Prevalence and genotypes of *Campylobacter jejuni* from urban environmental sources in comparison with clinical isolates from children

Sigita Ramonaite,1 Egle Kudirkienė,1,2 Egle Tamulevičienė,3 Giedrija Želvienė,3 Alvydas Malakauskas,4 Greta Gölz,5 Thomas Alter5 and Mindaugas Malakauskas 1

Correspondence
Sigita Ramonaite
ramonaite@lva.lt

This study aimed to investigate the prevalence of *Campylobacter jejuni* in potential contamination sources that are not regularly monitored such as free-living urban pigeons and crows, dogs, cats and urban environmental water and to assess the possible impact on the epidemiology of campylobacteriosis in children using multilocus sequence typing (MLST). *Campylobacter* spp. were detected in 36.2% of faecal samples of free-living urban birds and in 40.4% of environmental water samples. A low prevalence of *Campylobacter* spp. was detected in dogs and cats, with 7.9% and 9.1%, respectively. Further identification of isolates revealed that environmental water and pet samples were mostly contaminated by other *Campylobacter* spp. than *C. jejuni*, whereas *C. jejuni* was the most prevalent species in faecal samples of free-living birds (35.4%). This species was the dominant cause of campylobacteriosis in children (91.5%). In addition, the diversity of *C. jejuni* MLST types in free-living birds and children was investigated. Clonal complex (CC) 179 was predominant among free-living urban birds; however, only two isolates from children were assigned to this CC. One dog and one child isolate were assigned to the same clonal complex (CC48) and sequence type (ST) 918. The dominant two clonal complexes among the child clinical isolates (CC353 and CC21) were not detected among *C. jejuni* strains isolated from environmental sources examined in this study. As only two CCs were shared by environmental and child *C. jejuni* isolates and a high number of novel alleles and STs were found in *C. jejuni* isolated from free-living urban birds and environmental water, there is probably only a limited link between urban environmental sources and campylobacteriosis in children, particularly in rather cold climatic conditions.

INTRODUCTION

*Campylobacter jejuni* is the leading cause of human gastroenteritis worldwide. Campylobacteriosis was the most commonly reported zoonosis in the European Union (EU) in 2011 (Anonymous, 2013). Poultry meat is considered the major source of human campylobacteriosis in most countries (Anonymous, 2012; Mughini-Gras et al., 2012; Wilson et al., 2008). However, recently published data indicate that other sources such as wild birds, environmental water and pets may also contribute to the human infection burden (Carter et al., 2009; French et al., 2009; Mughini Gras et al., 2013; Parsons et al., 2009; Tenkate & Stafford, 2001). A high *Campylobacter* frequency in wildbird faecal samples and environmental water samples has been shown by several studies (Brown et al., 2004; Hughes et al., 2009). It was also demonstrated that *C. jejuni* strains...
associated with human infections occurred on children’s playgrounds contaminated with faeces of wild birds (French et al., 2009). This is an important aspect of campylobacteriosis epidemiology, as the highest rates of this zoonosis were observed among children of up to 5 years of age in all EU member states (Anonymous, 2011). According to recent data of the Centre for Communicable Diseases and AIDS (Lithuania), campylobacteriosis cases in children younger than 18 years of age accounted for 76.6% of the registered human cases in Lithuania.

Investigation of environmental sources that are not regularly monitored will generate more knowledge on the prevalent population structure of C. jejuni in the environment. Therefore, we investigated the prevalence and genetic diversity of C. jejuni in environmental sources such as free-living birds, environmental water and pets, and compared them with clinical isolates from children in a specified geographical location and period of time. Such data can contribute to a better understanding of the probable role of these sources in the epidemiology of campylobacteriosis in children.

Several methods are available to perform molecular epidemiological studies and to carry out source attribution (Foley et al., 2009). Among these, multilocus sequence typing (MLST) has been used successfully to identify the link between C. jejuni isolated from different sources and human C. jejuni infection (Colles et al., 2003; Dingle et al., 2001; Kwan et al., 2008).

To our knowledge, there are only a few reports indicating the risk for humans including children to acquire campylobacteriosis from environmental sources, including free-living birds (de Haan et al., 2013; French et al., 2009). Therefore, in this study we aimed to investigate the prevalence and genotype diversity of C. jejuni isolated from different environmental sources (free-living urban crows, pigeons, environmental water, dogs and cats) and compared them with the genetic diversity of clinical C. jejuni isolates from children in Lithuania.

**METHODS**

**Sample collection.** In total, 408 samples (96 pet samples, 52 environmental water samples, 260 samples from free-living birds in urban areas) were tested for the presence of Campylobacter spp. All samples were collected twice a month in the period of October 2011 to March 2012. Faecal samples of pets were taken at the ‘Dr L. Kriaučiūnienė Small Animal Clínics’ in Kaunas, Lithuania. Campylobacter spp. from environmental water samples were obtained from the four most popular outdoor swimming places (Neris, Nemunas, Kauno marios and Lampėdžiai). Fresh faecal samples of crows and pigeons were sampled in parks and other public places in Kaunas. Additionally, 54 C. jejuni isolates of confirmed clinical campylobacteriosis cases in children were collected within the same period by the Microbiological Laboratory of Kaunas Clinical Hospital and included in this study. The age of the children varied from 2 months to 18 years. All of the confirmed campylobacteriosis cases were of domestic origin.

**Campylobacter isolation.** All samples were analysed individually. Fresh faecal samples from free-living birds and from pets were collected using sterile cotton swabs. Generally, 1-litre water samples were obtained from each of the four natural water sources. All samples were transferred immediately to the laboratory. Thermophilic Campylobacter spp. were isolated by direct plating and by plating after selective enrichment. To detect campylobacters, faecal swabs of free-living birds and pets and 100 μl of the water samples were streaked onto modified charcoal cefoperazone deoxycholate agar (mCCDA; Liolfilchem) with CCDA selective supplement (Liolfilchem). Inoculated mCCDA plates were incubated in a microaerobic atmosphere (85% nitrogen, 10% carbon dioxide and 5% oxygen) generated by Campygen (CN25; Oxoid) or a CO2 incubator (CB; Binder) at 37 °C for 48 h. After incubation, colonies grown on mCCDA agar were examined on the basis of colony morphology, typical cell morphology and motility (by phase-contrast microscopy). An oxidase test was used for primary confirmation of isolated Campylobacter spp. Two suspected Campylobacter spp. colonies per sample were subcultured onto blood agar plates (Blood Agar Base No. 2; Liolfilchem) supplemented with 5% laked horse blood and incubated at 37 °C for 48 h under microaerobic conditions generated as described above. The purified isolates were subsequently stored at −80 °C in brain–heart infusion broth (Oxoid) with 30% glycerol.

A selective enrichment was performed to detect stressed or low numbers of thermophilic campylobacters in the samples. For the selective enrichment, the swab samples were placed in a tube containing 10 ml Bolton selective enrichment broth (Oxoid) with Bolton broth selective supplement (Oxoid) and 5% laked horse blood (Oxoid). The 100 ml water samples were filtered through 0.45 μm pore-size nitrocellulose membrane filters (Control biopgen, EC) and transferred into bags with 20 ml Bolton broth. Subsequently, tubes with swabs and the bags with filters were incubated microaerobically at 42 °C for 24 h. After incubation, 10 μl enriched faecal samples and 100 μl enriched water samples were streaked onto mCCDA agar. The identification and purification of Campylobacter isolates were performed as described above.

**DNA isolation.** A 1 μl loop of bacterial culture grown on blood agar plates was collected and suspended in 200 μl PrepMan Ultra (Applied Biosystems, Life Technologies). The suspension was vortexed for 10–30 s to dissolve the culture and subsequently heated at 100 °C for 10 min for lysis. Afterwards, samples were centrifuged at 16 000 g for 3 min. The supernatant containing bacterial DNA was transferred to a new tube and placed in the freezer at −20 °C for later use.

**Identification of thermophilic Campylobacter spp. by multiplex PCR.** Campylobacter isolates were identified to the species level by a minor modification of a multiplex PCR assay described by Wang et al. (2002). Primer mix targeting Campylobacter spp. (23S rRNA), C. jejuni (hipO) and Campylobacter coli (glyA) gene regions was used. The 25 μl PCR mixture contained 15.75 μl Milli-Q water, 2.0 μl 2 mM dNTP mixture, 2.5 μl 10× reaction buffer, 2.5 μl 25 mM MgCl2, 0.25 μl HotStart Taq DNA polymerase (Thermo Scientific) and 1 μl 100 μM primer mixture containing 0.5 μM each 23S rRNA and glyA primers and 1 μM hipO primers. Finally, 1 μl chromosomal DNA was added to the prepared PCR mixture. DNA amplification was carried out in a thermocycler using an initial denaturation step at 95 °C for 6 min, followed by 30 cycles of amplification (denaturation at 95 °C for 0.5 min, annealing at 53 °C for 0.5 min and extension at 72 °C for 0.5 min) and ending with a final extension at 72 °C for 7 min. Each PCR product (11 μl) was loaded into a 1.3 % TopVision LM GQ agarose gel (Thermo Scientific) containing 0.05 μl ethidium bromide ml−1 and analysed by gel electrophoresis. The PCR products were visualized on an UV board. The GeneRuler 100 bp DNA Ladder (Thermo Scientific) was used as the molecular size marker.
MLST. One randomly selected isolate from each *C. jejuni*-positive sample was genotyped. In total, 54 stool isolates from children and two dog, 52 crow, 35 pigeon and two environmental water isolates were examined. MLST was carried out as described by Dingle et al. (2001). Amplifications of seven housekeeping genes (aspA, glnA, gltA, glyA, pgm, tkt and uncA) were performed in separate tubes in a final volume of 25 μl PCR mix composed of 12.5 μl Green Dream Taq PCR Master Mix (Thermo Scientific), 8 μl Milli-Q water, 1 μM (2.5 μl) each forward and reverse primer mix and 2 μl *C. jejuni* DNA (~30 ng). The amplified PCR products were purified with a GeneJET PCR Purification kit (Thermo Scientific). Purified PCR products were sent to GATC Biotech (Cologne, Germany) for sequencing using internally separated nested primer pairs. The obtained sequence data were analysed with BioNumerics v.7.0 (Applied Maths). Allele numbers for each housekeeping gene, sequence types (STs) and clonal complexes (CCs) were assigned by submitting the DNA sequence to the *C. jejuni* MLST database (http://pubmlst.org/campylobacter).

**Similarity index.** The Czekanowski index or proportional similarity index (PSI) was used to compare ST distribution among *C. jejuni* isolates from various sources. The PSI is calculated by:

\[
\text{PSI} = 1 - 0.5 \sum_{i} |p_i - q_i| = \sum_{i} \min(p_i, q_i).
\]

where \(p_i\) and \(q_i\) represent the proportion of strains belonging to ST \(i\) out of all strains typed from sources \(p\) and \(q\) (Feinsinger et al., 1981; Rosef et al., 1985). The values for PSI range between 1 for identical frequency distributions and 0 for distributions with no common types.

**RESULTS**

**Campylobacter prevalence**

Overall, *Campylobacter* spp. were isolated from 123 (30.1 %) of the 408 samples collected from free-living birds, pets and environmental water samples (Table 1) within a 6-month period in the Kaunas region, Lithuania. Out of 52 environmental water samples, 40.4 % were positive for *Campylobacter* spp. *Campylobacter* spp. were also frequently found in free-living birds (36.2 %), with higher rates occurring in crows (43.2 %) than in pigeons (29.2 %). Lower prevalences of *Campylobacter* spp. were detected in faecal samples of dogs and cats (Table 1). Identification to the species level revealed *C. jejuni* as the most common species among the wild-bird samples (35.4 %). In contrast, most environmental water, dog and cat sample isolates were identified as *Campylobacter* spp. other than *C. jejuni* (Table 1).

Within the same period, 54 *C. jejuni* strains were isolated from 59 registered campylobacteriosis cases in children in the Kaunas region.

**Diversity of *C. jejuni* MLST types**

Among the 145 *C. jejuni* isolates included in the MLST analysis, 89 distinct STs were identified (Table 2). Forty-four STs representing 91 isolates (62.8 %) were assigned to 18 previously described CCs. The remaining 54 isolates were assigned to 45 different STs, which could not be assigned to any of the known CCs registered at PubMLST database (http://pubmlst.org/campylobacter). Altogether, 62 (42.8 %) *C. jejuni* isolates representing 48 (53.9 %) STs were previously unreported. Interestingly, ST-6379, represented by one water isolate, had new allele sequences detected in all seven genes. Most of the new STs (83.3 %) were represented by a single isolate. After submission to the database, 33 of the new STs could not be assigned to any known CC (Table 3).

### Table 1. *Campylobacter* spp. prevalence among different sources

<table>
<thead>
<tr>
<th>Source</th>
<th><em>Campylobacter</em> prevalence (%)*</th>
<th><em>C. jejuni</em> prevalence (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs</td>
<td>5/63 (7.9)</td>
<td>2/63 (3.2)</td>
</tr>
<tr>
<td>Cats</td>
<td>3/33 (9.1)</td>
<td>1/33 (3.0)</td>
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<tr>
<td>Crows</td>
<td>56/130 (43.2)</td>
<td>54/130 (41.7)</td>
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<td>Pigeons</td>
<td>38/130 (29.2)</td>
<td>38/130 (29.2)</td>
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<tr>
<td>Environmental water</td>
<td>21/52 (40.4)</td>
<td>3/52 (5.8)</td>
</tr>
</tbody>
</table>

*Number of positive samples/number of examined samples.

### Table 2. *C. jejuni* isolates genotyped by MLST

<table>
<thead>
<tr>
<th>Source</th>
<th>No. isolates selected for MLST</th>
<th>No. STs detected</th>
<th>No. CCs detected</th>
<th>No. STs not assigned to CCs</th>
<th>No. new STs</th>
<th>No. new alleles</th>
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<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Crows</td>
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<tr>
<td>Children</td>
<td>54</td>
<td>26</td>
<td>14</td>
<td>3</td>
<td>5</td>
<td>–</td>
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<tr>
<td>Total</td>
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<td>89</td>
<td>18</td>
<td>48</td>
<td>48</td>
<td>24</td>
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</table>
Table 3. Distribution of CCs, STs and alleles among *C. jejuni* isolated from different sources

H, human isolates; D, dog isolates; W, environmental water; P, pigeon isolates; C, crow isolates. Novel STs and alleles are indicated in bold.

<table>
<thead>
<tr>
<th>No. of isolates (source)</th>
<th>ST</th>
<th>CC</th>
<th>aspA</th>
<th>glnA</th>
<th>gltA</th>
<th>glyA</th>
<th>pgm</th>
<th>tkt</th>
<th>uncA</th>
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Out of 18 identified CCs, five (CC179, CC353, CC21, CC952 and CC48) were the most prevalent, and 72 (49.7 %) of our examined isolates were attributed to these CCs. At least five individual isolates were assigned to one of these five CCs. Two (CC353 and CC21) were specific to children and included 32 (22.1 %) C. jejuni isolates. Three CCs were identified among C. jejuni isolates from different sources: 21 (14.5 %) of the tested isolates were assigned to CC179, 14 (9.7 %) to CC952 and five (3.4 %) to CC48 (Fig. 1). The remaining 13 CCs were identified sporadically among the tested C. jejuni isolates (Table 3).

Comparison of C. jejuni MLST types isolated from different environmental sources and children

In total, 26 STs were identified after MLST genotyping of 54 C. jejuni isolates from children. Most of these STs were assigned to 14 previously described CCs, and one ST was assigned to a previously unknown CC. Five C. jejuni isolates from children were assigned to three novel STs. The highest proportions of C. jejuni isolates from children were assigned to CC353 (31.5 %) and CC21 (27.8 %) (Fig. 1). The population structure of the isolates was also evaluated with a minimum-spanning tree (Fig. 2) using allelic data with the program BioNumerics v7.0. Only two out of 14 CCs identified among C. jejuni isolates from children were detected in strains isolated from free-living birds and dogs. Two C. jejuni isolates from children were assigned to CC179 identified in pigeon and crow isolates. This CC was predominant among wild pigeon isolates (40 %), and was also detected in 9.6 % of crow isolates. One C. jejuni isolate from a child’s sample was classified as ST-918 (CC48). This ST was also found in a faecal sample taken from a dog (Fig. 2).

CC45 was identified among dog and pigeon isolates, but this CC was not observed among child isolates. Four CCs (CC692, CC1287, CC179 and CC952) were identified among crows; however, only CC952 was predominant among crow isolates (25 %). MLST genotyping revealed

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that two C. jejuni strains isolated from environmental water samples were assigned to novel STs and did not match any other strain characterized in this study.

The PSI was calculated to evaluate the similarity of genotypes from child cases and the different tested sources. This analysis revealed that STs from crows, pigeons and environmental water isolates were dissimilar to STs from clinical isolates of children (PSI = 0). The greatest similarity was observed between crows and pigeons (PSI = 0.08).

**DISCUSSION**

The characterization of C. jejuni isolates from multiple sources is necessary for a better understanding of campylobacteriosis epidemiology focusing on the impact of different sources on the disease burden. Our study revealed a high overall prevalence of Campylobacter spp. (30.1%) in samples collected from free-living birds, pets and environmental water (Table 1). Similar prevalence rates have been found by other studies; however, the prevalence revealed in free-living birds in the Kaunas region is among the highest found in wild bird populations (Waldenström et al., 2002; Weis et al., 2014).

A low prevalence of Campylobacter spp. was found in faecal samples from dogs and cats (7.9 and 9.1%, respectively). However, other studies have shown that Campylobacter spp. can be present in up to 43% of dog faecal samples, and up to 13% of these isolates were identified as C. jejuni (Procter et al., 2014).

Identification to the species level revealed that C. jejuni was the most common species among the wild-bird samples (35.4%). C. jejuni was found in up to 93% of infected free-living birds (American crows) (Procter et al., 2014). In contrast, in our study, most environmental water, dog and cat sample isolates were identified as Campylobacter spp. other than C. jejuni (Table 1).

MLST analysis of 145 C. jejuni isolates (91 environmental and 54 from children) identified 89 distinct STs (Table 2).

Forty-four STs representing 91 isolates (62.8%) were assigned to 18 previously described CCs. The remaining 54 isolates were assigned to 45 different STs, which could not be assigned to any of the known CCs registered at the PubMLST database (http://pubmlst.org/campylobacter). Altogether, 62 (42.8%) C. jejuni isolates representing 48 (53.9%) STs were previously unreported. Interestingly, ST-6379, represented by one water isolate, had new allele sequences detected in all seven genes. Most of the new STs (83.3%) were represented by only a single isolate. After submission to the database, 33 of the new STs could not be assigned to any known CC (Table 3).

Out of 18 identified CCs, five (CC179, CC353, CC21, CC952 and CC48) were the most prevalent and 72 (49.7%) of our examined isolates were attributed to these CCs. At least five individual isolates were assigned to one of these five CCs. Two of these CCs, CC353 and CC21, were specific to children and 32 C. jejuni isolates (22.1%) were attributed to these CCs. Three CCs were identified among C. jejuni isolates from different sources: 21 (14.5%) of the tested isolates were assigned to CC179, 14 (9.7%) to CC952 and five (3.4%) to CC48 (Fig. 1). The remaining 13 CCs were identified sporadically among the tested C. jejuni isolates (Table 3).

Our study shows that C. jejuni is the most prevalent species in clinical campylobacteriosis cases in children as well as in faecal samples of free-living birds. Fresh faecal samples of pigeons and crows were taken in parks in Kaunas where small children frequently play. As C. jejuni was highly prevalent in these sources, recreational outdoor activities could be the possible transmission route for infection with C. jejuni in children. Several studies that compared genotypes from wild birds and human isolates showed that genotypes of C. jejuni in wild birds are distinct from and generally not related to human genotypes. This indicates that wild birds are not a significant direct source of human campylobacteriosis (Colles et al., 2003; Griekspoor et al., 2013). One CC (CC179) found among free-living birds was also found among isolates from children, but only two paediatric isolates were assigned to this CC. However, these isolates clustered to different STs than isolates from free-living birds.

Interestingly, CC179 was the most common CC in the faecal samples collected from pigeons. The prevalence of this CC in pigeons and environmental water was shown previously by Hughes et al. (2009) and Meinersmann et al. (2013). According to the PubMLST database, CC179 is frequently found in environmental sources such as sand, environmental water or free-living birds, and is occasionally reported in humans. Another CC (CC952) found in free-living birds was predominant among C. jejuni isolated from crows (23.1% of all isolates). It was genetically not related to C. jejuni strains in children or C. jejuni isolated from other sources investigated in the present study. In contrast, CC952 has been detected previously in rabbits and environmental sources (water and soil) with marginal association to free-living birds (Kwan et al., 2008).

**Fig. 1.** CC distribution among different sources of C. jejuni.
In contrast to the high *Campylobacter* prevalence in free-living birds, a low prevalence of *Campylobacter* spp. was detected in pets, where *C. jejuni* was not the dominant campylobacter species. STs of only two *C. jejuni* isolates from dogs matched *C. jejuni* isolates from children. MLST analysis showed that one dog isolate and one child isolate belonged to ST-918 of CC48. This indicates that children can acquire campylobacteriosis from dogs. It is noteworthy that previous studies suggest that CC48 is related to the consumption of meat (especially beef) (Kärenlampi et al., 2007). However, other studies have revealed that CC48 is one of the most common CCs in dogs and humans (Amar et al., 2014).

Despite the high prevalence of *Campylobacter* spp. in environmental water samples, *C. jejuni* isolation rates from this source were the lowest (Table 1). In the present study, only two *C. jejuni* isolates were examined and STs of both...
were not related to \textit{C. jejuni} strains from children. This might indicate that environmental water is negligible for the epidemiology of campylobacteriosis in children in Lithuania. In contrast to our results, Meinersmann \textit{et al.} (2013) found that \textit{C. jejuni} was frequently detected in river water in the USA. However, in that study, the genetic relationship between human and river isolates did not show a significant overlap (Meinersmann \textit{et al.}, 2013).

CC353 and CC21 were dominant among campylobacteriosis cases in children in the Kaunas region in Lithuania. Other studies have reported that CC21 is also highly prevalent in the UK, the Netherlands and Finland (Colles \textit{et al.}, 2003; de Haan \textit{et al.}, 2010; Dingle \textit{et al.}, 2001, 2002; Smid \textit{et al.}, 2013). Interestingly, CC21 is often associated with poultry, cattle, wild birds, sheep and water (Colles \textit{et al.}, 2003; de Haan \textit{et al.}, 2010; Magnússon \textit{et al.}, 2011; Sopwith \textit{et al.}, 2008), yet this CC was not identified among \textit{C. jejuni} isolated from environmental sources examined in our study. The second CC that was commonly associated with campylobacteriosis in children was CC353. Whereas CC353 is rarely found in Europe (Dingle \textit{et al.}, 2002; Duim \textit{et al.}, 2003; Manning \textit{et al.}, 2003), it is highly prevalent among poultry in Senegal and China (Kinana \textit{et al.}, 2006; Zhang \textit{et al.}, 2010). The PubMLST database reveals that the majority of \textit{C. jejuni} CC353 are found in infected humans.

In conclusion, our study suggests that the high prevalence of \textit{Campylobacter} spp. and especially \textit{C. jejuni} in free-living birds (crows and pigeons) could be associated with the spread of these bacteria in urban areas. The high numbers of novel alleles and STs found among \textit{C. jejuni} isolated from free-living urban birds and samples from environmental water reveal that these potential \textit{Campylobacter} sources should be investigated further to provide more knowledge on the population structure, genetic diversity and epidemiology of \textit{C. jejuni}. Only two CCs and one ST were common among both urban environmental and child isolates. This probably indicates limited transmission of \textit{C. jejuni} from urban environmental sources to children, particularly in rather cold climatic conditions. Further long-term studies are needed to evaluate the potential spread of campylobacters between environmental sources and humans, including the characterization of isolates by whole-genome sequencing for a better understanding of campylobacteriosis epidemiology.

**ACKNOWLEDGEMENTS**

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**REFERENCE**


