MOLECULAR TYPING OF *CAMPYLOBACTER JEJUNI*

PhD thesis

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Pécs
2009

INTRODUCTION

Campylobacter jejuni is one of the most common cause of bacterial diarrhea. It is not infrequently followed by late autoimmune neurological sequelae, *i.e.* Guillain-Barre syndrome. In the light of its relatively low infective dose most of the human cases are sporadic, although food- and water-borne outbreaks do occur.

Traditionally, fluoroquinolones have played a prominent role in the treatment of *Campylobacter* infections, however, due to wide-spread resistance this therapeutic option quickly loses its grounds. By mechanisms yet to be elucidated, fluoroquinolone resistance also appears to coincide with increased fitness of the organism. In most of the cases it is due to point mutation in the gyrase A gene. The majority of highly resistant clinical isolates carry the Thr-86 to Ile substitution in *gyrA*. Other common changes reported are the Pro-104 to Ser, the Thr-86 to Lys or to Ala, the Ala-70 to Thr, the Asp-90 to Asn substitutions, respectively. Codons between 69 and 120 contain all mutations described so far conferring resistance represent the Quinolone Resistance Determinant Region (QRDR).

To understand the epidemiology of pathogens it is necessary to type the organisms. The most common phenotypic methods are serotyping, biotyping, protein or fatty acid profile analysis and phage typing. Serotyping has been used extensively together with molecular typing techniques. The molecular techniques usually further divide isolates within serogroups, however, sometimes the opposite may occur *i.e.* isolates exhibiting one molecular fingerprint type can further distinguished based on serotyping of the heat stable (HS) antigens.

A major disadvantage of serotyping is the high number of untypeable strains. Contrary to the apparent uniformity observed it was not unreasonable to assume that this non-typable group in fact consists of a mixture of types: a hypothesis we intended to expose to scientific scrutiny within the current study. *C. jejuni* is one of the most prominent agents of traveler's diarrhea. Countries on the Arabian Peninsula, especially the United Arab Emirates, are highly frequented tourist destination. The region recently was scored as a high risk area for *C. jejuni* infections. This fact and the paucity of local epidemiological and antibiotic resistance data prompted the initiation of some studies of this thesis.

In the recent decades, just like for other pathogens, numerous molecular methods were developed to type *C. jejuni*. The most frequently used ones are the pulsed filed gel electrophoresis (PFGE), the flagellin A gene typing by polymerase chain reaction followed by restriction fragment length profile analysis (PCR-RFLP), the random amplified fragment length polymorphism (RAPD) and the amplified fragment length polymorphism (AFLP) analysis in sequencing gels. All of these methods detect variation indirectly in the DNA sequence, however, direct information on the genetic diversity of the *Campylobacter jejuni* genome was not available. Multi locus sequence typing (MLST) is one of the methods which could provide information on the genetic diversity. The method 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 MLST is useful in studying questions of "long term" or "global" epidemiology. MLST is based on sequencing ~500 bp fragments of a few, usually seven, internal housekeeping genes. MLST of various pathogens revealed different population structures, level of genetic diversity and different rate of mutation and recombination which played role to achieve the detected sequence variations. 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 and describe the genetic variation of the pathogen, and furthermore, to reveal the role of recombination in the development of genetic diversity in *C. jejuni*.

AIMS

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

2. To survey the serotype distribution of human *Campylobacter jejuni* strains isolated in Hungary

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

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

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

MATERIALS AND METHODS

STRAINS AND CULTURE CONDITIONS. Thirty three strains were investigated to assess the allelic diversity of *C. jejuni*, 18 from Germany (5 from Wurzburg, 13 from Freiburg), 6 from Hungary, 5 from Thailand, 3 from the United States and one from the United Kingdom. To investigate Hungarian isolates 92 random selected strains were used isolated in 1999-2000 in county Veszprém (59), in county Pest or in Budapest (33). In the United Arab Emirates 41 strains were collected in Tawam Hospital, Al Ain, Abu Dhabi Emirate between 2002 September and 2004 September.

The identification of the strains was always confirmed by using standard biochemical tests and the MAST-ID CAMP identification system (MAST Group Ltd., Merseyside, UK) in case of Hungarian isolates or API Campy Kit (bioMerieux, France) in the UAE. Strains were routinely grown on Columbia agar (Oxoid, Basingstoke, UK) containing 10% of sheep blood in jars with Campy*Gen* (Oxoid, Basingstoke, UK) gas generating inserts at 36^{o}C for 48 hours and were stored in Tryptic Soy Broth (TSB, Oxoid, Basingstoke, UK) containing 10% glycerol at -80^{o}C.

ANTIBIOTIC SENSITIVITY TESTING. Sensitivity testing – for screening purposes – was carried out by disc diffusion. The minimal inhibitory concentrations (MICs) of nalidixic acid, ciprofloxacin and erythromycin were determined by the agar dilution method using Mueller-Hinton agar (Oxoid) supplemented with Sheep blood 5% (v/v) and incubated under microaerophilic conditions at 36^{o}C for 48 h. The final antibiotic concentrations in the agar plates ranged from 0.25-512 mg/L, according to the CLIS standards, formerly known as the NCCLS (NCCLS, 2003 and 2005). Reference strain *Campylobacter jejuni*, ATCC 33560, was included in each run as control.

SEROTYPING. Serotyping, based on the heat stable antigen of the strains according to Penner and Henessy was carried out by passive hemagglutination using the set of antisera produced by Denka Seiken (Tokyo, Japan) according to the manufacturer's instruction.

DNA PURIFICATION. Genomic DNA was purified with QiaAmp DNA Tissue Kit (Qiagen) according to the manufacturer's instruction.

MULTI LOCUS SEQUENCE TYPING (MLST). Seven fragments of housekeeping genes were selected. Details of the fragments sequenced are shown in **Table 1**. The genes were selected based on the following criteria: they encode housekeeping genes, are widely separated on the chromosome, and are not located in the vicinity of putative virulence genes and outer membrane protein genes. The amplification and direct sequencing of gene fragments was performed using primers shown in **Table 2**.

PHYLOGENETIC STUDIES. Sequences were aligned by using SEQLAB and PILEUP from the Genetics Computer Group (Madison, Wis) Wisconsin Package, version 9.1. All sequences for one gene fragment were reduced to a common length and exported to MSF format. Where necessary, sequences were converted to .meg format. The non-synonymous (K_A) and synonymous (K_S) mutation rate values were calculated with DNASP 3.0. The homoplasy test was performed with HOMOPLASY. The UPGMA (unweighted pair group mean average) tree was constructed by START software.

Table 1. Sequenced gene fragments

Gene	Gene numbering*	Position*	Lenght (bp)	Gene product	GenBank accession code
asd	Cj1023c	954448	564	Aspartate-semialdehyde dehydrogenase	AJ292175–AJ292188
atpA	Cj0105	111788	660	ATP synthase, F1a	AJ292166–AJ292174
ddlA	Cj0798c	748473	480	D-Alanine-D-alanine-ligase	AJ290352–AJ290363
eftS (*tsf*)	Cj1181c	1108052	444	Elongation factor TS	AJ290337–AJ290351
fumC	Cj1364c	1296454	645	Fumarate hydratase	AJ290322–AJ290336
nuoH	Cj1572c	1502750	423	NADH dehydrogenase H	AJ290377–AJ290387
yphC	Cj0386	352112	570	GTP-binding protein	AJ290364–AJ290476

* Numbering and position is based on the *C. jejuni* NCTC 11168 published sequence by Sanger Centre (http://www.sanger.ac.uk/Projects/C_jejuni/).

MISMATCH AMPLIFICATION MUTATION ASSAY (MAMA) PCR. Detection of the Thr-86-to-Ile mutation in the *gyrA* gene was carried out according to previously described method of Zirnstein *et al.* In the system the reverse primer anneals to the target in the presence of ACA→ATA mutation only.

Table 2. Primers used for amplification and for sequencing

Primer	Application*	Sequences
Asd		
CJasd1(1)	A, S	GCA-GGT-GGA-AGT-GTG-AGT-G
CJasd2(2)	A, S	TTT-GTT-GCA-GCA-CCT-ACA-CG
CJasd3(2)	A	ACG-AAT-TTG-ATC-CGC-CAC-AC
CJasd4	A, S	GCC-ATT-GTG-GGT-GCT-ACT-GG
CJasd5	A, S	CGC-TAG-TCA-TTA-AAG-GCA-TAG-G
atpA		
CJatpA1(1)	A, S	GAG-AAG-GTT-TAA-AAG-AAG-GTG-C
CJatpA2(2)	A, S	TGT-AGC-TTT-AAT-TTG-AGC-AGC
ddlA		
CJddla1(1)	A, S	GAT-CAA-TCT-TAT-CCA-TGG-TAG
CJddla2(2)	A, S	AGC-CAA-AGA-ACC-AGG-GTT-TG
eftS (tsf)		
CJefts1(1)	A, S	AAA-GCA-GAT-AGA-CTT-GCT-GC
CJefts2(2)	A, S	TTT-TCA-GGT-TTA-CCT-TGA-GC
fumC		
CJfumC1(1)	A, S	TCG-TGC-CAC-TGA-AAT-CAT-GG
CJfumC2(2)	A, S	ACC-TAT-GTG-TGG-ATT-TAG-AGC
nuoH		
Cjnadh1(1)	A, S	GCA-GCT-ATT-CCT-ATG-CTA-CC
CJnadh2(2)	A, S	TTG-ATC-TGG-ACG-CAA-TTG-CG
yphC		
CJyphc1(1)	A, S	TAT-CAG-AGT-GGG-TAT-TGT-AGG
CJyphc2(2)	A, S	AAT-CAC-TAA-AGG-CAC-ACC-TTC

***** A, amplification; S, sequencing

SEQUENCING OF THE QRDR REGION. A 673 bp long part of the *gyrA* gene, containing the QRDR region, was amplified using primers GZ*gyrA5* and GZ*gyrA6* and directly sequenced by fluorescent-based direct sequencing using the ABI Prism Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer) and an ABI Prism 310 genetic analyzer (Perkin-Elmer) automated sequencing system. For the analysis of the sequences the program MEGA v3.1 was used.

PCR-RFLP. The amplification of the *flaA* gene using consensus primers, and the digestion of the Amplicon with DdeI were carried out as described by Wassenaar & Newell. The digested products were run in 2% agarose gel at 1 volt/cm for 120 minutes.

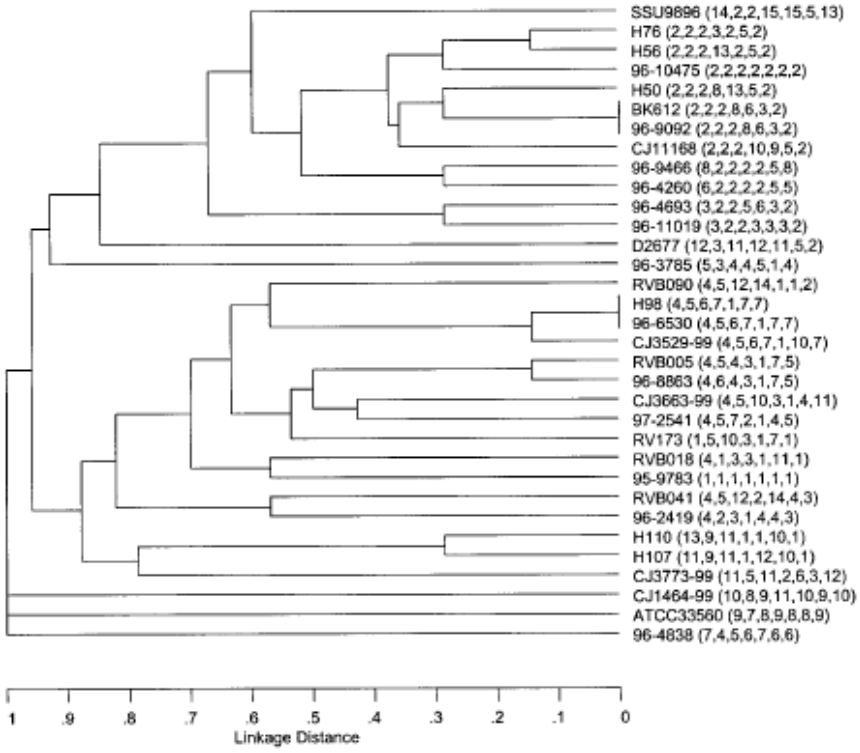
MACRORESTRICTION ANALYSIS AND PULSED-FIELD GELE ELECTROPHORESIS (PFGE). C. jejuni genomic DNA was digested with *Sma*I following the formaldehyde-inactivation of DNase, and the fragments were subsequently separated on a CHEF DRII apparatus (Bio-Rad) as described by "Campynet" PFGE Subtyping Group.

ANALYSIS OF THE PCR-RFLP AND PFGE PATTERNS. The PCR-RFLP and the PFGE macrorestriction patterns of the isolates were compared according to the Dice similarity index (1-1% tolerance interval) using the GelCompare II software. A cluster was arbitrarily defined as strains exhibiting band patterns with at least 95% similarities on the UPGMA tree.

RESULTS

POPULATION GENETIC STUDIES OF CAMPLYOBACTER JEJUNI. Thirty-tree *C.jejuni* strains collected from different continents were analyzed by MLST of seven housekeeping genes. The number of alleles of each genes varied between 9 and 15 (Table 3.), however, the 33 strains were divided into 31 different allelic profile with two pairs exhibiting the same variant, only (**Figure 1.**).

Figure 1. UPGMA dendogram showing the genetic relatedness of the *C. jejuni* examined in the study based on the allelic profile



The numbers in parentheses are showing the allele numbers in the following order: *asd, atpA, ddlA, eftS, fumC, nuoI* and *yphC.*

As it was expected in housekeeping genes, the ratio of non-synonymous mutation was markedly lower than the number of synonymous ones (**Table 3**.). In case of *atpA* gene this ratio could be compared to the fragment of *H.pylori atpA* gene. Twenty strains of that species exhibited 20 allelic variant of *atpA* gene, and the mutation rate of synonymous (K_S=12,3) as well as non-synonymous mutations (K_A=0,26) was noticeably higher.

In order to assess the intraspecies recombination rate Homoplasy test was performed. The H values were compared with other species homoplasy values (*Borrelia burgdorferi* 0,06, *Escherichia coli* 0,26, *Streptococcus pneumoniae* 0,3, *Neisseria meningitidis* 0,34, *H. pylori* 0,65). Genes of *C.jejuni* showed relatively high homplasy altough that value did not reach the one experienced in *H.pylori* (**Table 3**.).

Table 3. The number of allelic variants, mutation rate and homoplasy ratio in housekeeping genes of *C. jejuni (n=33)*

Gene	No. of allels	K_S *	K_A	H ratio**
asd	14	3,8	0,4	0,4
atpA	9	0,86	0,01	
ddlA	12	8,9	0,8	0,42
eftS(tsf)	15	7,1	0,2	0,36
fumC	15	6,4	0,14	0,48
nuoH	11	3,18	0,6	
yphC	13	1,9	0,1	0,47

* Synonymous (K_S) and non-synonymous (K_A) mutation rate (%)

** Homoplasy ratio

SEROTYPE DISTRIBUTION OF THE HUNGARIAN C. JEJUNI ISOLATES. Of the 92 strains tested 64 (69,5%) were typable with the serum set used. The strains could be divided into 17 serogroups (**Table 4**.). The four most frequent serogroups, i.e. HS3, HS2, HS1,44 and HS 4,13,16,43,50, respectively, represented 46,8 % (30 strains) of all typable isolates.

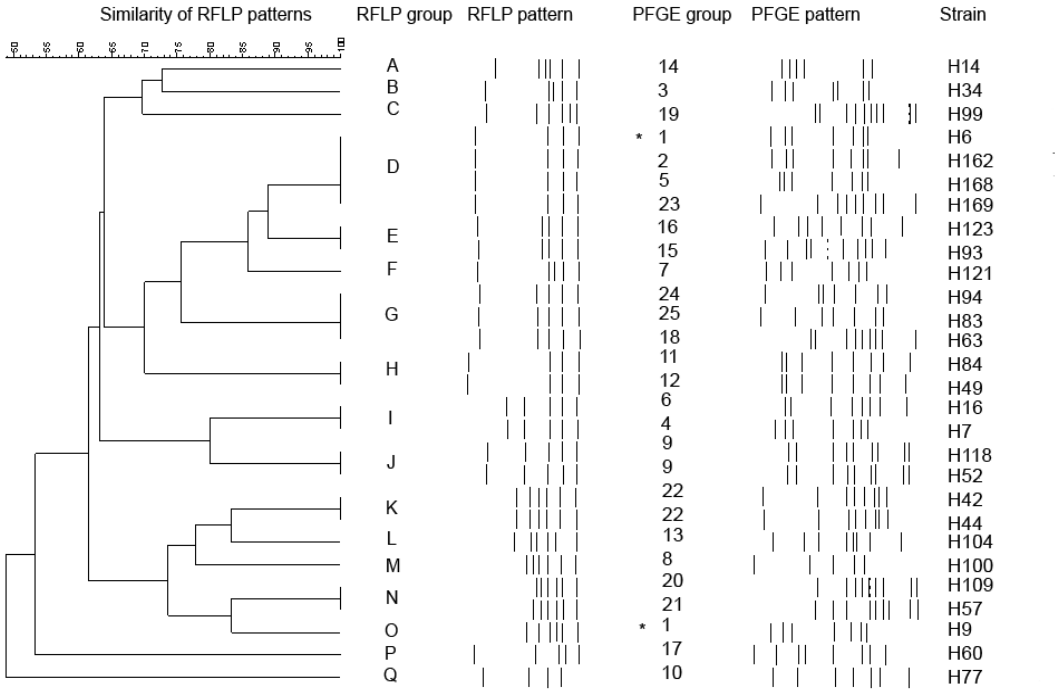
Table 4. Serotype distribution of Hungarian *C. jejuni* isolates

Serogroup	Number of isolates	All strains (*n=92*) percentage	Typable strains (*n=64*) percentage
HS3	9	9.8	14.0
HS2	8	9.7	12.5
HS1,44	8	9.7	12.5
HS4,13,16,43,50	5	5.4	7.8
HS15	4	4.3	6.3
HS31	4	4.3	6.3
HS37	4	4.3	6.3
HS41	4	4.3	6.3
HS5	3	3.3	4.7
HS6,7	3	3.3	4.7
HS18	3	3.3	4.7
HS8	2	2.2	3.1
HS12	2	2.2	3.1
HS23,36,53	2	2.2	3.1
HS11	1	1.1	1.6
HS19	1	1.1	1.6
HS38	1	1.1	1.6
NT*	28	30.4	-

* NT – non-typeable

INVESTIGATION OF STRAINS NON-SEROTYPABLE BASED ON THEIR HEAT-STABLE ANTIGEN. As it is shown in **Table 4**., the largest group was the NT group with 28 isolates (30.4% of all strains studied). This was true irrespective of the area of isolation, i.e. 16 strains from Veszprém County, and 12 from Pest County (27.1% and 36.3% of the local isolates) were NT (data not shown). 17 RFLP groups (A-Q), and 25 PFGE groups (1-25) were established (**Figure 2**.). Accordingly, when the results of the two typing systems were combined the 28 NT strains could be divided into 26 molecular types.

Figure 2. PCR-RFLP and PFGE patterns of non-serotypable isolates



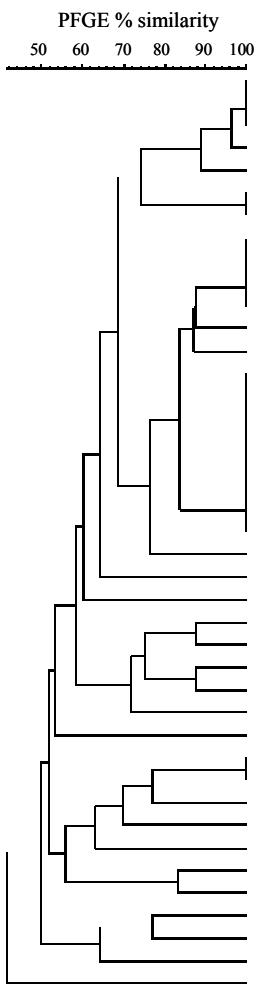
***** indicates a pair of strains with different PCR-RFLP but identical PFGE pattern

ANTIBIOTIC SUSCEPTIBILITY TESTING AND TYPING OF C. JEJUNI ISOLATED IN THE UNITED ARAB EMIRATES. All isolates were sensitive to erythromycin (MIC 0.5-4 mg/L), but 85.4% were resistant to nalidixic acid and ciprofloxacin with MIC values of between 128-512 mg/L and 8-64 mg/L, respectively. A total of 31 (75.6%) strains were serotypable, representing 10 serogroups. Serotype complex group HS4,13,16,43,50 was the most frequently encountered (22%), followed by serotype HS2 (14.6%) (**Figure 3**).

All strains were typable by both PFGE and PCR-RFLP (**Figure 3**). The forty-one isolates belonged to 26 distinct PFGE groups (P1-P26); twenty-one of them consisting of only single isolates. Of the 23 RFLP types (R1-R23) 15 were represented by single isolates, only. Combining the results of the two typing methods strains could be assigned into 30 distinct molecular types (MT1-30). It was noteworthy that the eight isolates with identical PFGE patterns (P7) also exhibited identical RFLP patterns (R13) and formed a homogenous molecular type (MT1) representing 19.5% of the ciprofloxacin resistant strains (**Figure 3**)

Sequencing the *gyrA* between nucleotides 64 and 654 all resistant strains carried the Thr-86-to-Ile mutation as the only amino acid change within the QRDR region (i.e. between codons 69 and 120) (**Figure 3**).

Figure 3. Molecular-, sero-, allel types, antibiotic sensitivity and isolation dates of *Campylobacter jejuni* strains isolated in Al Ain, UAE



Strain	PFGE	RFLP types*	MT	Serotype	MIC (mg L^{-1})† NA	MIC (mg L^{-1})† CIP	*gyrA* allel	Date of isolation
#1437	P1	R12	MT10	NT‡	512	32	G3	2002 Sep
#1576	P1	R1	MT19	HS3	256	32	G6	2003 Oct
#1804	P1	R1	MT19	HS3	256	32	G6	2003 Dec
#127	P1	R10	MT18	NT	512	8	G6	2004 Mar
#1978	P2	R12	MT14	NT	512	64	G3	2003 Dec
#920	P3	R19	MT12	HS15	256	16	G3	2003 May
#1011	P3	R19	MT12	HS15	256	16	G3	2003 Jun
#1525	P4	R6	MT21	HS2	256	32	G8	2003 Oct
#1532	P4	R6	MT21	HS2	256	32	G8	2003 Oct
#773	P4	R12	MT23	HS1,44	256	32	G10	2004 May
#1968	P4	R12	MT23	HS1,44	256	32	G10	2004 Sep
#1731	P5	R20	MT13	NT	256	8	G3	2002 Oct
#5171	P6	R7	MT3	HS4,13,16,43,50	256	16	G1	2004 Jun
#94	P7	R13	MT1	HS4,13,16,43,50	512	64	G1	2003 Mar
#40	P7	R13	MT1	NT	512	64	G1	2003 May
#1395	P7	R13	MT1	NT	512	64	G1	2003 Oct
#118	P7	R13	MT1	HS4,13,16,43,50	128	32	G1	2003 May
#1917	P7	R13	MT1	HS4,13,16,43,50	256	32	G1	2002 Nov
#1498	P7	R13	MT1	HS4,13,16,43,50	256	64	G1	2003 Sep
#1747	P7	R13	MT1	HS4,13,16,43,50	128	32	G1	2003 Nov
#576	P7	R13	MT1	HS4,13,16,43,50	512	64	G1	2003 Apr
#256	P8	R14	MT25	HS8	16	0.5	ND§	2004 Mar
#1536	P9	R21	MT22	HS4,13,16,43,50	512	32	G9	2002 Sep
#102	P10	R4	MT2	NT	256	32	G1	2004 Jan
#1635	P11	R17	MT15	HS2	256	64	G4	2002 Sep
#4941	P12	R10	MT9	HS2	512	64	G3	2004 Jun
#2448	P13	R11	MT6	NT	256	64	G3	2002 Sep
#304	P14	R16	MT26	HS2	16	0.5	ND	2003 Apr
#964	P15	R8	MT11	HS2	256	8	G3	2003 Jul
#1846	P16	R7	MT17	HS1,44	256	16	G5	2002 Nov
#987	P17	R23	MT8	HS21	128	16	G3	2003 Jun
#999	P17	R22	MT24	HS21	256	32	G10	2004 Jun
#1424	P18	R9	MT20	HS4,13,16,43,50	256	16	G7	2002 Sep
#915	P19	R15	MT27	NT	16	0.25	ND	2003 May
#1618	P20	R18	MT28	HS31	8	0.125	ND	2002 Sep
#968	P21	R17	MT4	HS15	256	32	G2	2003 May
#1670	P22	R17	MT5	HS15	256	16	G2	2003 Oct
#25	P23	R2	MT29	HS19	8	0.125	ND	2003 Jun
#370	P24	R3	MT30	NT	16	0.25	ND	2003 Mar
#786	P25	R5	MT16	HS15	256	32	G5	2004 May
#1779	P26	R7	MT7	HS10	512	64	G3	2003 Nov

[1] PFGE: pulsed-field gel electrophoresis, RFLP: restriction fragment length polymorphism of the flaA gene, MT: molecular type (i.e. the combination of PFGE and RFLP types); [2] MIC: minimal inhibitory concentration, NA: nalidixic acid, CIP: ciprofloxacin; [3] NT: not typable; [4] ND: not done; strains marked with thick line represent members of a uniform clone

Surveying the GenBank, only nucleotide sequences identical to allelic variants G3 and G10 were found (AJ567826.1 and AJ567825.1, respectively), the other 8 alleles were deposited in the GenBank (DQ449657-DQ449664). Allelic types G1 and G3 were carried by 10 isolates. Further 2 groups (G6 and G10) contained three strains; three groups (G2, G5, G8) consisted of 2 strains and tree allelic variants (G4, G7, G9) were represented by single isolates, only. The MT1 molecular type carried the same allelic variant of the *gyrA* gene (**Figure 3**).

DISCUSSION

*POPULATION GENETICS OF CAMPYLOBACTER JEJUNI.*The results presented here show that *C. jejuni* has a relatively small pool of unique alleles with few polymorphic nucleotides (**Table 3**.). However, these sequences occur in many different combinations, *i.e.* we observed 31 unique sequence types among the 33 strains (**Figure 1**.). The homoplasy test gave strong evidence that intraspecies recombination plays a major role in generating genetic heterogeneity of *C. jejuni*. The mean *H* ratio observed was higher than values previously reported for highly clonal *Borrelia burgdorferi* or for partially clonal *Neisseria meningitidis*, *Escherichia coli* and *S. pneumoniae.* The only organism in which higher homoplasy ratios were reported is *H. pylori*.

The observation of recombination in *C. jejuni* is in agreement with previous analyses of the two tandem flagellin genes in which both intragenomic recombination and interstrain recombination have been shown to occur. *C. jejuni* and *H. pylori* are phylogenetically closely related gastrointestinal pathogens. Both have comparatively small, AT-rich genomes, are naturally competent for DNA uptake, and contain abundant hypermutatable simple nucleotide repeats that permit switching of genes on or off. However, some features of the population structure of *C. jejuni* described here differ markedly from those of *H. pylori*. In *H. pylori*, it is most unusual to find two unrelated strains with the same nucleotide sequence in any given gene. In a multilocus sequencing study of 20 *H. pylori* strains that included seven housekeeping genes, the number of unique alleles per locus was between 18 and 20. However the number of allelic variants were much lower in *C. jejuni* (**Table 3**.). In *C. jejuni* the frequency of recombination (or, more precisely, the probability that recombinant genotypes remain in the population) is lower than in *H. pylori*. Whether this is due to a lower frequency of DNA transfer events or to ecological differences that imply more effective purification mechanisms is not known. However, recombination in *C. jejuni* occurs frequently enough to create many different combinations of alleles as reflected by the large number of unique sequence types (**Figure 1**.).

A practical implication of this finding is that an MLST approach bears considerable promises to be an efficient typing method for *C. jejuni* molecular epidemiology, despite the overall very low level of sequence diversity. However, the number of strains for any serotype was too small to allow definite conclusions. In fact, another MLST study of 154 strains of *C. jejuni* coming almost exclusively from the United Kingdom has provided evidence for an association of some serotypes with certain clonal lineages.

One possible explanation of low sequence diversity in the *C. jejuni* isolates studied is that *C. jejuni* is a young species and therefore has not yet had enough time to accumulate sequence diversity. Total lack of sequence diversity in the etiological agent of plague, *Y. pestis*, has recently been explained by a very recent emergence of this "species" as a new clone of *Yersinia pseudotuberculosis*.

Another explanation for the relative paucity of sequence variation is that the *C. jejuni* population has recently undergone rapid expansion, possibly driven by the changes in food animal husbandry and slaughtering practices in the last one or two centuries. Such an expansion of clones particularly fit to survive under the conditions of industrialized animal husbandry could explain the limited number of alleles and the low frequency of synonymous nucleotide polymorphisms. Frequent recombination between strains is likely to greatly facilitate the spread of favorable traits such as antibiotic resistance genes in the population.

INVESTIGATION OF THE HUNGARIAN C. JEJUNI ISOLATES. A previous study using home made set of antisera found that one third of the strains were un-typable, a figure higher compared to studies conducted in other countries. The typing set used in that study was made of 60 individual typing sera, while our, commercially available kit contained several pooled groups of antibodies. However, it was still evident that some serogroups, e.g. 2, (**Table 4**.) were frequently encountered in both studies. This is in agreement with previous observations recording that while changes do occur, dominating serotypes can be relatively stable over time in a given geographical area.

The most remarkable similarity between the results of the previous and our investigations was that over 30% of the isolates belonged to the NT group in both studies (**Table 4**.). Using molecular typing techniques, our findings clearly showed that, as expected, the NT group is far from homogenous (**Figure 2.**). The fact that PFGE divided strains into more groups than PCR-RFLP of the *fla*A gene did was not surprising. Macrorestriction followed by PFGE reports on the entire genome while the PCR-RFLP pattern reflects mutational changes in a short stretch of DNA, only. Genomic variations influencing the PFGE patterns may not affect the single locus containing the *fla*A gene.

Serotyping is a useful tool in studying the epidemiology of *C. jejuni* infections, especially when it is combined with molecular typing methods. Our results indicate that due to the high frequency of non-serotypable strains in Hungary, conducting molecular typing of *C. jejuni* is necessary.

CHARACTERIZATION OF C. JEJUNI ISOLATES FROM THE UNITED ARAB EMIRATES. The high incidence of fluoroquinolone resistance found in this study has only been reported from a few locations, like 88%-75% in Spain, 96% in Thailand, 85.9% in Hong Kong, and 77.1% in India.

Data from the Middle East are sparse. A study conducted in 1998 in Lebanon reported 39%, while a recent study from Kuwait found 53%. It should be noted that the results of both the Kuwait and our studies were based on strains collected at single hospital laboratories. Further and more expansive larger scale studies should reveal whether or not they reflect real differences between the Northern-, and South-Eastern parts of the Arabian Peninsula. Earlier, Jumaa & Neringer had reported a 50% fluoroquinolone resistance rate among the *Campylobacter* spp. isolated from humans between 1999 and 2002, from the same hospital laboratory in UAE. Although their data were based on the results utilizing a non-validated disc diffusion assays. However it does not exclude the possibility that a sharp rise in fluoroquinolone resistance had actually taken place in the 2 years that followed their report.

Serotype complex group HS4,13,16,43,50 was the most frequently encountered (22%), followed by serotype HS2 (14.6%); both are the commonly encountered groups in Hungary at other geographical locations, as well (**Table 4**.). Similar to the results of others, we found that the overlap between the results of sero-, and molecular typing was not complete. Identical serogroups were encountered in different P or R groups (*e.g.* HS2 in P4, P11, P12, P14 and P15 or in R6, R8, R10, R16 and R17), while different serogroups could be detected within the same molecular groups (HS2 and HS1,44 in P4 or HS10 and HS1,44 and HS4,13,16,43,50 in R7). The six strains sensitive to fluoroquinolones exhibited no clustering by either the molecular typing methods or by serotyping (**Figure 3**).

All quinolone resistant strains, but none of the sensitive ones, carried the Thr-86-to-Ile replacement which was confirmed by partial sequence determination of gyrase a gene. These data show that, similar to other geographical locations, this mutation is the most commonly encountered one in fluoroquinolone resistant isolates, in the Middle East.

Extensive polymorphism of the *gyrA* gene in *C. jejuni* within, as well as outside of the QRDR region, is well documented. Based on partial sequences, the 35 fluoroquinolone resistant strains encountered in this study carried ten allelic variants of the *gyrA* gene. Only nucleotide sequences identical to two allelic variants were found in the GenBank database. It was noteworthy that all the eight members of the MT1 molecular type carried the same allelic variant of the *gyrA* gene with the unique Ala-206-to-Thr amino acid replacement. The lack of available epidemiological data prevented us from confirming or excluding the possibility of an undetected outbreak in the past involving this clone. Nevertheless, the fact that strains with completely identical genotype *i.e.* PFGE and *flaA*-RFLP patterns and partial *gyrA* sequences, have been isolated during a time period of over one year (Figure 3) suggests that a highly stable ciprofloxacin-resistant clone was identified in this study. These strains of a diverse species were identical even in the non-synonymous mutations not under selective pressure. Clonal complexes can be associated with reservoirs, e.g. poultry, however stable clone of *C. jejuni* from unrelated sourrces was described as well. The reason of such stability, otherwise not charachteristic to this species, is unknown.

NEW RESULTS

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.

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.

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.*

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.

5. It was clearly shown that the non-serotypable group is 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.

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.

7. The genetic basis of fluroquinolone resistance was found to be the Thr → Ile replacement at codon 86 of the *gyrA* gene.

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 ciprofloxacin resistant 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.