Published in final edited form as:

J Appl Microbiol. 2010 February ; 108(2): 591–599. doi:10.1111/j.1365-2672.2009.04444.x.

MLST clustering of *Campylobacter jejuni* isolates from patients with gastroenteritis, reactive arthritis and Guillain–Barré syndrome

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Abstract

Aims—To determine the diversity and population structure of *Campylobacter jejuni* (*C. jejuni*) isolates from Danish patients and to examine the association between multilocus sequence typing types and different clinical symptoms including gastroenteritis (GI), Guillain–Barré syndrome (GBS) and reactive arthritis (RA).

Methods and Results—Multilocus sequence typing (MLST) was used to characterize 122 isolates, including 18 from patients with RA and 8 from patients with GBS. The GI and RA isolates were collected in Denmark during 2002–2003 and the GBS isolates were obtained from other countries. In overall, 51 sequence types (STs) were identified within 18 clonal complexes (CCs). Of these three CCs, ST-21, ST-45 and ST-22 clonal complexes accounted for 64 percent of all isolates. The GBS isolates in this study significantly grouped into the ST-22 clonal complex, consistent with the PubMLST database isolates. There was no significant clustering of the RA isolates.

Conclusions—Isolates from Denmark were found to be highly genetically diverse. GBS isolates grouped significantly with clonal complex ST-22, but the absence of clustering of RA isolates indicated that the phylogenetic background for this sequela could not be reconstructed using variation in MLST loci. Possibly, putative RA-associated genes may vary, by recombination or expression differences, independent of MLST loci.

Significance and Impact of the Study—MLST typing of *C. jejuni* isolates from Danish patients with gastroenteritis confirmed that the diversity of clones in Denmark is comparable to that in other European countries. Furthermore, a verification of the grouping of GBS isolates

compared to RA isolates provides information about evolution of the bacterial population resulting in this important sequela.

Keywords

Campylobacter jejuni; Danish isolates; Guillain–Barré syndrome; multilocus sequence typing (MLST); reactive arthritis

Introduction

Campylobacter jejuni is the leading bacterial cause of gastroenteritis in the industrialized world causing almost 500 million annual cases worldwide (Friedman et al. 2000). In Denmark, the prevalence of campylobacteriosis was 71 cases per 100 000 in 2007 (<http://www.ssi.dk>). The number of laboratory-confirmed cases has increased in Denmark over a 10-year period from 2665 cases in 1997 to 3868 cases in 2007 and currently accounts for more than twice that of notifications of *Salmonella*, consistent with other developed countries. Most *Campylobacter* infections are sporadic and several risk factors have been identified including consumption of raw milk or contaminated water, red meat and poultry, contact with pets (especially birds and cats), and international travel (Kapperud et al. 1992).

The symptoms of campylobacteriosis vary from diarrhoea to severe invasive disease and sequelae including Guillain–Barré syndrome (GBS), a demyelinating disorder resulting in acute muscular paralysis. Affected people develop weakness of the limbs and the respiratory muscles and areflexia (Nachamkin et al. 1998). Approximately one case of GBS occurs for every 1000 cases of campylobacteriosis and of these, 20% are left with some disablement, sometimes needing mechanical ventilation. Approximately 2–3% of cases result in death with many more occurring in the developing countries of world (Willison and O’ Hanlon 2000). GBS is believed to be a result of molecular mimicry of lipooligosaccharide, a part of *C. jejuni* cell envelope, and sugar moieties on nerve gangliosides. Antibodies raised during *Campylobacter* infection containing such ganglioside mimics and can cross-react with gangliosides in the patients and lead to demyelination of nerves and degeneration of axons (Nachamkin et al. 1999; Ang et al. 2004).

Campylobacteriosis is also associated with another immune-mediated sequela, reactive arthritis (RA), a reactive arthropathy. It occurs in between 0.6% and 24% of the patients (Pope et al. 2007). Multiple joints can be affected, in particular the knee joint, with symptoms of pain and incapacitation usually resolving completely within several months (Jansen et al. 2002).

Discriminatory typing methods to study the population genetics of *C. jejuni* isolates are crucial to improve our understanding of the epidemiology and genetic background of this pathogen. Multilocus sequence typing (MLST) has proved to be a valuable typing tool for discriminating *Campylobacter* isolates and defining population structure (Dingle et al. 2001a). MLST is based upon an allelic profile obtained by sequence analysis of seven housekeeping genes. The allelic profile is summarized by a sequence type assigned using an online database (PubMLST). Relatedness can be inferred and isolates can be grouped as clonal complexes. The advantages of MLST, compared to other molecular methods such as

pulse-field gel electrophoresis, are standardized nomenclature, free access to the database and direct comparability of results between different studies/laboratories.

Previous studies have shown that certain genotypes are more common cause of disease in humans while others may be less important (Manning *et al.* 2003; Siemer *et al.* 2004; Sheppard *et al.* 2009a,b). In this study, the genetic diversity among isolates from human disease in Denmark was investigated and the relationship between Sequence types (STs) and Clonal complexes (CCs) and different clinical symptoms of the patients were determined.

Material and methods

Strain collection

During the period 2002–2003 consecutive faecal isolates were obtained in the diagnostic laboratory at Statens Serum Institut (SSI). On a follow up study for sequelae cases of human gastroenteritis ($n = 96$), reactive arthritis ($n = 18$) and GBS ($n = 0$) were defined (Schiellerup *et al.* 2008). Because no GBS cases were detected during the investigation, eight previously described GBS isolates originated from China, Japan and Mexico were included in the study (Engberg *et al.* 2001; Nachamkin *et al.* 2002; Leonard *et al.* 2004). In the phylogeny analysis, additional GBS MLST types were further extracted from the MLST database (PubMLST).

Bacterial growth and preparation of chromosomal DNA

The isolates were cultured on *Campylobacter* blood free medium (mCCDA) agar plates (SSI, LAB112), and subsequently grown on blood agar plates with 5% yeast for 24–48 h at 37 °C under microaerophilic conditions. For isolation of chromosomal DNA, a suspension of *C. jejuni* cells was prepared in 250 µl PBS in a 0.5 ml eppendorf tube. The suspension was vortexed briefly, heated at 100°C for 10 min and centrifuged at 10 000 g for 10 min. The supernatant was removed and stored at –20°C until it was required for PCR amplification.

PCR amplification and sequencing

Internal fragments of seven gene targets (*aspA*; *glnA*; *gltA*; *glyA*; *pgm*; *tkt*; *uncA*) were amplified by PCR with primers stated at the MLST database (PubMLST). The amplification reaction mixture comprised c. 10 ng *Campylobacter* chromosomal DNA, 1 µmol l⁻¹ each PCR primer, 1× PCR buffer, 1.5 mmol l⁻¹ MgCl₂, 0.8 mmol l⁻¹ deoxynucleoside triphosphates and 1.25 U of Amplitaq polymerase. Reaction conditions were 95°C for 3 min, 35 cycles of 94°C for 20 s, annealing temperature for each primer set at 50°C for 20 s, and extension at 72°C for 1 min, with a final extension step for 5 min. PCR product was confirmed by agarose gel electrophoresis. PCR products were purified by precipitation with 20% polyethylene glycol–2.5 mol l⁻¹ NaCl and their nucleotide sequences were determined on each strand with BigDye reaction mix (Applied Biosystems) in accordance with the manufacturers' instructions.

Allele and ST assignment

Sequences were commercially determined on both DNA strands and assembled from resultant chromatograms using the Staden suite of computer programmes (Staden, 1996). Consensus sequences for each allele were assigned an allele number and the 7-locus (3309 bp) ST by interrogation of the *Campylobacter* MLST database (<http://pubmlst.org/campylobacter/>). Novel alleles and STs were submitted to the MLST database to obtain new numbers.

Phylogeny analysis

Profiles of 7-locus allelic were concatenated and used to construct genealogies using two methods for inferring evolutionary relationships among *C. jejuni* STs. First relatedness of isolates was represented by a dendrogram constructed by cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) in the programme START2, available at <http://www.pubmlst.org> (Jolley *et al.* 2001). The second phylogenetic analysis estimated the clonal genealogy of STs using the model-based approach to determine bacterial microevolution: ClonalFrame (Didelot and Falush 2007). This is a model that calculates clonal relationships with improved accuracy as it distinguishes point mutations from imported chromosomal recombination events – the source of the majority of allelic polymorphisms. Analysis was carried out on concatenated sequences representing 51 STs, from 122 isolates from RA, GBS and gastroenteritis. The programme was run with 50 000 burn-in followed by 50 000 subsequent iterations. The consensus trees represent combined data from three independent runs with 50% majority rule consensus required for inference of relatedness.

Because of statistical limitations, we included and compared the typed GBS isolates with the GBS isolates in the PubMLST database that are distributed with the complexes ST-22 (30.5%), ST-21 (16.5%), ST-403 (8%), ST-508, 61 and 42 (5.5%), ST-48, 658, 52, 362, 607 and 206 (2.7%) and isolates currently unassigned to a lineage with 11% (<http://www.mlst.net>).

Statistical analysis

Association between the clonal complex and the clinical diagnosis was assessed by Fisher's exact test (also known as the Fisher–Freeman–Halton test) using software sas (SAS Institute, Cary, NC). Counts from the rare CCs were grouped into category Other.

Results

Diversity of sequence types (ST) and clonal complexes (CC)

Among the 122 isolates, 51 STs clustering in 18 clonal complexes were identified. Three clonal complexes, ST-21, ST-45 and ST-22, predominated and accounted for nearly 64% (78/122). In the ST-21 complex, ST-388 and ST-53 were the most common sequence types each representing 6.6%. The ST-45 complex, the second most common group, was dominated by ST-45 with 13.9%. The ST-22 complex represented only two groups, ST-22 and ST-567, with the ST-22 as main sequence type with 9.9%. Despite the fact that the ST-21 complex was the most frequent group, the subgroup ST-45 in ST-45 complex was

found to be the most common, accounting for 17 out of 122 isolates. A number of isolates were found in CCs only represented by one or two STs (Table 1).

Association between clonal complexes and clinical symptoms

The distribution of the 122 *C. jejuni* isolates and comparison of the gastroenteritis, GBS and RA isolates were investigated (Fig. 1). The isolates from gastroenteritis were represented in all CCs except ST-61. GBS isolates were found in ST-22, 42 and 354 complexes. Isolates from RA were found in the ST-21, 45, 42, 48 and 61 complexes. The isolates from gastroenteritis were most frequently represented with the ST-21 (34%) and ST-45 complexes (23%), GBS isolates with the ST-22 (63%) and ST-42 complexes and RA more evenly distributed with the complexes ST-21 (28%), ST-45 (28%), ST-22 (11%) and ST-48 (11%).

The clonal complex frequencies varied within the three clinical outcome groups. For most frequent CCs appearing in at least two different clinical diagnosis types, the significance of association with diagnosis was confirmed by Fisher's exact test. Both the observed frequency and the frequency expected under the hypothesis of no association (homogeneity) are given (Table 1). The cell frequencies show far more observed ST-22 and ST-42 complexes than expected among GBS patients. Fisher's exact test demonstrates a significant association between the CC and the diagnosis ($P = 0.0022$).

Phylogeny and clustering of the isolates

The UPGMA dendrogram cluster analysis (Fig. 2) showed a grouping of isolates in relation to clonal complexes (shown with brackets). The sequelae (GBS and RA), marked in bold, were not found to clearly cluster within these groups. There is, however, some evidence of clustering of GBS strains within the ST-22 complex even though there is a limited number of isolates. On the clonal frame genealogy (Fig. 3), the three different symptoms are found to be distributed evenly in the tree, not indicating any clustering of CCs or STs according to symptoms.

Discussion

We have examined the association between *C. jejuni* genotypes and different clinical sequelae. *Campylobacter jejuni* isolates from a range of sources, geographical locations in Denmark and patients diagnosed with either gastroenteritis or RA were discriminated and formed the basis of our study. GBS isolates were added to the dataset from previously described cases outside the studied population. By using MLST, the Danish isolates proved to be highly diverse with a total of 51 sequence types belonging to 18 clonal complexes. This finding is consistent with other studies examining *C. jejuni* isolates from a single geographic location (Duim *et al.* 2003; Mickan *et al.* 2007). However, our MLST data also identified a number of frequently described sequence types that have formerly been associated with human infection (Manning *et al.* 2003; Dingle *et al.* 2005; Sheppard *et al.* 2009b).

The ST-21 complex was particularly common with 38%, similar to previous studies, where it accounted for up to 20–33% of the isolates (Dingle *et al.* 2001a; Schouls *et al.* 2003;

Sopwith *et al.* 2006; Karenlampi *et al.* 2007). The prevalence of the ST-21 complex in Danish isolates is also found to be consistent with the MLST surveys of *Campylobacter* in other European countries, (Duim *et al.* 2003; Mickan *et al.* 2007; Kwan *et al.* 2008; McTavish *et al.* 2008). The other major clonal complex in our study, the ST-45 complex, accounted for approximately one quarter of the isolates and the ST-48 complex was found with the frequency of 6.6%. These complexes have also been identified in other countries and from multiple sources (Dingle *et al.* 2001a; McTavish *et al.* 2008). Isolates from patients diagnosed with RA were not found to be associated with one or few of the clonal complexes. Therefore, we suggest that specific RA features rather involve differential expression of virulence factors that might be revealed by expression analysis of RA isolates or because of rapid recombination of disease associated genes. Furthermore, host specific genetics might be involved.

The ST-22 complex accounted for 10% of the isolates and interestingly it was significantly overrepresented in the collection of GBS isolates both in our study as well as in the PubMLST database. The ST-22 complex was also described in isolates from several countries and animal sources (Duim *et al.* 2003; Kwan *et al.* 2008), but not as frequently as the ST-21 and ST45 complexes. A study by Dingle *et al.* 2001b suggested a possible relatedness between the ST-22 complex and GBS isolates and this notion is supported by our data. Furthermore, the authors found that the ST-45 complex was underrepresented among GBS isolates (Dingle *et al.* 2001b). Once more our data and the GBS collection in the MLST database confirm this by the fact that to date no GBS isolates carry the ST-45, despite the fact that this is the most common sequence type identified in our study. The association between GBS and the two clonal complexes ST-22 and ST-42 could be explained by a more frequently expression of the GBS-related Gm1 gangliosides, but we have no evidence of this. By comparison of the Danish isolates with the PubMLST database (including the global isolates), we found that the Danish isolates are similar to those obtained from other parts of the world and, therefore, geographical location of the isolates is not correlated with sequence type. In addition, the global GBS isolates have been compared with the GBS isolates in our study and shows the same results. In an earlier study, the GBS isolates were analysed by other methods and the population was found to be heterogenic (Engberg *et al.* 2001). Further analysis of the GBS isolates by DNA microarrays confirmed significant genomic heterogeneity among the isolates (Leonard *et al.* 2004). Despite these results, we believe that the results presented here together with those of others (Dingle *et al.* 2001b) suggest a possible correlation between certain complexes such as ST-22 complex, and the development of GBS.

Acknowledgements

We would like to thank Christina C. Vegge from University of Copenhagen and Frances Colles, Roisin Ure and Keith Jolley from University of Oxford for helpful advice. Furthermore, Azra Kurbasic, SSI, for suggestions regarding the statistical analysis.

This publication made use of the *Campylobacter* MultiLocus Sequence Typing website (<http://pubmlst.org/campylobacter/>) developed by Keith Jolley, sited at the University of Oxford, United Kingdom. Lene Nørby Nielsen was partially supported by the Research School for Biotechnology (FOBI). K.A. Krogfelt and L.N. Nielsen are members of MedVetNet, Network of excellence, supported by FP 7.

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Figure 1. Distribution of the CCs among the 122 isolates with different clinical outcomes in this study.

Gastroenteritis are shown in grey, Guillain-Barré syndrome (GBS) in white and reactive arthritis in black. The sequence types (ST)-21 complex are shown to be the most frequent clonal complex followed by the ST-45 and ST-22 complexes. The GBS isolates are represented in the ST-22, ST-42 and ST-354 complexes and found to be significantly overrepresented in the ST-22 complex.

Figure 2. Dendrogram demonstrating the phylogenetic relationship between the 122 isolates. Majority of strains were from patients with gastroenteritis, strains from patients with sequelae [Reactive arthritis (RA) and Guillain–Barré syndrome (GBS)] are marked in bold. The isolates clustered in relation to clonal complexes (marked with brackets). A minor clustering of GBS to ST-22 was observed.

Figure 3. Clonal frame tree demonstrating the genetic relationships of sequence types between *Campylobacter jejuni* isolated from patients with gastroenteritis (GI), Reactive arthritis (RA) and Guillain-Barré Syndrome (GBS).

GI are shown as light grey, GBS white and RA dark grey. Combinations of sequelae are shown as GI + RA + GBS with squares and GI + RA with lines. The clonal frame tree includes GBS isolates from the multilocus sequence typing database (PubMLST).

Table 1
Sequence types and clonal complexes among 122 human isolates grouped as clinical disease and results in parenthesis from Fisher's exact test ($P = 0.0022$) for the most frequent CCs (21, 22, 42 and 48).

For CCs with more than one isolate, the accumulated value is shown in the row for the last isolate

Isolate number	asp	gln	glt	gly	pgm	tkl	unc	ST [‡]	CC [§]	diagn.	ST freq. (FE)
1575, 1576, 1580, 1581	1	3	6	4	3	3	3	22	22	GBS	4
1577	1	3	6	4	3	3	1	567	22	GBS	1 (0-918)
1579, 1582	1	2	42	4	256	9	8	1672	42	GBS	2 (0-33)
1578	8	10	16	2	11	12	6	2049	354	GBS	1 (1 -90)
1319	7	1	52	83	2	3	6	3708	UN [¶]	RA	1
1372	33	160	30	79	19	62	5	3614	UN	RA	1
1209	33	160	30	79	104	62	5	UN	UN	RA	1
1152, 1336	2	1	1	3	2	1	5	21	21	RA	2
1142, 1149, 1172	2	1	21	3	2	1	5	53	21	RA	3(5-61)
1015, 1313	1	3	6	4	3	3	3	22	22	RA	2 (2-06)
1092, 1231	4	7	10	4	1	7	1	45	45	RA	2
1037	4	7	10	4	42	7	1	137	45	RA	1
1014, 1046	4	7	10	10	2	3	1	2589	45	RA	2 (4-13)
1093, 1094	2	4	1	2	19	62	5	2955	48	RA	2 (1-18)
1312	1	4	2	2	6	3	1	219	61	RA	1
1515	2	1	12	121	2	1	5	3709	UN	GI	1
1417, 1418, 1566	2	1	1	3	2	1	5	21	21	GI	3
1535, 1551	2	1	12	6	2	1	5	24	21	GI	2
1540, 1542	8	1	6	3	2	1	1	44	21	GI	2
1032, 1525, 1567, 1571	2	1	12	3	2	1	5	50	21	GI	4
1526, 1543, 1544, 1545, 1548	2	1	21	3	2	1	5	53	21	GI	5
1058, 1530, 1550	2	1	5	2	2	1	5	251	21	GI	3
1025, 1076, 1416, 1422, 1553 [*]	43	1	1	3	2	1	5	388	21	GI	8
1517	2	1	5	3	3	1	5	815	21	GI	1
1506	2		12	3	2	1	5	1461	21	GI	1
1520	2	1	1	4	1	62	5	1820	21	GI	1
1516	2	1	177	3	2	1	5	1823	21	GI	1
1565	2	1	4	3	2	1	5	2057	21	GI	1 (29-9)
1047, 1049, 1084, 1528, 1564, 1574	1	3	6	4	3	3	3	22	22	GI	6
1519	1	2	42	4	3	3	3	2615	22	GI	1 (11-02)
1023, 1533, 1539	1	2	3	4	5	9	3	42	42	GI	3 (3-93)
1006, 1091, 1507, 1508, 1510 [‡]	4	7	10	4	1	7	1	45	45	GI	15
1028, 1531, 1532	4	7	10	4	42	7	1	137	45	GI	4
1509, 1511	4	7	10	4	42	51	1	583	45	GI	2
1504	4	2	10	4	1	7	1	782	45	GI	1

Isolate number	asp	gln	glt	gly	pgm	tkl	unc	ST [‡]	CC [§]	diag.	ST freq. (FE)
1549	4	7	10	4	1	7	4	2406	45	GI	1 (22-03)
1057, 1537	2	4	1	2	7	1	5	48	48	GI	2
1524	7	4	5	2	11	1	5	429	48	GI	1
1500	2	4	1	4	19	62	5	475	48	GI	1
1033, 1043	43	4	1	2	7	1	5	505	48	GI	2 (6-29)
1523	3	1	5	17	11	11	6	49	49	GI	1
1569	9	25	2	10	22	3	6	52	52	GI	1
1534	2	21	5	3	23	1	5	290	206	GI	1
1027, 1522, 1554, 1562	9	2	4	62	4	5	6	257	257	GI	4
1513	9	17	5	62	4	5	6	2543	257	GI	1
1074	7	1	2	2	4	3	6	82	353	GI	1
1538	7	17	5	2	10	3	6	353	353	GI	1
1512	9	112	5	2	11	3	6	936	353	GI	1
1527	7	2	5	2	2	3	6	2132	353	GI	1
1071	7	17	5	2	13	3	6	3510	353	GI	1
1552, 1568	8	10	2	2	11	12	6	354	354	GI	2
1502, 1536	10	27	43	19	6	18	7	270	403	GI	2
1570	7	17	2	15	23	3	12	51	443	GI	1
1518	7	17	16	15	23	3	12	2687	443	GI	1
1298	1	6	60	24	12	28	1	508	508	GI	1
1501	10	1	50	99	120	76	52	678	677	GI	1
1503, 1561	10	81	50	87	120	76	52	794	677	GI	2
1573	2	1	29	28	58	25	58	1332	1332	GI	1

CC, clonal complexes; ST, sequence types; GBS, Guillain-Barre syndrome; GI, gastroenteritis; RA, reactive arthritis.

* +1557, 1563, 1572.

† +1514, 1521, 1529, 1541, 1547, 1555, 1556, 1558, 1559, 1560.

‡ Sequence type.

§ Clonal complex.

¶ UN, currently unassigned to a lineage.