Marked host specificity and lack of phylogeographic population structure of Campylobacter jejuni in wild birds

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Abstract

Zoonotic pathogens often infect several animal species, and gene flow among populations infecting different host species may affect the biological traits of the pathogen including host specificity, transmissibility and virulence. The bacterium Campylobacter jejuni is a widespread zoonotic multihost pathogen, which frequently causes gastroenteritis in humans. Poultry products are important transmission vehicles to humans, but the bacterium is common in other domestic and wild animals, particularly birds, which are a potential infection source. Population genetic studies of C. jejuni have mainly investigated isolates from humans and domestic animals, so to assess C. jejuni population structure more broadly and investigate host adaptation, 928 wild bird isolates from Europe and Australia were genotyped by multilocus sequencing and compared to the genotypes recovered from 1366 domestic animal and human isolates. Campylobacter jejuni populations from different wild bird species were distinct from each other and from those from domestic animals and humans, and the host species of wild bird was the major determinant of C. jejuni genotype, while geographic origin was of little importance. By comparison, C. jejuni differentiation was restricted between more phylogenetically diverse farm animals, indicating that domesticated animals may represent a novel niche for C. jejuni and thereby driving the evolution of those bacteria as they exploit this niche. Human disease is dominated by isolates from this novel domesticated animal niche.

Keywords: disease emergence, epidemiology, host associations, Zoonotic disease

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Introduction

Many pathogens that infect humans or domestic animals can inhabit multiple animal hosts and environmental reservoirs (Daszak et al. 2000; Woolhouse et al. 2001). A broad host range of a pathogen can increase the risk of disease emergence in novel hosts (Cleaveland et al. 2001). Limited gene flow among distinct pathogen populations inhabiting different hosts may lead to adaptations to specific niches and initiate allopatric speciation (Cohan & Koeppel 2008; Sheppard et al. 2008, 2011). The evolutionary implications of a pathogen with a multiple host population structure will depend on the transmission frequencies among and within host species, resulting in a range of outcomes from spillover infections to emerging infectious diseases in the novel host species (Fenton & Pedersen 2005). Understanding and quantifying the genetic structure of populations of pathogenic microorganisms could...
Campylobacter jejuni is a zoonotic multihost pathogen that has a substantial impact on human health. In humans, C. jejuni infections are primarily food-borne, the majority of which are caused by genotypes that are common in food animals, especially poultry (Dingle et al. 2001, 2002; Colles et al. 2003; Manning et al. 2003; Schouls et al. 2003; McCarthy et al. 2007). Commercially reared poultry are often colonized with C. jejuni and can carry high bacterial loads asymptomatically, suggesting commensal adaptations to the avian gut (Humphrey et al. 2007). However, although most studies have investigated C. jejuni isolates from domestic animals or human patients, the bacterium has a much broader host range. Campylobacter jejuni is common in a variety of species of wild birds (Luechtefeld et al. 1980; Kapperud & Rosef 1983; Waldenström et al. 2002) and has also been identified in several species of pet animals, rodents and insects (Rosef & Kapperud 1983; Sproston et al. 2010). In wild birds, C. jejuni prevalence rates vary between host species and seem to be linked to diet (Kapperud & Rosef 1983; Broman et al. 2002; Waldenström et al. 2002). Disease manifestation in wild birds has not been extensively evaluated, but a recent study showed a slight reduction in body mass in European robins, Erithacus rubecula, challenged with a C. jejuni isolate from another songbird species (Waldenström et al. 2010). In humans, the severity of infection varies profoundly from asymptomatic to severe gastrointestinal illness (Dasti et al. 2010). Although C. jejuni has been mainly studied in relation to its role as a human pathogen, its growth characteristics at different temperatures (i.e. able to grow at 37–42 °C) and frequent isolation from domestic and wild birds suggest that it is primarily an avian bacterium and that wild avian species may function as natural reservoirs for C. jejuni (Luechtefeld et al. 1980; Hermans et al. 2011; Sheppard et al. 2011).

To date, C. jejuni population genetic analyses have been largely dominated by isolate collections originating from food animals and human campylobacteriosis, although there is increasing interest in the analysis of isolates from environmental sources and wild animals (Broman et al. 2002, 2004; French et al. 2005, 2009; Colles et al. 2008a, 2009; Sheppard et al. 2011). The population structure of human and food animal C. jejuni isolates is essentially nonclonal and dominated by recombination (Dingle et al. 2001; Suerbaum et al. 2001; Manning et al. 2003), which acts to diminish genetic differentiation among bacterial subpopulations (Broman et al. 2002; Dingle & Maiden 2005). Within this population, however, genotyping with multilocus sequence typing (MLST) has identified clonal complexes, groups of related genotypes that are probable to share a common ancestor (Dingle et al. 2002). Because isolates from food animals and wild animals often show genetic subdivision, estimates of population genetic parameters obtained from analyses of poultry- or human-dominated data sets can obscure biological and epidemiological properties of C. jejuni (Meinersmann 2000).

Earlier studies of wild bird isolates indicate that C. jejuni has a high degree of host specificity with little overlap of genotypes between isolation sources including host species (Broman et al. 2002; Colles et al. 2008a, b, 2009). Recently, Sheppard et al. investigated niche segregation in C. jejuni and C. coli from wild and domestic animal sources in the United Kingdom (Sheppard et al. 2011) and found distinct C. jejuni genotype assemblages in different groups of birds, strengthening the notion of genotype by host associations. This contrasts to the situation seen among food animal C. jejuni genotypes, where host associations are weaker (Sheppard et al. 2011), possibly indicating a different ecology in the food animal niche. To further assess the natural ecology of C. jejuni and test the predictions from earlier studies at a larger geographical scale, we collected a large set of samples from various wild bird species, farm-related animals and humans, from Europe and Australia and employed MLST (Broman et al. 2002; Dingle & Maiden 2005; Mickan et al. 2007; Baker et al. 2010) to examine the population structure of C. jejuni by host and geography. By sampling taxonomically related, but geographically separated species pair, we could specifically address the existence of host-associated C. jejuni genotype assemblages among wild bird host and relate this to the ecology of C. jejuni in food animals and human disease.

Materials and methods

Bacterial isolates

The population genetic analyses were based on 2294 characterized C. jejuni isolates. Of these, 928 C. jejuni were isolated from wild birds sampled in Sweden (Waldenström et al. 2002; Broman et al. 2004), the United Kingdom (Colles et al. 2008a, 2009) and Australia (Table 1), while the remaining 1366 bacterial MLST genotypes came from published studies on C. jejuni in humans in Australia (Kinana et al. 2006; Mickan et al. 2007) and the United Kingdom (Dingle et al. 2008), and in farm animals (Kinana et al. 2006; McCarthy et al. 2007). The Australian wild bird samples were collected in 2004–2006 in the Newcastle and Melbourne areas. Australian shorebirds were trapped at Stockton Sandpit with funnel walk-in traps or mist nets, and faecal
Table 1 Host origin, sampling year and sampling site of investigated *Campylobacter jejuni* strains

| Host species | Latin name | No. of isolates | Year | Country |
|--------------|------------|----------------|------|---------|
| Mallard      | *Anas platyrhynchos* | 85 | 2002 | Sweden |
| Dunlin       | *Calidris alpina* | 21 | 2000 | Sweden |
| Black-headed gull | *Larus ridibundus* | 8 | 2001 | Sweden |
| Starling     | *Sturnus vulgaris* | 7 | 2000 | Sweden |
| Geese        |            | 166 | 2002-2003 | UK |
| Blackbird    | *Turdus merula* | 32 | 2000 | Sweden |
| Song thrush  | *Turdus philomelos* | 84 | 2000 | Sweden |
| Sharp-tailed sandpiper | *Calidris cuminata* | 33 | 2004 | Australia |
| Silver gull  | *Larus vuvellidae* | 10 | 2004 | Australia |
| Chicken      |            | 7 | 2000 | Senegal |
| Cattle       |            | 218 | 1984-2001 | UK |
| Sheep        |            | 158 | 1982-2002 | UK |
| Human        |            | 574 | 2003-2004 | UK |
| Total        |            | 2294 | | |

Assessment of association of genotype with host species, time and geography

The sequence alignment of the concatenated sequences of all seven housekeeping genes distributed around the bacterial chromosome were amplified by PCR (Dingle et al. 2001). The resulting PCR products were sequenced, and the nucleotide extension reactions products were separated and detected on automated DNA analysers. The sequences were assembled and edited, and allele numbers and STs and clonal complexes were assigned using the internet-based *Campylobacter jejuni* and *Campylobacter coli* MLST database (with accession numbers 21601-22077, http://pubmlst.org/campylobacter/).

Phylogenetic relationships were illustrated using two different approaches. In the first analysis, we used the ClonalFrame software to construct phylogenies that incorporate both mutation and recombination (Didelot & Falush 2007). A random set of 10 isolates from each of the 13 host species was drawn and run at default values with 50 000 burn-in iterations and 50 000 further iterations from which each 100th tree was sampled. Four independent runs were conducted, and convergence was estimated with Gelman Rubin statistics (Didelot & Falush 2007). A 75% consensus tree of the combined data from four runs was constructed with ClonalFrame and exported as a Newick tree for display and labelling in MEGA5 (Fig. 2).

In the second analysis, we used the software goeBURST (Francisco et al. 2009), which uses the same clustering rules as the frequently used eBURST (Feil et al. 2004) but which provides a global optimal solution, to determine the clonal relationships between STs of the entire data set. The software calculates a minimum spanning tree and

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identifies predicted founder STs and any single locus variant to those genotypes (Fig. S2, Supporting information).

Isolate assignment to hosts

Assignment of isolates from humans to food animal and wild bird populations was undertaken using the population genetic assignment software STRUCTURE (Falush et al. 2003). We reduced the number of reference populations by combining closely related C. jejuni populations from similar wild bird taxa. Thus, C. jejuni from sharp-tailed sandpipers and dunlins were treated as one group and C. jejuni from silver gulls and black-headed gulls as another group. The combining of host species harbouring similar C. jejuni populations was performed to ensure that reference populations were all of at least size 50. The STRUCTURE algorithm provides unbiased assignment based on the allele frequency assuming independence between alleles. The no-admixture model was used with default values and 5000 burn-in iterations followed by 10 000 sampled iterations in line with published approaches that have used the assignment of C. jejuni isolates to host species (McCarthy et al. 2007; Sheppard et al. 2009). The probability of origin in each of the reference populations is estimated for each human isolate, with a probability between zero and one inclusive for each possible source population summing to 1 across the possible sources (Fig. 3).

Results

We detected a broad diversity in the number of C. jejuni genotypes obtained from wild birds. Approximately one new ST was obtained for every fourth sequenced isolates with 251 unique STs detected from wild birds (Table S1, Supporting information). However, most of these of genotypes were grouped into 22 clonal complexes comprising related genotypes. The majority of the STs found in the wild bird species have not been associated with human disease in the Campylobacter jejuni and Campylobacter coli MLST database. The most common were ST-1020, which was represented by 63 isolates (all from UK starlings), and ST-177, which was represented by 49 isolates mostly from UK starlings, but also from bird species in the genus Turdus (thrushes). A further 26 STs were represented by ten or more isolates in this data set (Table S1).

Population structure among hosts

Campylobacter jejuni genotypes from the different wild bird host species were genetically distinct from each other, as well as from the genotypes typically recovered from humans and food animals. With nucleotide-based analyses, the values of genetic subdivision (F_{ST}, which has possible values between 0 = no and 1 = complete subdivision) were on average 0.420 for the concatenated sequence from all loci (Table S2, Supporting information). The genetic differentiation among isolates from
different host sources was well resolved in a neighbour-
joining tree (Fig. 1). Isolates from food animals and
humans clustered together and showed relatively little
genetic subdivision (mean $F_{ST}$ among these populations
was 0.064, SD ± 0.025), while wild bird $C.\textit{jejuni}$ popula-
tions showed high genetic subdivisions and long branch
lengths (Fig. 1). Notably, host species taxonomy rather
than geographic origin was the main informative crite-
rion for clustering, where populations of bacteria from
related wild bird host species had low levels of
genetic differentiation, for example, black-headed gulls
vs. silver gulls ($F_{ST} = 0.019$), and Dunlins vs. sharp-tailed
sandpipers ($F_{ST} = 0.051$). Also, Mallards and geese had
genetically similar $C.\textit{jejuni}$ populations ($F_{ST} = 0.078$;
Table S2). In fact, for these three comparisons, the
$F_{ST}$ values were in similar ranges to those observed in the
population sets of domestic animals and humans.

Population differentiation occurred also at the level of
allele distribution, although the pattern was not as clear
as for nucleotide polymorphisms (Fig. S1). Again, popu-
lations of $C.\textit{jejuni}$ from related wild bird species tended
to cluster together, and humans and food animals were
most closely related, an exception being chicken $C.\textit{jejuni}$
from Senegal that clustered away from the other farm
animal subsets. However, the $F_{ST}$ values were generally
lower than the nucleotide-based values (overall $F_{ST} = 0.070$)
and the resulting tree less resolved.

The CLONALFRAME algorithm was used for genealogi-
cal reconstructions. This evolutionary-based approach
reconstructed a genealogy with a topology consistent
with the relationships deduced on the basis of popula-
tion genetic subdivision, which is suitable for these

![Fig. 2 A ClonalFrame genealogy of Campylobacter jejuni STs from wild birds, food animals and humans. From each of the 13 host species, 10 random $C.\textit{jejuni}$ isolates were drawn and included in the analysis. The source of isolates is indicated with different colour (see inset in the figure), and STs are given in numbers at the tip of branches.](image)

![Fig. 3 Assignment of human isolates to food animals and wild bird populations. The probabilistic assignment of the Campylobacter jejuni host population was based on allele frequencies using the software STRUCTURE. Each allelic profile is represented by a vertical bar, showing the estimated probability that it comes from each of the source identified. The horizontal coloured bar above the vertical bars indicate whether human isolates were from UK (blue) or from Australia (pink). Food animal related isolates are shown in shades of grey, while the isolates of different wild birds are denoted in colours: silver gull (light green), black-headed gull (dark green), blackbird (red), song thrush (purple), European starling (pink), goose species (yellow), mallards (orange), sharp-tailed sandpiper (dark blue), dunlin (light blue).](image)
Types of recombinant bacteria. Generally, wild bird genotypes clustered away from farm animal–related genotypes, and wild bird species genotypes from taxonomically related species tended to cluster together (Fig. 2). A similar pattern was observed for clonal relationships in the entire data set, where the resulting minimum spanning tree showed clear separation of farm animal–associated genotypes and genotypes from wild bird hosts (Fig. S2).

Finally, when using STRUCTURE analysis, most human isolates were assigned to farm animal origin (Fig. 3). Over 98% assignment of the 153 Australian isolates from humans was attributed to a farm animal source (cattle, sheep or chicken) and less than 2% to wild bird species. Just over 75% of UK isolates from humans were attributed to farm animals with 15% attributed to shorebirds, 7% to mallards and 1% each to gulls and geese.

Temporal and geographical variation in genotypes

To assess the temporal stability of C. jejuni genotypes recovered from the different hosts in more detail, analysis of molecular variance (AMOVA) was performed for isolates from wild bird species where data had been collected in different years, in different migration seasons within a year (spring vs. fall migration) or in different geographical places (Table S1). Due to trapping and sampling effects, as well as in the case of the song thrush where prevalence rates differed between seasons, only two of the major wild bird host species could be analysed for season and year within a country. In the first species, the analysis of black-headed gull C. jejuni incorporated the effects of sampling year and season within the year. Year or season accounted for nearly 0.7% (F\textsubscript{ST} = 0.007, P < 0.001) and 0.9% (F\textsubscript{ST} = 0.009, P = 0.37) of the variation, respectively, and nearly, all molecular variation was found within season within year (98.4%, F\textsubscript{ST} = 0.016, P < 0.001). The analysis of isolates from Swedish blackbirds investigated the effect of season of the year (spring or autumn) and the sex of the birds (male or female). There were large differences among C. jejuni genotypes depending on whether they had been sampled during the spring or autumn migrations of the birds, accounting for 11% of the total molecular variance (F\textsubscript{ST} = 0.118, P < 0.001). No effect of gender was seen, and the remaining variance was best explained by variation within the sexes within the seasons (F\textsubscript{ST} = 0.116, P < 0.001).

Data from Swedish starlings and mallards were compared with the data from 50 starling and 50 goose isolates collected in Oxford, UK. These UK samples were randomly chosen from the larger collection of samples used in the genetic distance analyses and had been collected in three and two different years, respectively (Colles et al. 2008a, 2009). A structured AMOVA on starlings, incorporating sampling country (Sweden and UK) and year of sampling, showed that there was hardly no effect of sampling country (F\textsubscript{ST} = 0.022, P = 0.24), moderate effects of sampling year (6.3% of the variance explained, F\textsubscript{ST} = 0.061 P < 0.001) and large effects within population within year (95.6% of the variance explained, F\textsubscript{ST} = 0.040 P < 0.001). Similar patterns were seen in the comparison between UK geese and Swedish mallards; no effects of host species, which in this case also equals country of origin (F\textsubscript{ST} = 0.002 P = 0.34), moderate year effects (explaining 13.0% of the molecular variance, F\textsubscript{ST} = 0.130 P = 0.002) and large within population within year effects (87.2% of the variance, F\textsubscript{ST} = 0.130 P < 0.001).

Discussion

The C. jejuni populations investigated showed strong patterns of genetic subdivision, with distinct genetic subsets associated with particular hosts. The most genetically distinct C. jejuni populations were isolated from different wild bird species, with farm animal isolates much more similar to each other (Fig. 1). Furthermore, the genetic population structure was clearly associated with host taxonomy, with C. jejuni populations from taxonomically closely related bird species being most similar to each other (Fig. S2). The host associations were clearly evident in F\textsubscript{ST} analyses, where values based on the nucleotide sequences between C. jejuni populations from some of the wild bird species were several times larger than the corresponding values between C. jejuni populations in different farm animal species (Table S1). A similar, but not as strong pattern was observed at the allele level (Fig. S1), and a phylogenetic consensus tree of randomly sampled genomes from a reduced set of source populations also supported the notion of strong separation of C. jejuni populations by wild bird host species and not by geographic location (Fig. 2).

Strong differentiation at the nucleotide level and weaker differentiation at the allele level suggests that the observed population genetic structure is fairly old and not the result of recent introduction to particular hosts. The overall sequence diversity was 3.1%, well within the subspecies range for Campylobacter spp., and only few alleles were unique to a specific wild bird host. The population structure of C. jejuni comprises multiple clonal complexes of related genotypes, which exhibit high rates of recombinaction, but with little deeper clonal structure evident among clonal complexes in the analysis of 7 locus MLST data (Dingle et al. 2001; Manning et al. 2001; Suerbaum et al. 2001). The data collected here suggest that host associations are important.
determinants of the genotypes isolated from nonfood animal sources, and raise the question whether the structure observed in food animals is the consequence of more recent introductions and expansions of certain genotypes in this novel niche. The limited host range for some of the genotypes obtained from wild birds, as well as the general genetic subdivision detected in these analyses, suggests restriction to gene flow dependent on host species or the existence of host-adapted strains of *C. jejuni*. Some of the sampled bird species co-occur in time and space in such a way that transmission of bacterial genotypes should be possible. For instance, blackbirds and song thrushes occupy slightly different ecological niches in forested areas in Europe, but occur, side by side, during migration and on wintering sites. Similarly, shorebirds and gulls (both in Europe and in Australia) feed intensively in the littoral zone, at times together in large aggregations. Some shared genotypes were seen between the two species of thrushes, as well as between gulls and shorebirds, but the general picture was that these hosts served as different bacterial niches. Most remarkably, among blackbirds sampled in Australia, two of the four detected genotypes (ST-1324 and ST-1342) were shared with blackbirds sampled in Sweden, and the remaining two (ST-3067 and -3068) were very similar. We do not know whether the blackbird-associated genotypes occur in other Australian birds, or whether they are restricted to this single host species. If the former case is true, then the shared polymorphisms in *C. jejuni* strains occurring in an allopatric single host species strongly indicate host species adaptations, especially because the Australian blackbirds date back to the European colonization of Australia in the 19th century (Higgins *et al.* 2006).

Most studies that have compared serotypes or genotypes from wild birds with poultry and patient isolates have typically identified predominantly unique strains of *C. jejuni* in wild birds, for example, in herring gulls *Larus argentatus* from Scotland (Whelan *et al.* 1988), feral pigeons from Japan (Fukuyama *et al.* 1986), various wild bird species in Norway (Rosef *et al.* 1985) and starlings in the UK (Colles *et al.* 2003, 2009), indicating that wild birds are not a significant direct source of human campylobacteriosis. Recently, Sheppard and co-workers performed attribution analyses on *C. jejuni* strains from food and wild animal sources that were not associated with a single host species. If the differentiation by host is old relative to current genotype distribution and that host species–genotype associations are robust in wild bird *C. jejuni*. A zoonotic, multiple host bacterial pathogen, such as *C. jejuni*,
has many different selection forces operating on its epidemiology. Not only has it to evolve mechanisms to persist and multiply in different enteric environments, which could differ in temperature, structure and biochemical and immunological habitats, but it also has to evolve means to successfully survive in environments during transmission. In such a setting, two survival strategies can be postulated (i) adaptations favouring the colonization and persistence in one certain host species; or (ii) adaptations favouring transmission and colonization of several hosts. There is evidence for both these strategies in C. jejuni (i) genotypes that are widespread among hosts (multihost lineages), for example, the ST-45 complex and several other food animal-associated genotypes; and (ii) genotypes that are confined to single hosts (host specific lineages) such as the ST-1264 and ST-1347 complexes found in song thrushes. Strain-specific properties in colonization abilities have been noted previously in chickens, both in terms of host (Stern et al. 1990) and of bacterial genotypes (Stern et al. 1988; Cawthraw et al. 1996; Payne et al. 1999), and similarly, strain-specific differences in survival times in water environments have been observed (Obiri-Danso et al. 2001; Cools et al. 2003). It is interesting to note that multihost genotypes dominate in food animals and in human infections (Sheppard et al. 2011), suggesting that a broad host range is associated with the emergence of human campylobacteriosis. This study thus demonstrates that C. jejuni isolated from wild birds are in general distinct from those isolated from human campylobacteriosis and food animals. Further, there is strong differentiation by wild bird host species, which may be the biologically important niche for this bacterium. The population diversity for this niche described here, when compared to that found in C. jejuni isolated from food animals, demonstrates the small portion of the whole, which is visible from the anthropocentric view of the ecology of C. jejuni.

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Data accessibility

Data on Swedish and Australian wild bird \textit{C. jejuni} isolates have been deposited in the \textit{Campylobacter
jejuni\textsubscript{coli} PubMLST database (http://pubmlst.org/campylobacter/) with accession numbers 21601-22077, and an alignment can be downloaded at Dryad (doi:10.5061/dryad.8p2f7). Other analysed sequence data were gathered from published studies on \textit{C. jejuni} in humans and farm animals (Kinana \textit{et al.} 2006; McCarthy \textit{et al.} 2007; Mickan \textit{et al.} 2007; Dingle \textit{et al.} 2008).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Unrooted neighbor-joining tree displaying the pairwise genetic distances ($F_{ST}$ values) between \textit{Campylobacter jejuni} populations from different hosts and geographical areas. The $F_{ST}$ values were calculated from observed MLST allele distributions in the 2294 \textit{C. jejuni} isolates.

Fig. S2 (A) Full Minimum Spanning Tree of 2294 \textit{Campylobacter jejuni} isolates typed with MLST. The size of each circle is proportional (on a logarithmic scale) to the number of isolates with that particular ST in the total sample. STs are indicated by the number in each circle. Black lines connect STs that differ from each other at a single locus, while dark grey and light grey lines connect STs differing at two and three loci, respectively. The proposed founders of particular clusters are indicated by a light green outer circle. The subfounders (defined as having links to three or more STs) are indicated by dark green outer circles. Human and food animal related isolates are shown in grey while the isolates of different wild birds are denoted in colours: silver gull (light green), black-headed gull (dark green), blackbird (red), song thrush (purple), European starling (pink), goose species (yellow), mallards (orange), sharp-tailed sandpiper (dark blue), dunlin (light blue). For visualisation, the parts of the tree containing wild bird samples have been enlarged (B, C and D).