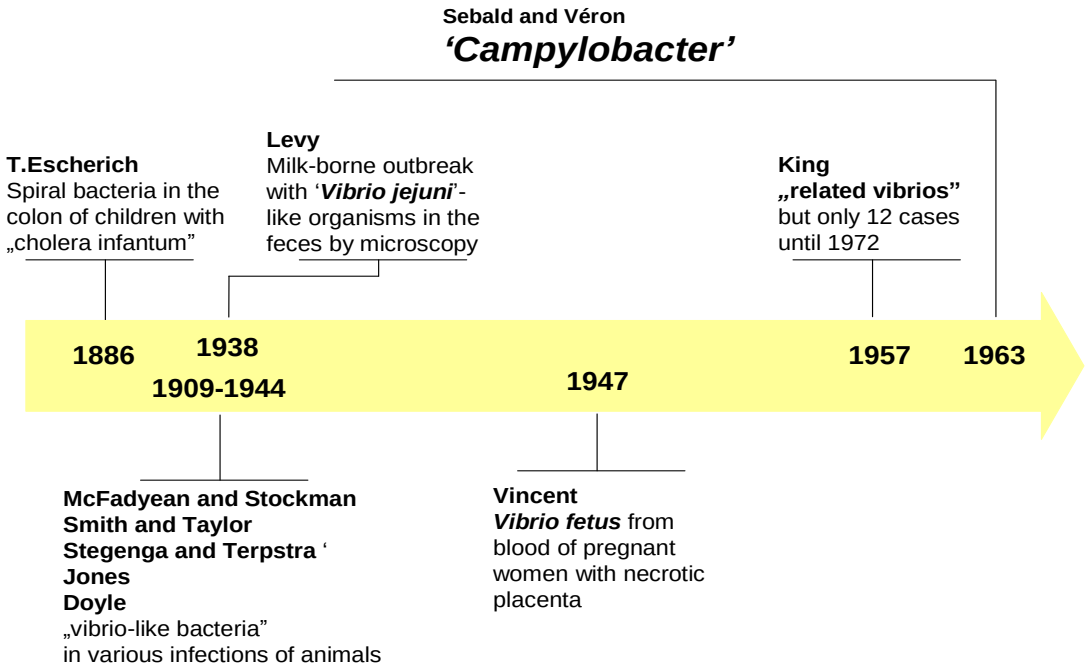


Figure 1. History of *Campylobacter* from its first description to being renamed

Almost 90 years after *Escherich*'s findings *Dekeyser*, *Butzler* and coworkers successfully isolated *C. jejuni* from specimens of a 20-year-old female patient suffering of severe diarrhoea and fever (40°C). The pathogen was recovered from blood and also from feces after differential filtration through 0.65-µm filters. This was the first faecal culture demonstrating an intestinal campylobacter infection as the origin of the bacteraemia (*Dekeyser et al., 1972*) (**Figure 2.** – next page).

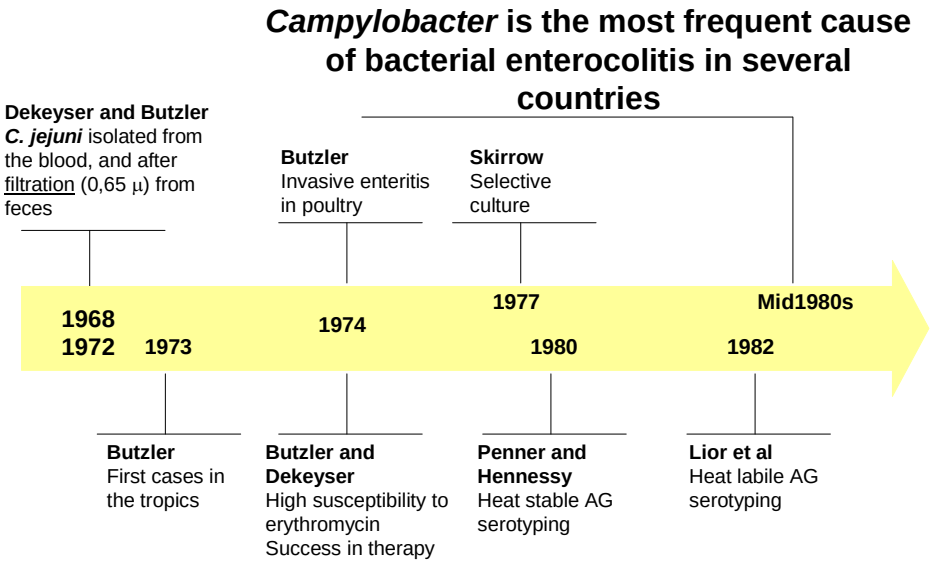


Figure 2. History of *Campylobacter* enteric infections

In 1977, *Skirrow* introduced a simple method for culturing campylobacter from stool specimens (*Skirrow, 1977*). This method, and the subsequent development of selective media allowed the pathogen's relatively easy isolation in diagnostic microbiology laboratories. Nowadays, *Campylobacter* is routinely sought for in stool samples of enteritis patients by selective culture on antibiotic containing agar at elevated temperature (42^{o}C), under microaerophilic conditions. While these techniques are clearly contributing to the increased rates of isolation of the pathogen, points have also been raised to be "over-selective" when using these approaches (*Silley, 2003*). Nevertheless, the pathogen reached the position of being one of the most frequent causative agents of human bacterial gastroenteritis in the developed world.

1.3 EPIDEMIOLOGY OF *CAMPYLOBACTER* INFECTIONS

Campylobacteriosis is a zoonotic and mainly food-borne disease. The source of infections is most frequently poultry, but other animals have been implicated as reservoirs, as well. The list of common reservoirs of the different *Campylobacte*r species is provided in **Table 2.** based on *Fitzgerald and Nachamkin, 2007*.

Interestingly, *C. jejuni*, a rather "demanding", fastidious species in the laboratory survives well in the environment (*Murphy, 2006*). Taking this into account together with its relatively low infective dose (approx. 1000 cells) (*Black, 1988*) it is surprising that most of the cases are sporadic, although outbreaks caused by contaminated food or water occur, as well (*Meusburger et al., 2007; CDC, 2007; Yoda et al., 2006*).

Table 2. Animal reservoirs of different *Campylobacter* species

Species	**Humans**	**Cattle**	**Sheep**	**Pigs**	**Wild birds**	**Poultry**	**Pets**	**Rodents**
C. jejuni subsp. *jejuni*	X	X			X	X	X	
C.jejuni subsp. *doylei*	X							
C.coli				X	X	X		
C.fetus subsp.*fetus*		X	X					
C.lari					X		X	
C.upsaliensis						X	X	
C.hyointestinalis		X		X		X	X	X
C. helveticus							X	
C. sputorum	X	X	X					
C. concisus	X							
C. curvus	X							
C. rectus	X							
C. showae	X							
C. gracilis	X							
C.mucosalis				X				

In the industrialised countries campylobacters are the most, or the second most frequently isolated bacterial agents of diarrhoea (**Table 3.,** Data from *Rautelin and Hänninen, 2000; Murphy et al., 2006).*

Table 3. Incidence of confirmed cases of campylobacteriosis in 2004

Region	Cases per 100,000 population
USA	12,9
United Kingdom	73
Northern Europe	60-90

There are estimates that, not unlike that in case of most enteric bacterial infections, the true incidence is 35-fold higher than the reported one (*Samuel et al., 2004*). In the developing countries where *C. jejuni* is affecting mainly children younger than 3 years of age campylobacter infections are hyperendemic due to the poor hygienic conditions and close contact with animals in the homes (*O'Ryan et al., 2005*). In Hungary campylobacteriosis has been a notifiable disease since 1998. The incidence has been steady with some drop in 2006 which can be attributed to the sudden change in the organization of enteric laboratories in the country (**Table 4.**) (Data obtained from National Centre for Epidemiology, Hungary).

Table 4. Human campylobacteriosis in Hungary

Year	Number of cases reported
1998	9222
1999	8968
2000	8664
2001	8775
2002	9234
2003	8274
2004	9086
2005	8293
2006	6829

1.4. PATHOGENESIS OF *CAMPYLOBACTER JEJUNI* INFECTIONS

The encounter of man with *Campylobacter jejuni* may result in a wide range of outcomes from a symptomless event through serious dysentery like symptoms to systemic infections. The outcome of the disease depends largely on the virulence of the particular infecting strain, as well as on the response of the host to infection.

1.4.1. VIRULENCE FACTORS

The invasive ability of certain campylobacters, as well as the capability of the bacterium to survive challenging environmental conditions have been extensively studied. *Campylobacter* cells do not multiply in the presence of air or under 30^{o}C temperature neither in food or water. Yet, these organisms are still one of the leading causes of bacterial gastroenteritis. They were shown to survive in water or at low temperatures for up to 4 months (*Rollins et al., 1986*). During processing of poultry meat (*Cools et al., 2005*) campylobacters survive the unfavorable environmental conditions partly by entering a viable but non-culturable (VBNC) stage. Here cells change from flagellated, spiral into a coccoid form, but data are conflicting as far as the reversion from VBNC stage to infective cells are concerned (*Murphy et al., 2006*). Campylobacters express several two-component regulatory systems, partly regulated by temperature changes that are involved in adaptation to different conditions (*Stintzi, 2003*). The temperature regulated protein expression is not only necessary to survive unfavourable environmental conditions but also plays an important role in colonizing the natural host, *i.e.* poultry, or causing infections in man. Genes encoding heat-shock proteins, chaperons and chaperonins showed upregulation in a study examining the gene expression changes to temperature variation in *C. jejuni* (*Stintzi, 2003*).

Several other putative virulence factors had been postulated to participate in various steps of infection. Motility plays an important role in colonization and in disease causing capacity since the bacteria penetrate the mucous layer covering the intestinal epithelium using their characteristic „cork screw" motion. The flagellar export apparatus is not only responsible to put together the flagellum but invasion antigens,

termed Cia proteins, are co-secreted using the same system to be released from the bacterial cell (*Malik-Kale et al., 2007; Rivera-Amill et al., 2001*).

Invasion of host cells, an important virulence property of *C. jejuni* (*Newell et al., 1985*), is not possible without prior adherence to the intestinal cells. Certain *C. jejuni* strains indeed possess adhesins, such as the CadF outer-membrane protein, the PEB1 protein and the CapA autotransporter protein (*Monteville et al., 2003; Pei et al., 1998; Ashgar et al., 2007*). Apparently not only proteins, but lipooligosaccharide (*Fry et al., 2000*) and capsular polysaccharide structures (*Bacon et al., 2001*) also contribute to this multistep process involving both specific and non-specific adhesin-receptor interactions. The most important putative virulence factors of *C. jejuni* are summarized and shown on **Figure 3**.

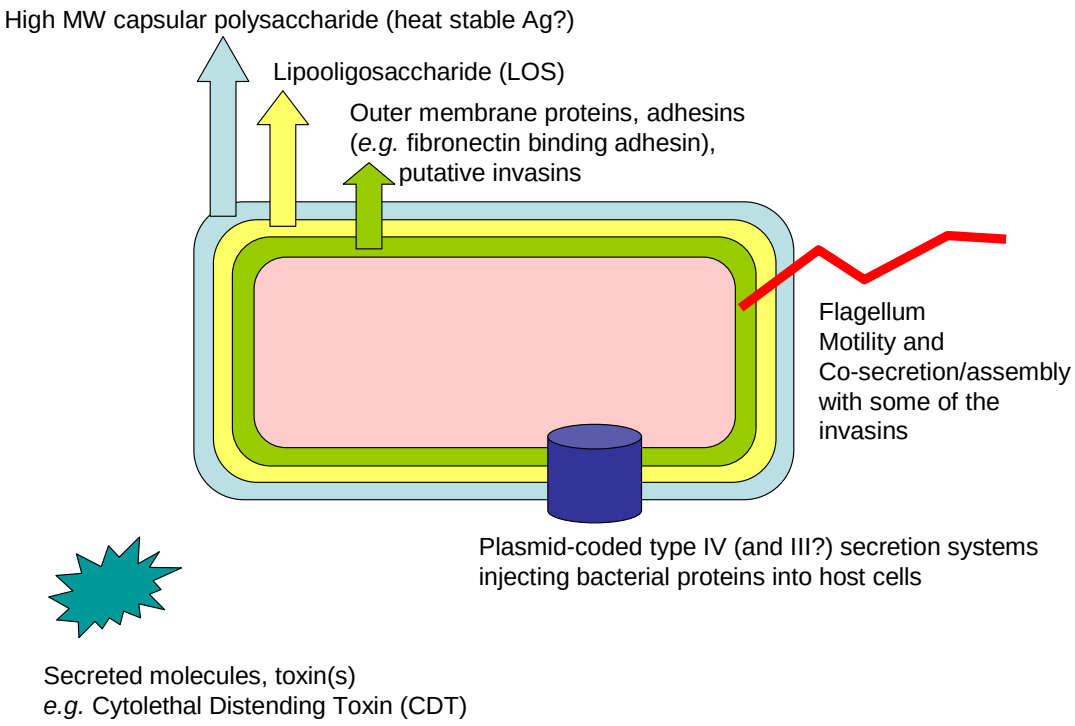


Figure 3. Putative virulence factors of *Campylobacter jejuni*

Experiments conducted with various clinical isolates showed conflicting results concerning the direct link between invasive ability and disease causing capacity of different *C. jejuni* strains. Several detailed *in vitro* studies were carried out using the highly virulent strain 81-176 expressing a type IV secretion system coded on a virulence plasmid, pVir (*Bacon et al., 2000*). However, studies examining the presence of this plasmid in human clinical isolates yielded controversial data concerning its role in the development of bloody diarrhoea (*Tracz et al, 2005; Louwen et al., 2006*). Others, as an alternative, proposed a serine protease encoded by the *Cj1365* gene as a factor involved in the development of dysentery syndrome (*Champion et al., 2005*).

Interestingly, it has been suggested that invasive campylobacters do not enter epithelial cells through the apical membrane only but, rather like *Shigella* spp., via the basolateral membrane (*Monteville and Konkel, 2002*). This mechanism is illustrated on **Figure 4.** Observations demonstrating the paracellular passage (*Monteville and Konkel, 2002*) and the M-cell-mediated transcytosis of campylobacter (*Kopecko et al., 2001*) certainly corroborate this hypothesis.

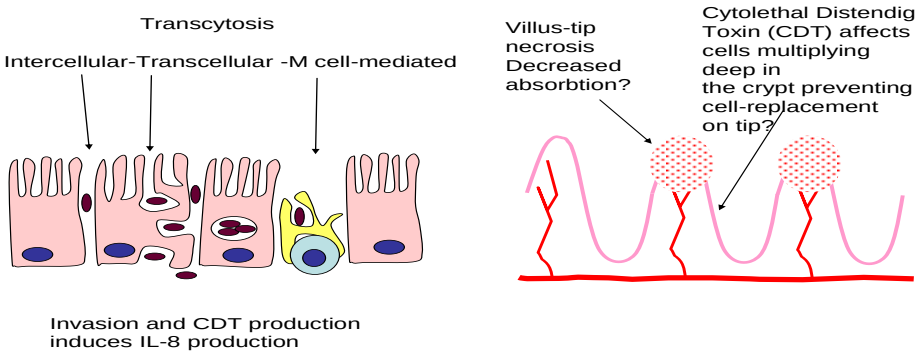


Figure 4. The traffic of campylobacter and its effect on the gut epithelium

In vitro, the internalisation of *C. jejuni* is triggered by the bacterium and was shown to be associated with the combined effect of microfilaments and microtubules of the host cells (*Biswas et al., 2003*). During internalisation the bacterium induces Ca^{++} mobilization from host intracellular stores, which is an essential step in the invasion.

(*Hu L. et al., 2005*). Once internalised, the bacterium is able to survive within epithelial cells and is moved by dynein while inside the endosome (*Hu et al., 1999*). Campylobacter induces a self-destructing cytotoxic response of the gut epithelium during infection (*Konkel et al., 1992*). Formerly, cytotoxicity was attributed to the production of cytolethal distending toxin (CDT), only (*Whitehouse et al., 1998; Purdy et al., 2000*) but a recent study found that strain dependent cytotoxicity correlated more with invasive ability and with the production of flagella (*Kalischuk et al., 2007*).

1.4.2. HOST DEFENCE

The lack of direct correlation between virulence of the infective strain and the severity of the illness caused, as well as the broad spectrum of clinical diseases seen suggest a role of individual susceptibilities, and in particular, the role of the host immune response in determining the infection's outcome. However, compared to other enteric infections, data in this field are particularly scarce for campylobacteriosis. What is clear is that *C. jejuni* triggers an innate inflammatory response, as well as initiates Th1 polarized adaptive immune response (*Hu et al., 2006*). Several studies demonstrated activation of NF-κB (*Chen et al., 2006; Johanesen and Dwinell, 2006; Zilbauer et al., 2005*) and production of IL-8, an effective chemoattractant for polymorphonuclear leukocytes during infection (*Hickey et al., 1999; Hu et al., 2006*). It has been shown that epithelial β-defensins, *i.e.* a group of endogeneous antimicrobial peptides expressed at the mucosal surfaces, exhibit potent bactericidal activity against *C. jejuni*. This effect may contribute to the clearing of bacteria in the immunocompetent host (*Zilbauer et al., 2005*). Although, it has also been shown that the innate response alone cannot block colonization persistence, it is sufficient to orchestrate marked gut inflammation. It was clearly the adaptive phase of the immune response found to be critical to mediate the clearance of the pathogen from the colonized gut (*Chang and Miller, 2006*).

1.5. CLINICAL ASPECTS OF *CAMPYLOBACTER JEJUNI* INFECTIONS

Infections caused by thermophilic campylobacters are usually indistinguishable from the ones caused by other enteric bacterial pathogens and mostly present as painful inflammatory gastroenteritis and diarrhoea (*Butzler, 2004*, *Murray, 2007*). Approximately half of the patients develop fever, abdominal pain, sometimes even mimicking acute appendicitis preceding the diarrheal phase. Fresh blood may occur in the stool by the third day of the illness. Bacteraemia is detected in less than one percent of the patients, most frequently in those with a seriously compromised immune system is.

Due to its world-wide distribution and to its capacity to spread by food and water *C. jejuni* is also one of the most prominent agents of traveller's diarrhoea (*Schrotz-King et al., 2007*). From the point of view of the current dissertation (**III.**) it is of particular importance that countries on the Arabian Peninsula, especially the United Arab Emirates, are highly frequented tourist destination. However, the region recently was scored as a high risk area for *C. jejuni* infections (*Ekdahl and Anderson, 2004*). This fact and the paucity of local epidemiological and antibiotic resistance data (*Sjogren et al., 1989; Albert et al., 2005; Jumaa and Neringer, 2005*) prompted the initiation of some studies (III.) of this Thesis.

C. jejuni infections are not infrequently followed by autoimmune or neurogical sequelae, such as reactive arthritis, erythema nodosum, Reiter syndrome or the most severe one, Guillain-Barré syndrome (GBS) (*P. Murray, 2007; Yu et al., 2006; Ternhag et al., 2008*). GBS is the most frequent cause of acute flaccid paralysis in man and the triggering agent most commonly identified is *C. jejuni* accountable for approximately one third of the cases (*Hughes and Comblath, 2005*). It is estimated that 0.3 to 3 per 1000 cases of symptomatic campylobacteriosis develops into GBS (*Bereswill and Kist, 2003*).

Guillain-Barré syndrome consists of at least four subtypes of acute peripheral neuropathy: acute inflammatory demyelinating polyradiculoneuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor and sensory axonal neuropathy

(AMSAN) and Fisher's syndrome subtype, respectively. In general GBS is considered to be an autoimmune disease, with the immune system mistakenly attacking myelin or axons. The "mistake" occurs because of the surface structure of certain pathogens contains polysaccharides that resemble gangliosides present in the nervous system. This resemblance is termed "molecular mimicry". For a long time strains carrying the heat-stable antigen HS19 were considered to be almost exclusively the only serotype associated with GBS. However, it is clear now that isolates expressing a variety of antigens might be responsible (*Yu et al., 2006*).

Each subtype of GBS is associated with an immune response mounted against different types of gangliosides. Antibodies to GM1, GM1b, GD1a, and GalNac-GD1a are particularly implicated in acute motor axonal neuropathy and, with the exception of GalNacGD1a, in acute motor and sensory axonal neuropathy. The Fisher's syndrome subtype is especially associated with response to GQ1b. In **Table 5**. (adapted from *Yu et al., 2006*) the heat stable polysaccharide antigens of different *C. jejuni* serotypes cross-reacting with various ganglioside antigens of the host are listed.

Table 5. Molecular mimicry between campylobacter HS antigens and gangliosides

Heat stable (Penner) serotype	**Mimicry with ganglioside antigen**									
	GM1	**GD1a**	**GQ1b**	**GT1a**	**GD3**	**GM2**	**GD2**	**GM3**	**GD1b**	**GA 1**
HS:1	X	X			X	X	X			
HS:2	X	X	X	X	X	X	X			
HS:4	X	X			X	X	X			
HS:10	X	X			X	X	X			
HS:19	X	X	X	X	X	X				
HS:23	X	X	X	X	X	X	X			
HS:23/HS:36 (81-176)								X	X	
HS:36	X	X			X	X	X			
HS:41	X	X			X	X	X			X

The molecular structure of the heat stable antigens of several of these serotypes has been studied in detail. The structures revealed explain why these antigens induce autoaggressive responses: they contain sialic acid linked terminally and/or laterally to residues of galactose, which makes them similar to the terminal regions of several human gangliosides (*Moran et al. 1996a and 1996b*).

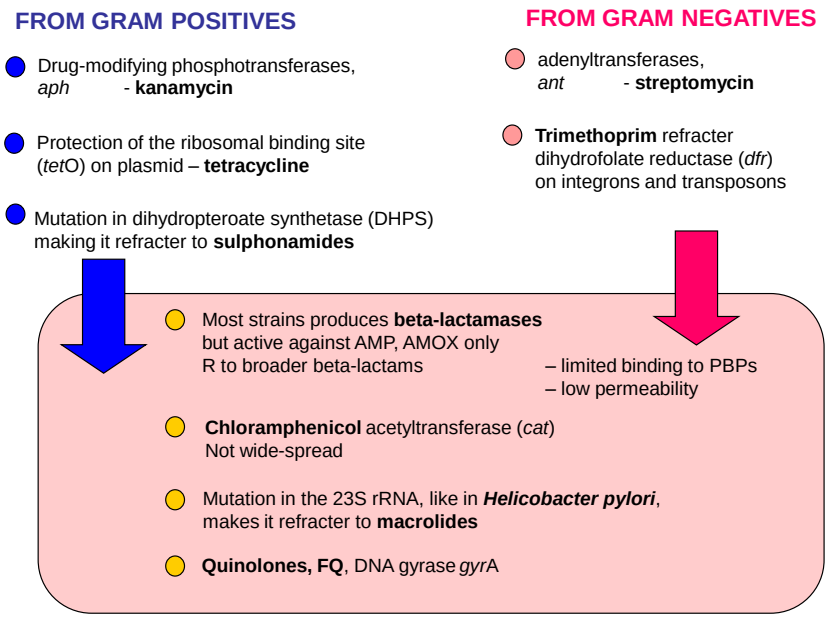
1.6. ANTIBIOTIC RESISTANCE

Campylobacter enteritis is a self limiting disease and usually has a good prognosis without antibiotic therapy. However, immunocompromised hosts or patients with persistent fever, bloody diarrhoea and significant fluid loss require antimicrobial drugs. Methods of antibiotic susceptibility testing of campylobacter has been controversial until recently when the Clinical and Laboratory Standards Institute (formerly NCCLS) suggested the use of the agar dilution method when testing these organisms (*NCCLS, 2005*), practically disqualifying the disc diffusion method routinely used before.

C. jejuni shows variable susceptibility to antimicrobial drugs. Resistance to these agents can be caused by a broad variety of mechanisms such as target modification (*e.g.* fluroquinolones, macrolides), target protection (*e.g.* tetracycline), expression of permeability barriers (some beta lactam antibiotics) and/or efflux pumps (*e.g.* quinolones) and production of drug-destroying enzymes (*e.g.* beta lactams, aminoglycosides, and chloramphenicol). Strains of *C. jejuni* can display broad sets of this arsenal. It is noteworthy that the organism is capable of acquiring resistance genes not only form Gram negative, but also form Gram positive organisms, see **Figure 3**. (based on *Moore JE et al., 2005* – next page).

Resistance to two classes of drugs is of particular importance: macrolides and fluoroquinolones. Although the epidemiology of antibiotic resistance in *C. jejuni* has recently been widely studied at several, although not all, parts of the world, surprisingly none of the numerous studies reported high incidence of macrolide resistance, so far. Based on this fact and on the proven clinical efficacy of macrolides against campylobacter makes erythromycin or its derivatives the primary drugs of choice to treat infections of known aetiology (*Butzler, 2004*).

Figure 3. Summary of genes and mechanisms of antibiotic resistance in *C. jejuni*



However, due to practical considerations, there is another class of drugs with utmost importance, *i.e.* fluoroquinolones. While initially quinolones and their fluorinated derivatives had been very active against these pathogens, resistance has been increasing alarmingly fast (*Butzler, 2004*). Consequently, once the aetiology is proven, these drugs are usually not indicated to treat campylobacteriosis any longer. However, as the causative agents of most infectious enteritis remains undetermined, often fluoroquinolones are given "ex iuvantibus". While it may work for cases of some alternative aetiologies, the resistance of campylobacters is prone to result in therapeutic failures (*Guerrant et al., 2001, Saunders et al., 2002*). This fact clearly justifies the effort to gain more knowledge on the mechanisms and distribution of fluoroquinolone resistance among *C. jejuni*, *i.e.* the very goals of some of the studies in this dissertation (III.).

Commonly, fluoroquinolone resistance in *C. jejuni* is attributed to target modification due to various mutations in topoisomerase II gene, *gyrA*, or/and to an active efflux pump coded by the *cmeABC* operon (*Ge et al., 2005*). The importance of this efflux mechanism is two-folded: Since it is not specific to fluoroquinolones strains expressing it are usually multidrug resistant. Furthermore, although fluoroquiolone resistance caused by efflux pumps alone is usually characterized by relatively low

MIC values, it has been shown that it contributes significantly to high level fluoroquinolone resistance. First, it may complement the resistance caused by various *gyrA* mutants further increasing the MIC. Perhaps even more importantly the elevated (albeit on its own not very high) MIC level provided by the efflux facilitates the selection for topoisomerase mutants with a much higher level of resistance (*Yan et al., 2006*).

"Real" high level ciprofloxacin resistance in *C. jejuni* is most often due to point mutations in the gene of DNA gyrase (*gyr*A). This type II topoisomerase catalyses DNA supercoiling and as such, it is involved in DNA replication, recombination and transcription. The part of this gene between codons 69 and 120 is called the Quinolone Resistance Determinant Region (QRDR) since it contains all known point mutations described sofar conferring nalidixic acid and fluoroquinolone resistance (*Hakanen et al., 2002*). While the exact role of all these mutations in fluoroquinolone resistance has not been established, the fact is that the majority of highly fluoroquinolone-resistant clinical isolates of *C. jejuni* carry the Thr-86 to Ile substitution in *gyrA* (*Piddock et al., 2003*). Other common changes reported are the Pro-104 to Ser (*Zirnstein et al., 1999; Hakanen et al., 2002*), the Thr-86 to Lys (*Ruiz J et al., 1998*) or to Ala (*Bachoual et al., 2001*), the Ala-70 to Thr, the Asp-90 to Asn substitutions, respectively (*Charvalos et al., 1996; Hakanen et al., 2002; Hanninen ML & Hannula M, 2007*).

No changes in the B subunit gene, *gyrB*, have yet been documented in campylobacter. There was one report suggesting the role of mutations in topoisomerase IV (*parC*) associated in fluoroquinolone resistance in *C. jejuni* (*Gibreel et al., 1998*), but further studies could not confirm it and the published sequence of *C.jejuni* NCTC 11168 completely lacks the entire *parC* gene (*Piddock et al., 2003*).

For a long time detection of mutations in the QRDR of *gyrA* was possible only by DNA sequencing or, indirectly, by single-strand conformation polymorphism analysis (*Charvalos et al., 1996*). Recently, *Zirnstein* and his coworkers developed a rapid and specific PCR based method to replace these costly and labour-intensive techniques, *i.e.* a mismatch amplification mutation assay (MAMA), *i.e.* an assay extensively used

in this thesis work (**III.**). The assay detects the Thr-86-Ile mutation by using a conserved forward primer and a reverse, mutation detection primer which anneals in the presence of the ACA→ATA point mutation, only. Amplification of the targeted 265 bp product is a clear indication that primer annealing indeed took place, *i.e.* the region carries the mutation looked for (*Zirnstein et al., 1999*).

In the past decade fluoroquinolone resistance of *C.jejuni* has became an emerging problem worldwide. (*Chu et al., 2004; Saenz et al., 2000; Jain et al., 2005; Gupta et al., 2004; Gaudreau, 2003; Wagner et al., 2003; Dingle et al., 2005*). Moreover, by mechanisms yet to be elucidated, fluoroquinolone resistance appears to coincide with increased fitness of the organism. In humans, fluoroquinolone resistant strains were reported to be shed longer than sensitive isolates (*Nelson et al., 2004; Engberg et al., 2004*) and they were found to out-compete their sensitive counterparts, at least in certain genetic backgrounds, when colonising poultry, *i.e.* the natural reservoir of the pathogen (*Luo et al., 2005*). All these findings underscore the necessity for close monitoring of the incidence and spread of fluoroquinolone resistance in *C. jejuni*, an important aim of the current study (**III.**).

1.7. TYPING TECHNIQUES

The fact that most *C. jejuni* enteritis cases present as sporadic ones does not make strain-typing obsolete. First of all outbreaks with epidemiological connections between cases and sources to be revealed do occur. Furthermore, even between seemingly sporadic cases there could be hidden links not obvious for descriptive epidemiology working by a case-based approach. This actually could easily be the case with a pathogen, like *C. jejuni*, where several, yet to be explained phenomenon exist: The low infective dose would theoretically suggests a higher rate of related, *i.e.* "outbreak", cases, and the *in vitro* fastidiousness is in sharp contrast with its remarkable survival skills in the environment. These all point towards the fact that there is still much to learn on the epidemiology of *C. jejuni* – an endeavour prone to fail without strain typing. Finally, strain comparison carried out with appropriate typing techniques could answer questions concerning the evolution of pathogens. This, in case of a pathogen apparently still holding important surprises (*Sheppard et al., 2008*) justifies the efforts for its typing.

Typing systems for epidemiologic studies involving *Campylobacter* vary according to the features examined, to their discriminatory power and complexity, as well.

1.7.1. Phenotyping of *Campylobacter jejuni*

The phenotypic typing techniques most widely used are serotyping, biotyping, protein and fatty acid profile analyis and phage typing. Serotyping is the most common phenotyping method for *C. jejuni*. There are two well-established and evaluated serotyping schemes described. One is based on the study of the heat-stable (HS) antigens using the passive hemagglutination technique (*Penner and Hennessy, 1980*), while the other one is based on heat-labile (HL) antigens applying bacterial agglutination (*Lior et al., 1982*). The significance of HS based serotyping is emphasized by the fact that the incidence of serious neurological sequelae following infection caused by certain HS serotypes might be higher (*Nachamkin et al., 1998; Butzler, 2004*).

The structure of HS antigens has extensively been studied (*Tsai and Frasch 1982; Mills et al 1985; Preston and Penner 1987, 1989*) and found to be lipo-oligoscaccharide (LOS)-like molecules containing the keto-deoxyoctulonosic acid (Kdo) characteristic to the bacterial LPSs and LOSs. Subsequent, more detailed analysis revealed that these molecules showed marked difference to both LPS and LOS. Some of them contain the polysaccharide polymer analogous to the O-antigen of LPS, but has outer core OS regions displaying structural diversity resembling those of LOSs (*Moran and Penner, 1999*).

Among the major disadvantages of serotyping techniques are the high number of untypeable strains and the time-consuming and technically demanding requirements of the techniques (*Wassenaar and Newell, 2000*). Production and quality control of antiserum reagents for serotyping are costly; consequently, these reagents are not widely available. Some laboratories are using home-made antisera, although a set of HS antisera is available commercially, as well (*Rautelin and Hanninen 1999*; *Hanninen et al. 2001*). The serotype distribution of *C. jejuni* strains of animal origin have been extensively studied in Hungary (*Varga et al. 1986a, 1986b, Varga et al., 1990a, 1990b*). However, little is known about the occurrence of different serotypes among human isolates. Varga and Fodor conducted a study using home-made HS antisera on these isolates and found that 30 % of them was non-typeable (*Varga et Fodor, 1998*). Contrary to the apparent uniformity observed by serotyping, it was not unreasonable to assume that this non-typable group in fact consists of a mixture of types: a hypothesis intended to be exposed to scientific scrutiny within the current study (**II.**).

Although genotyping is rapidly gaining popularity and serotyping of *C. jejuni* is technically more complicated than that of most *Enterobacteriaceae*, it is still an important complementary method in the epidemiological investigations of infections caused by this pathogen. That prompted us to include this method among the technical approaches we used at different geographical locations (**II.** and **III.**). With sera obtained from a commercial source we used the typing scheme of Penner, since this is much more commonly employed in Europe than the one base on heat sensitive antigens.

1.7.2. Genotyping of *Campylobacter jejuni*

A considerable advantage of genotyping techniques is that they are universally available and the results yielded are comparable. A major group of these techniques relies on uncharacterized genomic differences affecting cutting sites of restriction enzymes by comparing enzyme-digested DNA fragment patterns after electrophoresis, mostly in agarose gels. An alternative, more costly, but more informative approach is to directly examine the genetic material by sequencing. Both groups of methods may sample the whole genome or, alternatively, can single out gene(s) and detect differences only in this restricted - ideally representative - part of the genome. Depending on the targeted gene(s) the genetic variation indexed by a molecular technique may aggregate relatively rapidly revealing "tactical", micro-evolutionary changes in response to pressures the microbe is or has been encountering promptly or recently. This makes these methods suitable to compare strains isolated within a short period of time, like during outbreak investigations. These studies usually, but not exclusively, use techniques based on banding pattern comparisons. Alternatively, the features (*i.e.* their genes) examined can be relatively stable, meaning that the changes accumulate relatively slowly shedding light onto long-term, strategic alterations just off, or even during the course of macroevolution. These methods are almost exclusively based on sequence comparisons.

Comparison of electrophoretic banding patterns. These typing methods are based on the comparison of DNA fragments of different length as they are separated by electrophoresis yielding an electrophoretic pattern characteristic to an isolate. It should be noted that these analyses strictly work on patterns of bands, irrespective of their "content", *i.e.* two bands are considered identical if their molecular weights are the same, irrespective of their sequences. The extent of similarity is expressed as similarity indexes using different mathematical formulas. The difference between the formulas (*i.e.* the indexes calculated) derives from whether the absence or presence of a band weighs more, or equal, during the calculation. The formula most widely used to calculate the similarity between two gel patterns (A and B) is the Dice coefficient which stresses the presence of elements in the patterns:

$$SD = \frac{2a}{2a + b + c}$$

a is the number of elements found in both patterns, *b* is the number the elements found only in pattern A, and *c* is the number of elements found only in pattern B (*Riley, 2004*).

When S_D equals 1 the two patterns are identical, when it is 0 there are no common elements in the two patterns. With all the pair-wise similarity calculations done a similarity matrix can be built. Clustering of patterns is based on this matrix using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (*Riley, 2004*). The UPGMA is the simplest method of tree constructions. It was originally developed for constructing trees that reflect phenotypic similarities between patterns. First, it identifies the two patterns of all that are the most similar to each other and then treat these as a new single operational taxonomic unit (OTU). Such a unit is referred to as a composite OTU. Subsequently from among the new group of OTUs it identifies the pair with the highest similarity, and the circle continues until only two OTUs are left. This method is often part of purpose-built softwares (like BioNumerics' – GelComparII, *i.e.* the one we used in our studies **II.** and **III.**) handling databases with large number of electrophoretic patterns. This allows the clustering of almost unlimited number of patterns. The main disadvantage of UPGMA clustering is that it shows the average distance between patterns or units whereas genetic divergence of these units is not necessarily taking place at the same speed. Hence, while it is useful to compare groups, it does not reflect directly their phylogenetic distance and relationship (*Riley, 2004*).

Comparison of sequences. These methods gained popularity in the last decade only, with high through-output automated DNA sequencing becoming available for daily routine work. Beyond their technical straightforwardness their main advantage is that they indeed reveal the very bottom of the line, *i.e.* the nucleotide sequence, instead of examining some of the indirect consequences of its changes (*i.e.* fragment size after amplification or digestion). Results thus generated are directly comparable between

laboratories to the level not achievable even with the most carefully standardised alternatives.

In bioinformatics sequence alignment is a way of arranging the primary sequences of DNA samples to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships. Alignment of two sequences is useful to find matching pairs in a certain database like using the National Institute of Health DNA sequence database and the BLAST program provided freely. However, molecular epidemiologic studies usually require alignment of multiple sequences. With the help of this method, based on the extent of similarities, it is possible to reveal evolutionary relationships and construct phylogenetic trees describing the evolutionary distance between certain sequences.

One of the most widely used and freely available software for multiple sequence alignment is ClustalW (*Thompson et al., 1994*). It builds the phylogenetic tree using the Neighbour-Joining method (NJ) (*Saitou et Nei, 1987*) which is more robust then the UPGMA against the effects of unequal evolutionary rates in different lineages. It gives better estimates of individual branch lengths and recently has been proved consistent, *i.e.* it reconstructs the correct tree even when the original distance matrix is perturbed by a small noise (rewiewed in *Gascuel and Steel, 2006*).

By the end of the 1990ies several molecular techniques have been tested to type campylobacter (*Wassenaar and Newell, 2000*). Amongst the several possible methods the following ones became standards and widely used in molecular epidemiologic studies.

1.7.2.1. Outbreak investigations – banding pattern analysis

1.7.2.1.1. Pulsed field gel electrophoresis (PFGE)

The term refers to the electrophoretic conditions used to separate large fragments of the DNA generated by digestion of the entire chromosome with a restriction endonuclease having a rare cutting site, *i.e.* resulting in relatively few, very large

fragments. The method's discriminatory power is high, the pattern obtained reflects even relatively small genetic changes. It was first used to type *Campylobacter* by Yan and coworkers in 1991 (*Yan et al., 1991*) and since then it has been used successfully in several studies investigating sporadic and outbreak *Campylobacter* isolates alike. Currently two standardized methods are internationally accepted, the Campynet and the PulseNet protocols (*Campynet 2000, PulseNet 2004*). In our studies (**II.** and **III.**) we used the Campynet version. It should be noted that the remarkable genetic instability of *C. jejuni* (see **I.**) can affect results obtained even with this robust genotyping method. Recently *Wassenaar* and coworkers reported about isolates of *C. jejuni* of clonal origin which showed divers PFGE pattern due to, most likely, genomic rearrangement (*Wassenaar et al., 1998*). Despite the phenomenon reported and the labor-intensive nature of the method, PFGE is still one of the "gold standards" when genotyping *Campylobacter*.

1.7.2.1.2. Flagellin gene (*fla*A) typing by restriction fragment length polymorphism (RFLP)

The FlaA protein, together with FlaB, is the main component of the flagellar filament. Genes coding for these two proteins are located on the flagellin gene locus, arranged in tandem both containing highly conserved, as well as variable regions. The conserved regions are located at both ends of the gene allowing the amplifications of most of the gene using primers flanking the middle part. Digestion of the amplified, variable product results in DNA fragments with sizes reflecting the number and locations of the cutting sites, *i.e.* the variation in its DNA sequence. The electrophoretic banding pattern can be compared either manually or by computerized techniques. Several flaA PCR-RFLP techniques has been published with different primer sets and different restriction enzymes used. In our studies (II. and III.) we used a consensus primer set suggested by *Wassenaar* and *Newell* (*Wassenaar and Newell, 2000*). Some studies of outbreak-related isolates suggested that differences in *fla*A genotypes may arise due to intergenomic and intragenomic recombination (*Harrington et al., 1997*) and hence the method cannot be considered stable for long term monitoring of pathogenic *Campylobacter* populations. However, later on other study groups have found the method feasible and useful even for long term

comparisons as well (*Fitzgerald et al., 2001; Djordjevic et al., 2007*). Since the technique is fast, easy and cost-effective, it is a useful tool, especially in combination with other typing methods.

1.7.2.1.3. The amplified fragment length polymorphism (AFLP)

The technique is based on the complete digestion of the chromosome with two restriction enzymes followed by amplification of the digested DNA using fluorescein-labelled primers. Primers are so designed that fragments flanked by both restriction sites are amplified only. The labelled PCR products thus are analyzed on denaturing polyacrilamide gels. This separates fragments as small as 50-500 bases long allowing the detection of single base differences (*Duim et al., 1999*). The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. This prevents over-interpretation or misinterpretation due to point mutations or single-locus recombinations, which may affect other genotypic characteristics. The main disadvantages of the technique are its complexity and the requirement for an electrophoresis apparatus used for gel-based sequencing studies and for appropriate software to perform banding pattern analysis.

1.7.2.2. Outbreak investigations – sequence based methods

1.7.2.2.1. Sequencing the short variable region of the *fla*A gene (fla-SVR)

Sequence analysis of the short variable region of the *flaA* gene grouped the strains examined very similarly to sequencing the entire gene. Consequently it was suggested that comparing a short variable region (SVR) of the *flaA* gene from base positions 450 to 600 could be a useful tool for epidemiologic studies (*Meinersmann et al., 1997*). Later this method was proven to be as discriminative as PFGE (*Sails et al., 2003*) and when investigating outbreak related isolates the fla-SVR-based clustering method, not surprisingly, correlated better with the actual epidemiological findings than the data produced by multi-locus sequence typing (*Clark et al., 2005*).

1.7.2.3. Evolutionary studies, methods for "global" epidemiology

1.7.2.3.1. Multi-locus sequence typing (MLST)

The method is the "molecular version" of multi locus enzyme electrophoresis, MLEE, a technique developed earlier to detect slowly accumulating differences between isolates' housekeeping genes, *i.e.* detects allelic variations in housekeeping enzymes. Since the changes accumulating in housekeeping genes are neutral, *i.e.* they are not under selective evolutionary pressure, the MLEE was useful in studying questions of "long term" or "global" epidemiology (*Maynard-Smith et al., 1993*). Following the same principle but at the level of genetic information instead of proteins, MLST is based on sequencing ~500 bp fragments of a few, usually seven, internal housekeeping genes (*Enright and Spratt, 1999*).

In an MLST scheme the different sequences of each gene are considered as alleles and each allele is given an arbitrary number. Sequences that differ even by a single nucleotide are considered different alleles without weighting the number of nucleotide differences existing. This is explained by the fact that it is unknown whether the differences at multiple nucleotide sites are results of multiple point mutations or that of a single recombination event. The alleles at the seven loci provide an allelic profile (the combination of allele numbers at the seven loci). Each unique allelic profile is assigned an arbitrary number, in order of their discovery, and this is commonly referred to as sequence types or STs (*Enright & Spratt, 1999*). The sequences of allelic variants are placed in a database accessible through the World Wide Web allowing the easy and reliable comparison of isolates even between distant geographical locations. The large number of alleles at each of the seven loci provides the ability to distinguish billions of different allelic profiles, and a strain with the most common allele at each locus would only be expected to occur by chance approximately once in every 10000 isolates (*Spratt, 1999*). Consequently, it is extremely unlikely that two unrelated isolates would have the same allelic profile giving the MLST the sufficient discriminatory power. Since the accumulation of nucleotide changes in housekeeping genes is a relatively slow process and the allelic profile of a bacterial isolate is sufficiently stable over a long period of time, the

MLST is more useful for global epidemiological and even for evolutionary studies than for investigating local outbreaks with a short time-span.

The MLST websites offer a range of tools from the initial characterization of strains to clustering based on differences in allelic profiles, to building phylogenetic trees based on concatenated sequences, and to displaying the population structure and identifying clonal complexes (*Aanensen and Spratt, 2005*). A strain can be compared to the database using its allelic profile. If a query strain exhibits a minimum level of similarity with those stored in the database, *i.e.* shares ≥4 alleles of the seven loci, an UPGMA dendogram is built based on the matrix of pair-wise differences between the allelic profiles of the strains to show the extent of relationship. The other option is to concatenate the seven sequences of a query strain, *i.e.* to arrange the seven fragments in a continuous line, and to compare this concatenated sequence to the database. This method is generally not recommended since a long history of recombination will preclude the recovery of the true phylogenetic relationships between distantly related strains. Even the relatedness between similar strains may be better represented by a tree based on differences in allelic profiles than by one based on differences in the concatenated sequences (*Feil et al., 2001*). However, in special cases like the correct speciation of *S. pneumoniae*, this approach proved to be useful when examining whether the concatenated query sequence clusters together with sequences of a reference *S.pneumoniae* strains (*Aanensen & Spratt, 2005*).

Recently, a third type of model for comparison, specific to MLST data was described and proposed to reveal the structure of populations (*Feil et al., 2004*). It is called eBURST and it provides an alternative way of displaying relatedness different from the regular tree-like structures. The model assumes that, due to selection or genetic drift, some genotypes will occasionally increase in frequency in the population and will then gradually diversify by the accumulation of mutation(s) and/or recombinational replacements, resulting in slight variants of the founding genotype. Initially, members of this emerging clone will be indistinguishable in allelic profile by MLST. However, eventually, as time passes, the clone will diversify to produce a number of variants in which one of the seven MLST loci has been altered yielding single locus variants (SLVs). Further diversification will produce variants of the

founder ST that differ at two out of the seven loci leading to double locus variants (DLVs). In this simple model bacterial populations will consist of a series of clonal complexes (sets of variants of a founding genotype) that can be distinguished from the allelic profiles of the strains within a MLST database (*Feil et al., 2004*). The eBURST can be used to explore how bacterial clones diversify and can provide evidences concerning the emergence of clones of particular clinical relevance.

Today, there are approximately 20 well established MLST systems to type pathogenic, as well as non-pathogenic bacteria and fungi, *e.g. Neisseria meningitides, Streptococcus pyogenes, Staphylococcus aureus, Candida albicans, C. jejuni* and *S. pneumoniae* (*Maiden et al., 1998; Dingle et al., 2001; Tavanti et al., 2003; Enright and Spratt, 1998, Enright et al., 2000*). However, when the work for this dissertation was initiated there was no MLST system for *C. jejuni*, so we felt that it is of utmost importance to develop one (**I.**).

1.7.2.3.1. Mutation and recombination analysis

In haploid bacteria changes in DNA sequence can occur due to mutation or recombination. Analysis of the frequency of these events provides deeper insight into the structure of a microbial population. In case of a pathogen, like *C. jejuni*, with a known high rate of change these investigations are to provide new insights into the genetic plasticity of the organism.

1.7.2.3.1.1. Mutation analysis

Nonsynonymous mutations cause a change in the translated amino acid sequence, whereas synonymous mutations do not. Mathematical analysis of mutation frequencies is usually computed using various softwares *e.g.* DnaSP (*Rozas et Rozas, 1999*). The analysis and comparison between the number of nonsynonymous mutations (Ka) and the number of synonymous mutations (Ks) can suggest whether, at the molecular level, natural selection is acting to promote the fixation of advantageous mutations (positive selection) or to remove deleterious mutations (purifying selection) (*McDonald & Kreitmann, 1991*). In general, when positive

selection dominates the Ka/Ks ratio is greater than 1. In this case, diversity at the amino acid level is favoured, which likely due to the gained fitness, *i.e.* an advantage, provided by the mutations. Conversely, when negative selection dominates the Ka/Ks ratio is less than 1 demonstrating that the amino acid changes are deleterious and, therefore, are selected against. When the positive and negative selection pressures balance each other, the Ka/Ks ratio is close to 1 (*Kimura, 1991*).

1.7.2.3.1.2. Recombination analysis

Genetic recombination is the process by which a strand of genetic material (usually DNA) is broken and then joined into a different DNA molecule, or into a different site of the same molecule. Bacteria can differ widely in terms of their recombination rate which defines the degree of clonality (*Maynard-Smith et al., 1993*). Some species *e.g. Borrelia burgdorferi*, is highly clonal, *i.e.* all sequence diversity in its genome has arisen by gradual accumulation of point mutations without recombination. Others, like *Neisseria gonorhoeae* are panmictic, with random association between loci due to frequent intraspecies recombination events. To test the frequency of repeated recombinations within a population by using DNA sequence data the Homoplasy Test was developed by *Maynard-Smith* and *Smith* (*Maynard-Smith et al., 1998*). Homoplasy, in general, means the appearance of similarities in separate evolutionary lineages. The Homoplasy Test, which can be applied to a set of sequences of a single gene or to several unlinked genes from the same strains, compares the observed number of homoplasies to the expected number. If the latter is greater than the former, the plausible explanation is recombination. The extent of recombination is measured by the Homoplasy ratio (H). The H value varies from 0 (clonal) to 1.0 indicating complete linkage equilibrium (*Maynard-Smith et al., 1998*).

The measurement of linkage equilibrium, *i.e.* the association between neighbouring nucleotides, is a statistical method of detection of clonality. The index describing the degree of association between loci is called Index of Association (I_A) (*Smith, 1999*). The I_A dependens on the number of loci analyzed which is taken into account in the calculation of the standardized I_A (sI_A). sI_A is expected to be 0 when alleles are in linkage equilibrium because of free recombination (*Smith, 1999*).

The close taxonomical relation of *C. jejuni* to *Helicobacter pylori*, *i.e.* to a species probably with one of the highest degree of intraspecies recombination, suggested that similar features might be revealed in the former pathogen as well. In general, it was thought that extended information on the details of molecular make-up of the *C. jejuni* genom will help to understand its unique pathological and epidemiological features – an idea which had a considerable impact on initiating some studies of this project (**I.**).

2. AIMS

2.1. To study the genetic diversity and population structure of *Campylobacter jejuni* by analysing sequence polymorphism and frequency of recombination in housekeeping genes of strains isolated at different geographical locations

2.2. To study the serotype distribution of human *Campylobacter jejuni* strains isolated in Hungary

2.3. To investigate the variation among non-serotypable *Campylobacter jejuni* strains in Hungary using different molecular typing methods

2.4. To investigate the antibiotic sensitivity and type distribution of *Campylobacter jejuni* isolated from human infections in the United Arab Emirates

2.5. To reveal the genetic background of fluoroquinolone resistance in *Campylobacter jejuni* found with high incidence in the United Arab Emirates

3. SHORT SUMMARY OF RESULTS AND THEIR BRIEF INTERPRETATIONS

A Multi Locus Sequence Typing scheme for *Campylobacter jejuni* was established and used successfully to type isolates from different geographical locations (I.).

Parts of seven housekeeping genes - aspartate-semialdehyde dehydrogenase (*asd*), ATP synthase F1a (*atpA*), D-Alanine-D-alanine-ligase (*ddlA*), elongation factor TS (*eftS*), fumarate hydratase (*fumC*), NADH dehydrogenase H (*nuoH*), GTP-binding protein (*yphC*) - were selected based on the published *C. jejuni* NCTC 11168 complete genome (GenBank AL111168). The criteria for selection were the following: the chosen genes were to be distributed widely around the whole chromosome, and no putative virulence genes, *i.e.* genes predicted to be under strong selection pressure, were to be located near to them. Primers were designed to amplify 400-660 bp long internal fragment of each gene, respectively. The length of the PCR products allowed sequencing them directly on both strand in one run. The sequence alignment did not demonstrate any gaps or insertions in any of the seven loci. For all the seven genes one allele was found in more than one isolate. For the different genes the number of alleles varied between 9 (*atpA*) and 15 (*eftS* and *fumC*). However, when comparing the allelic profile of the 33 strains only two pairs were indistinguishable.

Mutation and recombination analysis of the housekeeping genes of *Campylobacter jejuni* provided evidences that recombination, rather than a series of point mutation plays and important role in generating genetic heterogeneity between strains of this pathogen (I.).

The synonymous (K_S) and non-synonimous (K_A) mutations values were calculated. As it was expected, the frequency of mutations leading to amino-acid changes was very low for each fragments (K_A<1%) and the K_A/ K_S ratio

varied between 0.014 (*atpA*) and 0.17 (*nuoH*), clearly demonstrating the negative selection pressure for amino-acid changes.

When performing the Homoplasy Test (*Maynard-Smith et al., 1998*) only five of the seven fragments yielded sufficient number of informative sites, the diversity was not sufficient in the *atpA* and *nuoH* data set. In the remaining five loci the *H* ratios were the following: *asd* 0.40, *ddlA* 0.42, *eftS* 0.36, *fumC* 0.48 and *yphC* 0.47, respectively. These values are clear indicators of frequent intraspecific recombination, since the mean *H* ratio observed (0.42±0.05) was even higher than the one of *Neisseria meningitides* (11 genes, mean H= 0.34) which was considered as panmictic, *i.e.* lacking any clonality. (*Maynard-Smith et al.,1993*). The high recombination rate assures to create several combinations of allels evidenced by the number of unique sequence types observed. This clearly indicated that the MLST developed will be a useful tool in epidemiological investigations.

Serotyping based on the heat stable antigen of *Campylobacter jejuni* revealed that 30.4% of the strains isolated from sporadic human infection in different parts of Hungary were non-typable. The typable strains belonged to 17 different serogroups, amongst them HS serotype 3, 2 and serogroup 1,44 were the most prevalent ones (II.).

These findings were consistent with previous observations of *Varga* and co-worker (*Varga and Fodor, 1998*), although the strain collection of this previous study included human isolates from Budapest only and applied a set of diagnostic antisera different from the commercially available one used in our investigations. Nevertheless, in both studies the number of non-typeable strains was unusually high, such figures have not been found in any other parts of the world before (*Newell et al., 2000; Hänninen et al., 2001; Gibson et al., 1997*).

Genotyping revealed 26 distinct molecular types among the 28 non- serotypable (NT) strains proving that this group is extremely heterogeneous and serotyping alone is insufficient for studying the epidemiological connection of such isolates (II.).

Seventeen different PCR-RFLP patterns and 25 different PFGE patterns were found. Only one pair of strains with identical PFGE pattern exhibited different PCR-RFLP profile. The different discriminatory power exhibited by the two molecular typing methods used, *i.e.* PCR-RFLP and the PFGE, is not surprising. Unlike the *flaA* PCR-RFLP, the PFGE provides information based not only on changes in the gene coding for flagellin subunit but on the restriction of the entire chromosome. Our data clearly showed, that the non-serotypable group cannot be considered homogenous. Consequently, serotyping alone is not suitable for epidemiological studies. This is particularly true for a country, like Hungary, with such high number of non-serotypable isolates. Non-typable isolates, such isolates should be subjected to further molecular typing when evaluating their possible connections.

Investigation of antibiotic sensitivity of *Campylobacter jejuni* isolated from individual patients in a hospital of the United Arab Emirates revealed extremely high level, *i.e.* 85,4 %, of ciprofloxacin resistance (III.).

While the examined strains were all sensitive to erythromycin (MIC 0.5-4 mg/L), 35 isolates of the total 41 were resistant to ciprofloxacin (MIC 8-64 mg/L). Since the United Arab Emirates is an increasingly popular tourist destination and the risk of acquiring campylobacter gastroenteritis here is rather high (*Ekhdal and Andersson, 2004*), this fact should alert physicians to determine the causative agent of traveller's diarrhoea instead of treating the disease "blindly" by ciprofloxacin.

Mismatch amplification mutation assay (MAMA) PCR and sequencing of the *gyrA* gene showed that the genetic change behind the high fluoroquinolone resistance observed is the carriage of a point mutation leading to amino acid change at the position 86 from threonine to isoleucin (III.).

The MAMA PCR (*Zirnstein et al, 1999*) detected the ACA→ATA point mutation at nucleotide position 257 leading the Thr-86 to Ile amino acid change. Furthermore, sequencing the 673 bp long part of the *gyr*A genes confirmed that in all resistant isolates this was the only nonsynonimous mutation whitin the quinolone resistance determinant region. Although existence of other mechanisms leading to elevated fluroquinolone MIC values (*e.g.* efflux, target protection) cannot be excluded, the mutation detected in all resistant isolates is likely to be either the only, or the main reason of their high level of fluroquinolone resistance.

Heat stable antigen based serotyping and molecular typing, *i.e.* flaA PCR-RFLP and PFGE, of the isolates from the United Arab Emirates showed that although the majority of them were heterogeneous, a homogenous cluster representing 19.5% of all isolates was also present (III.).

Of the forty one isolates tested 75.6% were serotypable, representing 10 serogroups. Serogroup HS4,13,16,43,50 (22%) and serotype HS2 (14.6%) were the most frequent ones. The molecular typing techniques, when combined, divided the strains into 30 groups. Eight ciprofloxacin resistant strains isolated over a year-long period of time showed identical PCR-RFLP and PFGE pattern and displayed 100% similarity in the sequenced part of the *gyr*A gene. These facts strongly suggested the presence of a stable clone, *i.e.* a phenomenon rarely seen in this highly variable pathogen. To determine the genetic base of this stability *i.e.* measurement the rate of mutation and/or recombination in this specific group of strains, requires extensive investigation which stretches beyond the scope of this thesis.

4. NEW RESULTS

4.1. An MLST system, based on analysing the sequences of parts of seven house-keeping genes was developed for *Campylobacter jejuni* and successfully tested by investigating strains isolated at different geographical locations (**I.**).

4.2. It was established that although the allelic variations of the seven genes are limited in this species, the number of sequence types based on their combinations approaches the number of strains tested (**I.**).

4.3. Using the homoplasy test of five housekeeping genes we proved that the frequency of recombination in *C. jejuni* exceeds the respective figures found in most pathogens and approaches that seen in *Helicobacter pylori* (**I.**).

4.4. We proved, as suspected before based on studies conducted using home-made sera, that approximately one third of the Hungarian *C. jejuni* isolates are non-serotypable – a figure high by international comparison (**II.**).

4.5. Based on studying Hungarian isolates and ones collected in the UAE it was clearly shown that the non-serotypable group is in fact rather heterogenous when subjected to molecular typing. This bears important practical implications, *i.e.* for their epidemiological studies serotyping always must be complemented by other methods, particularly in countries with high incidence of non-serotypable isolates (**II.** and **III.**).

4.6. It was shown that the rate of fluroquinolone resistance of *Campylobacter jejuni* in the UAE, *i.e.* a frequented tourist destination and a high risk area for campylobacter infections, is one of the highest ever reported (**III.**).

4.7. The genetic basis of fluroquinolone resistance was found to be the Ala → Ile replacement at codon 86 of the *gyrA* gene (**III.**).

4.8. Based on molecular typing and on determination of sequence-based allelic variants of the *gyrA* gene it was found that approximately 20% of the isolates belong to a single strain cluster, probably clone – an unexpected finding in case of a highly variable species. The basis of this stability is yet to be determined (**III.**).

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7. APPENDICES

PAPERS I-III

I.

II.

III.

PUBLICATIONS NOT INCLUDED IN THE THESIS

1. Conlon JM, **Sonnevend A**, Patel M, Camasamudram V, Nowotny N, Zilahi E, Iwamuro S, Nielsen PF, Pal T. : A melittin-related peptide from the skin of the Japanese frog, Rana tagoi, with antimicrobial and cytolytic properties. 2003 *Biochem Biophys Res Commun.* **306**:496-500. (**IF:2.836**)

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CONFERENCE PRESENTATIONS RELATED TO THE THESIS

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2. **Sonnevend A**, Czirók É, Pál T: Molecular, sero-, and antibiotic susceptibility typing of *Campylobacter jejuni* strains isolated in Hungary, 2003 Congress of the Hungarian Society of Microbiology, Balatonfüred, Hungary
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