Molecular Epidemiology of *Campylobacter jejuni* Isolates from Wild-Bird Fecal Material in Children’s Playgrounds

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In many countries relatively high notification rates of campylobacteriosis are observed in children under 5 years of age. Few studies have considered the role that environmental exposure plays in the epidemiology of these cases. Wild birds inhabit parks and playgrounds and are recognized carriers of *Campylobacter*, and young children are at greater risk of ingesting infective material due to their frequent hand-mouth contact. We investigated wild-bird fecal contamination in playgrounds in a New Zealand city. A total of 192 samples of fresh and dried fecal material were cultured to determine the presence of *Campylobacter* spp. *Campylobacter jejuni* isolates were also characterized by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), and the profiles obtained were compared with those of human isolates. *C. jejuni* was isolated from 12.5% of the samples. MLST identified members of clonal complexes ST-45, ST-682, and ST-177; all of these complexes have been recovered from wild birds in Europe. PFGE of ST-45 isolates resulted in profiles indistinguishable from those of isolated obtained from human cases in New Zealand. Members of the ST-177 and ST-682 complexes have been found in starlings (*Sturnus vulgaris*) in the United Kingdom, and these birds were common in playgrounds investigated in New Zealand in this study. We suggest that feces from wild birds in playgrounds could contribute to the occurrence of campylobacteriosis in preschool children. Further, the *C. jejuni* isolates obtained in this study belonged to clonal complexes associated with wild-bird populations in the northern hemisphere and could have been introduced into New Zealand in imported wild garden birds in the 19th century.

Campylobacteriosis is a common disease of humans worldwide and a major burden on the health service in many nations. In New Zealand the reported rate of campylobacteriosis is one of the highest in the world, and a recent estimate is that there are around 300 cases per 100,000 people per year (2). Despite many years of concerted research effort, many questions related to the complex epidemiology and control of this disease remain unanswered. Much emphasis has been placed on controlling exposure pathways involving food, particularly poultry (1). This can be justified due to the high carriage rates in poultry (26) and due to evidence that similar strains are present on poultry and in human infections (26).

Other sources, both food and environmental, are also considered important contributors to human infection (7, 12, 19, 20). It is likely that there are multiple diverse other pathways and that further public health gains beyond those achieved by the control of poultry contamination will be possible only through systematic identification and control of these alternative pathways. These pathways include cattle and sheep meat, occupational exposure, and direct contact and contamination of the environment with wildlife and livestock fecal material.

In New Zealand the reported rate of campylobacteriosis is highest in children between 1 and 4 years old (578/100,000) (2). The potential environmental pathways for infection in this preschool age group include exposure to wild-bird feces in open playgrounds. Many playgrounds are located in parks that are natural habitats for wild birds; play equipment provides ideal perching sites for wild birds, and overhanging trees provide ideal roosting areas. Children’s behavior, particularly the frequent hand-mouth contact in this age group, is also likely to increase the risk of ingesting infective material (3, 13, 25).

In this study we investigated the possibility that wild-bird contamination of children’s playgrounds is a source of human infection by estimating the prevalence of *Campylobacter jejuni* in fresh and dried wild-bird fecal samples and comparing the genotypes isolated from these samples with human clinical isolates. As far as we are aware, this is the first study in New Zealand, and possibly elsewhere, to isolate *Campylobacter* spp. from children’s playgrounds and to examine the potential zoonotic risk from these organisms, and it is a unique use of molecular epidemiology for investigation of a potential environmental risk pathway. This study also provided the first opportunity to compare wild-bird genotypes obtained from an urban area in New Zealand with wild-bird genotypes from other countries recorded in the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/). This is particularly relevant given the historical transfer of urban-dwelling birds from the United Kingdom to New Zealand in the 19th century (24).
sphere (85% N₂, 10% CO₂, 5% O₂) for 48 h before an aliquot was plated onto M, Bury, England). The broth was incubated at 42°C in a microaerobic atmosphere. Cotton-tipped swabs that were immediately placed into 3 ml of Bolton broth (Lab M, Bury, England). The broth was incubated at 42°C in a microaerobic atmosphere. Isolates were characterized further using the pulsed-field gel electrophoresis (PFGE). Isolates were characterized further using the pulsed-field gel electrophoresis (PFGE) method of Ribot et al. (21), and the profiles were compared with profiles stored in the PulseNet Aotearoa New Zealand Campylobacter database (16) using an optimization value of 1% and a position tolerance value of 1.5%.

**Materials and Methods**

Samples were obtained from all 10 publicly accessible children's playgrounds in the southeastern sector of the city of Palmerston North, New Zealand, between 8 November 2004 and 2 February 2005. The maximum distance between playgrounds was 3 km.

**Isolation of Campylobacter spp.** Bird fecal samples were collected using sterile cotton-tipped swabs that were immediately placed into 3 ml of Bolton broth (Lab M, Bury, England). The broth was incubated at 42°C in a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) for 48 h before an aliquot was plated onto mCCDA agar (Fort Richard, Auckland, New Zealand). Plates were examined after 48 h of incubation at 42°C in a microaerobic atmosphere. Isolates were examined by dark-field microscopy to determine morphology and motility and tested to determine whether oxidase was produced. The isolates that had typical morphology and motility and for which the oxidase test was positive were frozen at −80°C in 15% glycerol broth (Oxoid, Basingstoke, England). Isolates were identified as *C. jejuni* by PCR (23).

**MLST.** Multilocus sequence typing (MLST) was performed as described by Dingle et al. (10). Chromosomal DNA was prepared from freshly grown cultures by boiling the cultures for 10 min, followed by centrifugation of the disrupted cells. The supernatant was decanted into a fresh tube and used for amplification. Amplification was performed in a 25-µl reaction mixture using Applied Biosystems AmpliTaq Gold master mixture (Applied Biosystems, Auckland New Zealand) and 5 pmol of each primer. Products were sequenced with an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1 (Applied Biosystems) according to the manufacturer's instructions. Sequence data were collated, and alleles were assigned using the Campylobacter PubMLST database (http://pubmlst.org/campylobacter). Novel alleles and sequence types were submitted for allele and ST designations when appropriate.

**PFGE.** Isolates were characterized further using the pulsed-field gel electrophoresis (PFGE) method of Ribot et al. (21), and the profiles were compared with profiles stored in the PulseNet Aotearoa New Zealand Campylobacter database (16) using an optimization value of 1% and a position tolerance value of 1.5%.

**Analysis of genotypes.** Data for the related wild-bird-associated ST-177 and ST-45 complexes were analyzed using the method of Didelot and Falush (8), who provided a Bayesian model-based method for inferring clonal relationships of bacteria based on MLST data. The method of these workers was implemented in the software ClonalFrame (version 1.1). We performed two independent runs of the Markov chain from different random configurations; each run included 100,000 steps, the first 50,000 steps of which were discarded as burn-in, and the state was sampled every 100 steps. Initial values and prior values for the parameters were all left at the default settings. ClonalFrame was used to assess the convergence of the Markov chain by comparing the results of the two independent runs using the method of Gelman and Rubin (15). All Gelman-Rubin statistics were less than 1.2, indicating that there was satisfactory convergence. The 500 post-burn-in trees from each of the two runs were concatenated and exported as a Newick file containing 1,000 trees.

We then used SplitsTree (18) to visualize the 20% threshold consensus network (17) of the 1,000 post-burn-in trees. The consensus network displayed all edges (splits) that appeared in more than 20% of the trees. A consensus network can display conflicting splits as boxed areas where sets of parallel edges all represent the same split. The edge lengths are drawn proportional to the mean length of the edge in all the trees where it appears.

**Results**

**Contamination of playgrounds with bird feces.** A total of 24/192 samples (12.5%) were positive for *C. jejuni*, including 4/60 dried samples (6.7%) and 20/132 fresh samples (15.2%). Positive samples were found on wood, concrete, soil, bark, plastic, and grass surfaces. The predominant species of birds observed in the playgrounds at the time of sampling were the European starling (*Sturnus vulgaris*), house sparrow (*Passer domesticus*), welcome swallow (*Hirundo tahitica*), blackbird (*Turdus merula*), Australian magpie (*Gymnorhina tibicen*), song thrush (*Turdus philomelos*), tuft (*Prosthemadera novaeseelandiae*), chiffinch (*Fringilla coelebs*), and goldfinch (*Carduelis carduelis*).

**Molecular typing.** Twenty-two isolates were typed by MLST. Two other isolates could not be resuscitated from frozen culture for further characterization. Sequence type 45, the founder sequence type of clonal complex ST-45, was the predominant type, accounting for 12/22 (54.5%) isolates. Two other isolates, designated ST-137 and ST-583, also belonged to clonal complex ST-45 (Table 1). The remaining eight isolates either belonged to clonal complexes ST-177 or ST-682 or were not assigned to any complex. Two of the unassigned isolates shared at least two alleles with ST-177 and/or ST-682.

Further analysis of the 12 ST-45 isolates using PFGE revealed four Smal profiles. The largest group (group A) contained five isolates, two groups (groups B and C) each contained three isolates, and one (group D) contained a single isolate. Compared with a database consisting of 318 Smal PFGE profile isolates from food, environmental, and human sources in New Zealand, which included 37 ST-45 isolates, the group B profile was indistinguishable from the profiles for two human ST-45 isolates from clinical cases in Christchurch and Palmerston North (the latter obtained in 2005). The group B profile was also indistinguishable from the profiles for three ST-45 isolates cultured from poultry meat in Wellington and Palmerston North, and the group C profile was indistinguishable from the profiles for two ST-45 isolates cultured from poultry meat in Auckland and Hamilton.

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**Table 1. Multilocus sequence types of *C. jejuni* isolates from wild-bird fecal material contaminating children’s playgrounds in New Zealand**

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Allelic nucleotide sequence no.</th>
<th>Sequence type</th>
<th>Clonal complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1 35 2 8 51 361 2 21 2538</td>
<td>45a</td>
<td>ST-45b</td>
</tr>
<tr>
<td>1</td>
<td>1 37 4 4 4 8 13 25 23</td>
<td>137a</td>
<td>ST-45b</td>
</tr>
<tr>
<td>1</td>
<td>1 37 4 4 4 8 13 25 23</td>
<td>583a</td>
<td>ST-682</td>
</tr>
<tr>
<td>1</td>
<td>1 37 4 4 4 8 13 25 23</td>
<td>681a</td>
<td>ST-682</td>
</tr>
<tr>
<td>1</td>
<td>99 128 91 125 170 146 111</td>
<td>1324a</td>
<td>U</td>
</tr>
<tr>
<td>1</td>
<td>99 128 91 125 170 146 111</td>
<td>2354a</td>
<td>U</td>
</tr>
<tr>
<td>1</td>
<td>99 128 91 125 170 146 111</td>
<td>2536</td>
<td>U</td>
</tr>
<tr>
<td>1</td>
<td>2 35 185 162 5 8 222 21 25</td>
<td>2537</td>
<td>ST-177b</td>
</tr>
<tr>
<td>1</td>
<td>2 35 185 162 5 8 222 21 25</td>
<td>2538</td>
<td>U</td>
</tr>
<tr>
<td>1</td>
<td>17 2 2 8 5 8 222 21 25</td>
<td>2539</td>
<td>ST-177b</td>
</tr>
</tbody>
</table>

a Sequence type previously associated with wild birds.

b Clonal complex previously associated with wild birds.

c U, unassigned.

d Allele nucleotide sequence numbers in the PubMLST database (http://pubmlst.org/campylobacter).
Of the remaining 10 isolates, 3 were not cut with the SmaI enzyme and produced a single band (ST-681, ST-2539, and ST-583), 5 had unique profiles (ST-137, ST-681, ST-1324, ST-2354, and ST-2357), and the 2 ST-2536 isolates were indistinguishable from each other and their profiles were different from all other profiles. The ST-137 isolate was indistinguishable from a single ST-45 poultry isolate in the PulseNet Aotearoa New Zealand Campylobacter database, and the ST-2354 isolate was indistinguishable from an ST-991 isolate from a wild duck; all other isolates were unique to this study.

Phylogenetic analysis based on MLST. Our New Zealand isolates were compared with other members of wild-bird-associated complexes ST-682 and ST-177 from Europe submitted to the Campylobacter PubMLST database (http://pubmlst.org/campylobacter/). Sequence types belonging to clonal complex ST-45 were not included in this analysis as they are very distantly related to the ST-177 and ST-682 complexes (there are 59- and 63-bp differences between ST-45 and ST-177 and ST-682, respectively, whereas there are only 8 differences between ST-177 and ST-682) and clearly represent a different introduction into the New Zealand wild-bird population. The consensus network shown in Fig. 1 contains nine separate areas with boxes, indicating parts of the tree that are uncertain. However, the boxes all indicate local rearrangements, and the overall structure of the tree is stable.

Figure 1 shows two groups of fairly closely related sequence types, one containing ST-177 and one containing ST-682. There are also other smaller groups of more divergent strains. Of the sequence types found in New Zealand, ST-2354 and ST-2357 fall into the group containing ST-177; ST-681, ST-2536, and ST-2539 fall into the group containing ST-682; and ST-2354 and ST-1324 belong to a more divergent group. This supports the hypothesis that there were multiple origins of the campylobacters in the wild-bird population of New Zealand.

DISCUSSION

This study demonstrated that fecal material deposited by wild birds frequenting children's playgrounds in New Zealand may contain C. jejuni, including strains associated with human disease. Approximately one-half of the strains recovered from the fecal material were ST-45 strains, a multilocus sequence type associated with many species of animals, including wild birds (4–6, 14), and frequently recovered from human cases of campylobacteriosis (http://pubmlst.org/campylobacter/). The SmaI PFGE profile of three of the ST-45 isolates in this study was indistinguishable from the profile of isolates recovered from human clinical cases, providing evidence that wild-bird feces in playgrounds cannot be ruled out as a potential source of infection of young children. Further typing using a second
enzyme, such as KpnI, or flaA short variable region (9) sequencing could be used to investigate these relationships in more detail.

Although exposure to wild-bird fecal material is a possible pathway for human infection, particularly in young children, it is unlikely to be a primary contributor to the overall burden of human clinical cases in New Zealand. Food-related exposure, particularly due to handling and consumption of fresh poultry, is the most probable source of human clinical cases (1, 2, 11). Other types of environmental exposure to fecal material from livestock, including ruminants (22), have also been cited as important contributors to human infection. Understanding the relative contributions of these alternative exposure pathways is critical for designing appropriate public health interventions.

Wild-bird strains isolated in this New Zealand study belonged to clonal complexes also associated with wild birds in the northern hemisphere. In addition to ST-45, the two other members of the ST-45 complex isolated in this study, ST-137 and ST-583, have been associated with wild-bird fecal material in the United Kingdom (14). The following three isolates have been isolated only from wild birds in previous studies: ST-681 in the United Kingdom, ST-1324 in Sweden, and ST-2354 in New Zealand (http://pubmlst.org/campylobacter/). The remaining isolates were all unique to this study. Two of these isolates (ST-2537 and ST-2539) have been assigned to clonal complex ST-177, a complex known to be associated with wild birds in Europe, and the other two isolates (ST-2536 and ST-2538) have not been assigned to any clonal complex but share many alleles with members of the ST-682 and ST-177 complexes, both of which are associated with wild birds in Europe (4–6).

Although the primary aim of this study was to examine the potential zoonotic risk due to wild birds in playgrounds, this study also identified some interesting relationships between the genotypes isolated in New Zealand and genotypes in Northern Europe. The isolation of members of clonal complexes also associated with wild birds in Europe (4–6), suggests that there was a common ancestor. Although exposure to wild-bird fecal material is a possible pathway for human infection, particularly in young children, it is unlikely to be a primary contributor to the overall burden of human clinical cases in New Zealand. Food-related exposure, particularly due to handling and consumption of fresh poultry, is the most probable source of human clinical cases (1, 2, 11). Other types of environmental exposure to fecal material from livestock, including ruminants (22), have also been cited as important contributors to human infection. Understanding the relative contributions of these alternative exposure pathways is critical for designing appropriate public health interventions.

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