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***Campylobacter* genotypes from food animals, environmental sources and clinical disease in Scotland 2005/6**

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Abstract

A nationwide multi-locus sequence typing (MLST) survey was implemented to analyze patterns of host association among *Campylobacter jejuni* and *Campylobacter coli* isolates from clinical disease in Scotland (July 2005–September 2006), food animals (chickens, cattle, sheep, pigs and turkey), non-food animals (wild birds) and the environment. Sequence types (STs) were determined for 5247 clinical isolates and 999 from potential disease sources (augmented with 2420 published STs). Certain STs were over represented among particular sample sets/host groups. These host-associated STs were identified for all sample groups in both *Campylobacter* species and host associated clonal complexes (groups of related STs) were characterized for *C. jejuni*. Some genealogical lineages were present in both human disease and food animal samples. This provided evidence for the relative importance of different infection routes/food animal sources in human disease. These results show robust associations of particular genotypes with potential infection sources supporting the contention that contaminated poultry is a major source of human disease.

Keywords

MLST; *Campylobacter jejuni*; *Campylobacter coli*; Host association

1. Introduction

Campylobacter is the most common bacterial cause of gastroenteritis in the industrialized world, with economic costs estimated at US \$4.3 billion in 1997 (Buzby et al., 1997). Human campylobacteriosis is caused principally by *C. jejuni* and *C. coli* (Friedman et al., 2000). Consumption of contaminated chicken, red meat, water, milk, and contact with pets and farm animals have been implicated as potential sources of human *Campylobacter* infection (Friedman et al., 2000; Kapperud et al., 2003). Recent work focuses upon characterizing the relative contributions of different sources to the human disease burden (Sheppard et al., 2009). Microbial typing techniques can allow isolates to be grouped on the

basis of genotype, potentially enabling the identification of host-associated lineages from possible food chain sources (Sheppard et al., 2009).

It is known that *Campylobacter* populations differ among host species and environmental niches (McCarthy et al., 2007). In the past this was poorly understood in large part because of the lack of uniform and common typing approaches allowing comparison of different studies. Population studies combined with epidemiological analyses of bacteria require the characterization of large numbers of isolates and to achieve this classification should be (i) unambiguous, (ii) reproducible among laboratories, (iii) rapid/scalable (high-throughput), and (iv) cost effective. Nucleotide sequences are the ideal data for detailed population and evolutionary analyses, being both highly discriminatory and reproducible (Maiden, 2006).

Multi-locus sequence typing (MLST) approaches have become increasingly common and have been effectively implemented for research on the population structure and molecular epidemiology of numerous bacterial species (Maiden et al., 1998) including *Campylobacter* (Dingle et al., 2001a,b; Miller et al., 2005). This information can be shared via publicly accessible online databases (Jolley et al., 2004a,b) and there is an increasing body of literature in which MLST is applied to *Campylobacter* isolates of human, animal and environmental origin (Clark et al., 2005; Colles et al., 2003; Dingle et al., 2001a,b; Dingle and Maiden, 2005; D'Lima et al., 2007; Fearnhead et al., 2005; French et al., 2005; Karenlampi et al., 2007; Kinana et al., 2006, 2007; Mickan et al., 2007; Miller et al., 2006; Ogden et al., 2007; Salis et al., 2003a,b; Sheppard et al., 2008; Sopwith et al., 2006). It is possible to differentiate between *C. jejuni* and *C. coli* populations from some food animal sources using MLST data (Colles et al., 2008; McCarthy et al., 2007; Miller et al., 2006) and there is, therefore, the potential to improve the understanding of *Campylobacter* ecology and to identify the sources of human infection.

The aim of this study was to implement a high-throughput MLST genotyping protocol to enable the national-scale characterization of *C. jejuni* and *C. coli* genotypes from clinical cases of campylobacteriosis, food animals and environmental sources. The data can then be used to test the extent to which host/source associated genetic lineages exists and describe genotypes that are principally found among certain isolate groups. Comparison of this data to clinical isolates provides insight into the potential reservoirs for human infection which will in turn allow the introduction of effective mitigation strategies.

2. Materials and methods

2.1. Microbiology

Clinical stool (5674), animal faeces (2644) and food samples (394) were collected as part of a Food Standard Agency Scotland funded *Campylobacter* MLST project in Scotland (CaMPS). Feed animal and environmental isolates represented a large dataset from a wide range of hosts, geographical areas and time periods. A structured sampling protocol involved the rotation of sampling in a 15-month period (July 05-September 06) between rural (North East and South West) and urban (Aberdeen, Edinburgh and Glasgow) locations. Rural farms were selected randomly and urban sampling centred on areas where animal faeces coincided with human activity (parks) and food sampling of various meat types (retail chicken and livers from ox, sheep and pig) from retail outlets including supermarkets and butchers.

Food and fresh faecal mammalian (25 g) and avian (<5 g) grab samples were collected. Smaller avian samples were collected using a sterile swab moistened with saline. Samples were transported chilled (4 °C) to the laboratory for immediate analysis. Faecal aliquots and swabs were homogenised (1:9) in *Campylobacter* enrichment broth (see below) and 0.1 ml

decimal dilutions plated directly onto charcoal cefoperazone deoxycholate (CCD, CM0739, Oxoid, UK) agar for target enumeration. The presence or absence of *Campylobacter* was ascertained by incubating the remaining enrichment broth microaerobically (2% H₂, 5% O₂, 5% CO₂, balance N₂) (100-ml volumes of nutrient broth base (Mast, Bootle, UK) with 5% horse blood, growth supplement (Mast Selectavial SV61), amphotericin (2 µg/ml), cefoperazone (15 µg/ml) and trimethoprim (10 µg/ml)) at 37 °C. After 6–8 h enrichment, two additional antimicrobials (polymixin B (2500 IU/l) and rifampicin (5 µg/ml) were added, and the broths incubated for a further 5 days. All antimicrobials were purchased from Sigma-Aldrich UK. Enrichment broths (0.1 ml) were plated, after 2 and 5 days, on CCD agar (Oxoid, UK) and incubated microaerobically at 37 °C as in previous studies (Humphrey et al., 2005). Colonies were presumptively identified as *Campylobacter* microscopically (Gram stain) and by agglutination with Microscreen latex (Microgen, Camberley, UK) followed by PCR amplification using the pgm primers specific for *C. jejuni* and *C. coli* (Dingle et al., 2001a,b). Individual colonies from direct stool culture (clinical isolates) and enrichments (animal faeces and food isolates) were stored (–80 °C, nutrient broth with glycerol added to 15% (v/v)) for MLST. *Campylobacter* presence was subsequently confirmed by MLST of the remaining 6 loci and ST was used to assign species designation (*C. jejuni* or *C. coli*).

Clinical *Campylobacter* isolates were collected in the 15 health board regions in Scotland (Fig. 1). Isolates were received on charcoal transport swabs from the 28 diagnostic laboratories. As for animal faeces and food samples, clinical isolates from faeces were incubated microaerophilically and cultured on mCCD. Cultured samples were divided providing cells for DNA extraction and archiving at –80 °C. in 15% glycerol. Sample information, for example source species or health board, were recorded and isolates were given a unique identifier code.

2.2. Multilocus sequence typing (MLST)

Isolates were recultured on CCD agar under microaerobic conditions (2% H₂, 5% O₂, 5% CO₂, balance N₂) for 48 h at 37 °C and DNA was prepared initially as a boilate in phosphate buffered saline or, later in the project, using a CHELEX resin method (BIO-RAD, USA). Template DNA arrays were prepared in 96-well polypropylene plates (Abgene, UK) and seven-locus MLST was carried with published primers (Miller et al., 2005) using a high-throughput protocol. The first phase was carried out using a Roboseq 4204 SE robot (MWG Biotech Inc.) and incorporated a disposable-tip liquid handling step in the transfer of template DNA and PCR reagents, to reduce the possibility of PCR template cross-contamination. Seven separate PCR amplifications, one per locus, were carried out using an incorporate thermal cycler as well as other PCR blocks using published primers, reagent concentrations and cycle parameters (Dingle et al., 2001a,b; Gormley et al., 2008; Miller et al., 2005). PCR products (5 µl) were visualised with ultraviolet transillumination following electrophoresis at 200 V (10 min) on a 1% (w/v) agarose gel in 1× TAE buffer (1 mM EDTA, 40 mM Tris-acetate). The amplification products were purified by precipitation with 20% polyethylene glycol-2.5 M NaCl (Embley 1991). A different robotic system was used for liquid handling in sequencing PCRs as there was no requirement for disposable pipette tips. Template DNA plates were transferred to a TheOnyx Liquid Performer Robot (MWGt—Genomic Technology, UK) with incorporated Cytomat™ plate transfer system. Nucleotide sequencing PCRs were performed in both directions with the same primers, diluted in water. Reactions were carried out in 10 µl volumes containing 2 µl of PEG precipitated DNA resuspended in water, 1.0 µl 5× buffer, 0.02 µl BigDye Terminator v3.1 mix (Applied Biosystems, UK) and 0.1 µM of either the forward or the reverse primer. The DNA and primer concentrations were adjusted according to the strength of the PCR product. Cycling parameters were as follows: 30 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for

2 min. Unincorporated dye terminators were removed by precipitation of the termination products with 95% ethanol, and the reaction products were separated and detected with an ABI Prism 3730 automated DNA sequencer (Applied Biosystems, UK). Forward and reverse sequences were assembled from the resultant chromatograms using the Staden suite of computer programs (Staden, 1996). Consensus sequences for each allele were assigned an allele number and the 7-locus (3309 bp) allelic profile was assigned a sequence type (ST).

2.3. Bioinformatics

A database, was developed in the framework provided by the existing *Campylobacter* profiles database [<http://pubmlst.org/Campylobacter/>] using mlstbNet (Jolley et al., 2004a,b), to provide access to genotype information.

Additional genotype information from food animal and environmental isolates was included from published sources (Dingle et al., 2005; McCarthy et al., 2007; Miller et al., 2006; Thakur and Gebreyes, 2005). These comprised 2420 STs.

2.4. Genealogical analysis

The clonal genealogy of the STs was estimated using a model-based approach for determining bacterial microevolution: CLONALFRAME (Didelot and Falush, 2007). Using this model, clonal relationships are calculated with improved accuracy compared with standard phylogenetic inference techniques for recombining bacteria because the two major sources of allelic polymorphisms (point mutation and recombination) are distinguished. This model has been used successfully to distinguish clades within *Campylobacter* species (Sheppard et al., 2008) and can describe the relationships between genotypes. Analysis was carried out on all the STs from clinical campylobacteriosis and on *C. jejuni* and *C. coli* separately. In each case, 7-locus STs were concatenated and the program run with 50,000 burn-in iterations followed by 50,000 data collection iterations. The consensus trees represent combined data from three independent runs with 75% consensus required for inference of relatedness.

3. Results

3.1. Food, animal isolates

A total of 999 isolates, from 3038 samples from potential source populations, were typed at 7 loci. These were augmented with archive data (2420) to give a total of 3419 isolates in the database. There were 170 wild bird (faeces) and 91 environmental (sand and surface waters) *C. jejuni* isolates included in the study. Different host groups have distinct sequence types (Colles et al., 2008; McCarthy et al., 2007) and, while there is overlap, the association of different STs with particular host sources makes it possible to estimate the origin of human infection. Here, the focus is upon sequence type associations that are robust to variation in sample collection and, as in previous studies (McCarthy et al., 2007), isolates from food animals were grouped under the heading of the animal species irrespective of the sample source (faeces/food swab), as in a previous study (Sheppard et al., 2009). 586 cattle, 1288 chicken, and 249 sheep *C. jejuni* isolates were typed and 86 cattle, 459 chicken, 57 sheep, 322 pig, and 111 turkey were typed for *C. coli*.

3.2. Clinical isolates

There were 5674 isolates from confirmed clinical cases of campylobacteriosis, received from 15 health board regions in Scotland between July 2005 and September 2006 (Fig. 1). Of these, 427 isolates were excluded because they comprised mixed cultures, incomplete typing or other *Campylobacter* species such as *C. lari* and *C. upsaliensis*. Of the remainder

(5247), MLST confirmed that approximately 90% were *C. jejuni* and 10% were *C. coli*. The attack rate varied between health boards (Table 1) ranging from 0–136 per 100,000 people based on 2006 population census data.

3.3. ST variation in food and animals

The total number of STs varied among *C. jejuni* (chicken=407, cattle=131, sheep=72, environment=72, wild bird=57) and *C. coli* (chicken=128, cattle=18, sheep=7, pig=146, turkey=29) isolates included in the study. The frequency of STs varied between sample groups (Figs. 2 and 3). ST-45 was common in all *C. jejuni* sample groups and all food animals also had STs from the ST-21 and ST-48 clonal complexes. The distribution of ST frequencies among *C. jejuni* isolates were distinctive with ST-1286 (environment) and ST-220 and ST-637 (wild birds) being uniquely abundant in these sample pools. Chicken isolates exhibited many of the same STs as cattle (at low levels) but were particularly characterised by the abundance of ST-50, ST-137, ST-257, ST-354, ST-443, ST-573, ST-574. ST associations were observed among cattle and sheep, in particular ST-262, ST-42, ST-61 and ST-206 were common among these sample groups. Among the *C. coli* there was less evidence of shared STs among sheep and cattle as different STs were abundant in all of the sample groups with the exception of ST-825 and ST-827 which are both common in chicken and sheep isolates. The highest diversity was observed among chicken and pigs but the sample size is lower than for *C. jejuni*. All of the species had abundant STs within the ST-828 complex but the ST-1150 complex is almost exclusively associated with turkey with only three chicken isolates with STs from this complex.

The proportion of each clonal complex among each host/source population varied for *C. jejuni* and *C. coli* (Fig. 4). In *C. jejuni* this provides evidence of the shared clonal complexes, such as ST-21 complex, and also demonstrates that the pattern of host association extends beyond the level of ST with distinct clonal complexes characterising chicken (ST-257, ST-443, ST-573, ST-574 complexes), wild bird (ST-179, ST-1275 complexes) and cattle/sheep (ST-61, ST-206, ST-403 complexes). Environmental isolates also have two clonal complexes (ST-177 and ST-682 complexes) that are not abundant in any other group but there were fewer isolates and it does not represent a discrete sample group, effectively representing faecal contamination from multiple sources, so these could originate in another host reservoir. STs from these clonal complexes have been isolated starling faeces (Colles et al., 2008).

3.4. ST variation in clinical isolates

The human isolates were diverse for both *Campylobacter* species (Fig. 5). With few exceptions, the *C. coli* STs exhibited only limited genotypic sub-structure reflecting the dominance of STs belonging to the ST-828 complex in the sample collection. Among the *C. jejuni* isolates, genealogical analysis provided evidence of population structure within species (Fig. 5). Clonal complexes were matched onto the genealogy showing good agreement and the proportion of human cases resulting from each clonal lineage was indicated (Fig. 6). The distribution of the abundance of each clonal lineage among the potential reservoir populations was uneven. Three (ST-21, ST-45 and ST-48 complexes) of the four most common clonal complexes among human disease were common among several host/source populations. The fourth, ST-257 complex is associated with chicken, as are ST-354, ST-443, ST-573, ST-283, ST-353, ST-574, ST-661, and ST-607 complexes. The ST-206, ST-61, ST-42, and ST-403 complexes are more common among cattle and sheep and there was no evidence of clades of related genotypes, among human disease, that are exclusively associated with wild bird or environmental sources.

4. Discussion

The analysis of these data, using genealogical approaches, gave results consistent with those using statistical attribution methods (Sheppard et al., 2009) confirming the association of *Campylobacter* STs with different host/source species. Among the *C. jejuni* isolates, several clonal complexes were associated with more than one sample population, for example the ST-21, ST-45 and ST-48 complexes. This may limit the ability to assign a genotype or lineage to a particular host without the use of quantitative probabilistic assignment model-based approaches (McCarthy et al., 2007; Sheppard et al., 2008) such as structure (Pritchard et al., 2000). However, in this study we show that variation in the frequency of several STs and clonal complexes allows the distinction to be made between isolates from chickens, ruminants and wild birds. The associations were determined despite the temporal and geographical differences in samples demonstrating the robustness of the host associations and providing a basis for epidemiological investigations.

Campylobacter, like other zoonotic bacteria, have the potential to migrate between hosts and for this reason complete separation of genotypes is unlikely. Furthermore, recent work suggests that host ecology can have a profound effect upon the evolution of these organisms and it has been suggested that recombination of *Campylobacter* in a farm setting is driving the convergence of *C. jejuni* and *C. coli* species (Sheppard et al., 2008). The distinction between *C. jejuni* STs from cattle and sheep is not as strong as between other host/source groups, as has been previously reported (McCarthy et al., 2007). This may be because isolates from these host groups share a common gene pool indicative of ancestral interdependence or that there are no complete barriers to recombination (physical, mechanistic and selective) between these groups. For example, cattle and sheep may occupy a similar space (field) and are physiologically more closely related to one another than to chicken so that an immigrant *Campylobacter* needs to adapt less to the relatively similar niche.

The number of unique STs for each sample group reflected the genetic diversity of *Campylobacter*. The dataset used in this study was large, particularly when augmented with published STs. Classifying STs into clonal complexes that group genotypes that have expanded in a particular niche and are preferentially recombining with one another, enables comparison between sample groups. *C. coli* had little differentiation at the level of clonal complex but in *C. jejuni*, clonal complexes exhibited host association. STs and complexes associated with particular sample groups have been previously reported (Colles et al., 2008; Dingle et al., 2001a,b) but the large reference population from a full range of potential disease reservoirs can enable comparison with genotypes from human disease.

Clinical bacterial isolates are routinely cultured to determine the cause of gastroenteritis but this analysis is usually limited to the identification of the disease agent. Genotyping of clinical isolates is an increasingly common component of disease investigation and here we show the practicability of routine genetic screening in a public health setting for enhanced surveillance of campylobacteriosis. The combination of comprehensive sampling of clinical isolates, typing of contemporaneous samples from potential source populations, and access to a large genotype data archive allowed inference of the source of disease. In our analysis, chicken associated genotypes were over-represented among isolates from clinical infection with 9 out of 19 of the major disease causing lineages being associated with chicken. In fact, with the exception of the ST-206, ST-61, ST-42, and ST-403 complexes (ruminant genotypes), all of the lineages that were responsible for more than 1% of human disease are common among STs from chicken isolates.

The observation that chicken-associated genotypes are common among human disease isolates is consistent with that from other studies where typing has indicated that strains from chicken carcasses are often linked to human campylobacteriosis (Kramer et al., 2000; Nielsen and Nielsen, 1999; Saito et al., 2005; Sheppard et al., 2009). There is also evidence from analytical epidemiology studies implicating contaminated poultry meat as a major route of infection. Risk assessment (Mylius et al., 2007; Nauta et al., 2007), outbreak investigation (Friedman et al., 2000) and case-control studies (Endtz et al., 2003; Ethelberg et al., 2005; Evans et al., 2003; Friedman et al., 2004; Neimann et al., 2003; Nygard et al., 2004; Potter et al., 2003; Rodrigues et al., 2001; Schonberg-Norio et al., 2004; Stafford et al., 2007) all point to poultry meat as a major source of campylobacteriosis as does a controlled food withdrawal program in Belgium where removal of poultry products from sale, due to food contamination, resulted in a 40% reduction in human *Campylobacter* infection (Vellinga and Van Loock, 2002).

In conclusion, we have demonstrated the feasibility of a high throughput typing approach to generate multi-locus sequence data from a range of food animal, wild bird and environmental sources and clinical isolates. Host association among STs and clonal complexes was identified providing resource for future investigation and supporting existing evidence that contaminated chicken meat is a major source of human infection.

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Fig. 1. Confirmed clinical cases of human campylobacteriosis in each NHS health board region in Scotland (2005/6).

The Argyll and Clyde region has since been subsumed into the Greater Glasgow health board. Infection caused by *Campylobacter jejuni* and *Campylobacter coli* are indicated by the light and dark grey regions on the graph.

Fig. 2. Sequence types (ST) among *Campylobacter jejuni* isolates from food animals: chickens (A), cattle (B), sheep (C), the environment (D), and wild birds (E). STs are ordered by clonal complex and frequencies are represented by vertical bars. The major clonal complexes are indicated.

Fig. 3. Sequence types (ST) among *Campylobacter coli* isolates from food animals: chickens (A), cattle (B), sheep (C), pigs (D), and turkeys (E). STs are ordered by clonal complex and frequencies are represented by vertical bars. The 828 and 1150 clonal complexes are indicated.

Fig. 4. Clonal complexes (CCs) among *Campylobacter* isolates from food animal and non food sources.

C. jejuni STs are from chickens (A), cattle (B), sheep (C), the environment (D), and wild birds (E) and *C. coli* STs are from chickens (A), cattle (B), sheep (C), pigs (D) and turkeys (E). The proportion of clonal complexes (shaded areas) among isolates from each host population is indicated. Coloured shading highlights clonal complexes that are predominantly associated with a particular host. 177 and 682 clonal complexes are common among isolates from starling faecal samples not included in this study.

Fig. 5. Clonal frame genealogy of *Campylobacter jejuni* and *Campylobacter coli* genotypes isolated from clinical cases of campylobacteriosis in Scotland (2005/6).

Fig. 6. Phylogenetic relationships among disease-causing *C. jejuni* (A) and *C. coli* (B) Sequence Types (STs) in 2005/6.

The genealogy was constructed using ClonalFrame software. ST-based clonal complexes responsible for 1% or more of human disease (constituting 97% of the total) are indicated. The diameter of each pie chart is proportional to the number of disease cases caused and the area of shaded segments indicates frequency that each clonal complex occurs in comparison to scaled data sets for *C. jejuni* (Bovine=586, Poultry=1288, Wild bird=170, Environmental=91, Ovine=249) and *C. coli* (Bovine=86, Poultry=459, Ovine=57, Porcine=322, Turkey=111). The scale bar represents the genetic distance. * indicates clonal complexes that are not well supported by the ClonalFrame genealogy.

Table 1
Clinical isolates received from health boards and reported to Health Protection Scotland (HPS).

Health Board	Isolates received	% of <i>C. jejuni</i> ^a	Cases reported to HPS	% of HPS cases received	Incidence per 100,000 ^b	
					2005	2006
Argyll and Clyde	132	92.7	112	>100	90.5	89.1
Borders	133	92.9	154	86.4	113.5	135.8
Dumfries & Galloway	219	86.4	221	99.1	107.5	131.5
Western Isles	9	88.9	9	<100	45.7	22.8
Fife	284	90.6	351	80.9	77.9	67.6
Forth Valley	174	92.7	256	68.0	69.6	69.3
Grampian	820	94.2	771	>100	122.8	114.5
Greater Glasgow	927	89.1	1070	86.6	60.7	80.5
Highland	310	89.6	281	>100	90.4	104.9
Lanarkshire	682	88.7	651	>100	90.8	104.6
Lothian	1150	89.1	1126	>100	116.7	110.6
Orkney	19	77.8	17	>100	82.0	91.9
Shetland	24	86.4	0 ^c	>100	4.6	0 ^c
Tayside	524	90.5	525	99.8	116.0	111.4
Ayrshire & Arran	265	93.6	287	92.3	50.6	71.7
Total	5672 ^d	90.4	5831	97.3	90.2	95.3

^a Species identity was inferred only for the 5247 *C. jejuni* and *C. coli* isolates with complete MLST data.

^b data from HPS weekly report "Gastro-intestinal and foodborne infections" Vol. 41, No. 2007/01. c Shetland did not report cases to HPS in 2006.

^c Shetland did not report cases to HPS in 2006.

^d Two of the isolates were split according to colony morphology into two isolates each, yielding a total of 5674 clinical isolates.