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Tracing isolates from domestic human *Campylobacter jejuni* infections to chicken slaughter batches and swimming water using whole-genome multilocus sequence typing

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ABSTRACT

Campylobacter jejuni is the leading cause of bacterial gastroenteritis and chicken is considered a major reservoir and source of human campylobacteriosis. In this study, we investigated temporally related Finnish human (n = 95), chicken (n = 83) and swimming water (n = 20) *C. jejuni* isolates collected during the seasonal peak in 2012 using multilocus sequence typing (MLST) and whole-genome MLST (wgMLST). Our objective was to trace domestic human *C. jejuni* infections to *C. jejuni* isolates from chicken slaughter batches and swimming water. At MLST level, 79% of the sequence types (STs) of the human isolates overlapped with chicken STs suggesting chicken as an important reservoir. Four STs, the ST-45, ST-230, ST-267 and ST-677, covered 75% of the human and 64% of the chicken isolates. In addition, 50% of the swimming water isolates comprised ST-45, ST-230 and ST-677. Further wgMLST analysis of the isolates within STs, accounting their temporal relationship, revealed that 22 of the human isolates (24%) were traceable back to *C. jejuni* positive chicken slaughter batches. None of the human isolates were traced back to swimming water, which was rather sporadically sampled. The highly discriminatory wgMLST, together with the patient background information and temporal relationship data with possible sources, offers a new, accurate approach to trace back the origin of domestic campylobacteriosis. Our results suggest that potentially a substantial proportion of campylobacteriosis cases during the seasonal peak most probably are due to other sources than chicken meat consumption. These findings warrant further wgMLST-based studies to reassess the role of other reservoirs in the *Campylobacter* epidemiology both in Finland and elsewhere.

1. Introduction

Campylobacteriosis is the most commonly reported cause of bacterial gastroenteritis in the European Union (EU), with 214,779 reported infections in 2013 (EFSA, 2014). In Finland, more than 4251 human *Campylobacter* infections were registered in 2012 (incidence rate 78.7/100 000) and in 2014 the number of cases increased to 4887 (incidence rate 90.1/100 000), and approximately 95% are caused by *C. jejuni* (www.thl.fi). Approximately half of these infections are associated with foreign travel, but in summer, domestically acquired infections increase during the seasonal peak from July to September (Vierikko et al., 2004). Furthermore, most cases are defined as sporadic, which complicates tracing the source of infection (www.thl.fi). Several studies have identified handling of raw or eating improperly cooked poultry meat as major risk factors for acquiring *Campylobacter* infection (Levesque et al., 2013; Mughini Gras et al., 2012; Strachan et al., 2013). Other potential sources are the consumption of contaminated beef or unpasteurized milk, drinking water from private wells, and swimming in natural waters, which a previous Finnish case–control study also identified as risk factors (Schönberg-Norio et al., 2004). Furthermore, surface waters have been shown to contain *Campylobacter* spp. (Hörman et al., 2004) and recreational swimming activities are common in Finland, especially in summer.

C. jejuni is a commensal of the gastrointestinal tract of most warm-blooded animals and birds including chicken (http://www.who.int/mediacentre/factsheets/fs255/en/). Due to EU-legislation, the occurrence of *Campylobacter* in broiler production has been monitored since 2007 (http://www.efsa.europa.eu/en/topics/topic/campylobacter.htm), revealing a high level of *Campylobacter*–positive slaughter batches as well as retail meat in several countries. However, the prevalence has remained low in Finland, (EFSA, 2014). Approximately 100 million kg

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of broiler meat is consumed annually in Finland and in 2012, of which the majority originates from domestic production. However, imported chicken meat (90% from Thailand and Brazil), approximately 1 to 3 million kg, is used in meat processing industry and is therefore not sold as raw meat products at retail (www.mmm.fi). Thus only domestically produced chicken meat contaminated with *C. jejuni* is reasonable to consider as a relevant source to Finnish consumers.

Multilocus sequence typing (MLST) has proved to be an essential tool in studies of the molecular epidemiology and population genetics of *C. jejuni* (Cody et al., 2013; de Haan et al., 2013; Sheppard et al., 2009). Our previous MLST studies have revealed that the most frequent sequence types (STs) among Finnish *C. jejuni* isolates, detected in human patients and broiler batches, are ST-45 and ST-230 (ST-45 clonal complex, [CC]), ST-50 (ST-21 CC), ST-267 (ST-283 CC) and ST-677 (ST-677 CC) (de Haan et al., 2014; Kovanen et al., 2014; Llarena et al., 2015). Whole-genome sequencing (WGS) and the use of comparative genomics tools such as whole-genome MLST (wgMLST) have become increasingly affordable, providing information from bacterial genomes with a much higher resolution than MLST (Bessell et al., 2012; Cody et al., 2013; Kovanen et al., 2014; Sheppard et al., 2012; Zhang et al., 2015). In combination with other epidemiological data, wgMLST enables the recognition of related isolates, providing more accurate information of the potential sources of infection.

Although several studies have compared *C. jejuni* isolates from the chickens and human patients, the data on temporal relationships between human patient and potential source of infection are rarely included, which would provide valuable knowledge on the epidemiology of the disease. We therefore performed this study (i) to analyze the association of MLST and wgMLST types, using recently developed software for wgMLST (Zhang et al., 2015), of Finnish *C. jejuni* isolates collected from patients with domestically acquired infections, chicken slaughter batches and swimming water during the seasonal peak in 2012 and (ii) to assess the ability of wgMLST to trace human *C. jejuni* infections to two different potential sources with simultaneous consideration of their temporal relationships.

2. Materials and methods

2.1. Bacterial isolates

The human patient *C. jejuni* isolates (n = 95) include all recovered isolates from domestically acquired enteric infections collected during the seasonal peak (June to September) from three hospital districts in Central Finland in 2012 as described in our previous study (Kovanen et al., 2014). The illness was defined as domestic if the patients had not been traveling outside of Finland in two preceding weeks prior to illness. The gender, age and the date of collection of the fecal sample were available to be included in the analysis. *C. jejuni* was isolated from the fecal samples by direct culture on modified charcoal cefoperazone deoxycholate agar (mCCDA) and confirmed to species level using species specific PCR as described by Kovanen et al. (2014). All clinical and microbiological data was provided by the three clinical laboratories of the districts. The districts center around four cities with populations of 279,000 (district 1), 121,000 (district 2 contains two cities), and 265,000 (district 3) inhabitants (http://www.stat.fi/tup/tilastotietokannat/index_en.html).

The included chicken-derived *C. jejuni* isolates (n = 83) were collected during 2012 in the Finnish *Campylobacter* monitoring program for poultry (Maa- ja Metsätalousministeriö, 2007) and are described in more detail in our previous study (Llarena et al., 2015). In brief, the surveillance program sampled all chicken batches slaughtered in Finland between June and October, and the detection of *C. jejuni* was done by direct plating of a pooled cecal sample (10 cecas/batch) on mCCDA. A single typical colony was selected for further analysis according to the method of The Food Safety Authority (Evira) 3512/5 (The Finnish Food Safety Authority Evira, 2012). Of the total 83 isolates included in the study (83 positive slaughter batches), 75 were isolated during the same period as the human patient isolates (June to September) and originated from a total of 37 farms indicating that a positive farm could have several positive batches (from one to four). According to the National Report to the European Food Safety Authority (EFSA), the total number of studied slaughter batches during the national reporting period from June to October was 1534, revealing that only 5.3% of all slaughter batches were positive for *C. jejuni* 2012 (EFSA, 2014; Llarena et al., 2015). Thus, the isolates represented 96.3% of the total *C. jejuni* population detected during the study period. Eight of the total 83 *C. jejuni* slaughter batch isolates were outside of the seasonal peak and were collected either in March, May or October. All 83 isolates were included in the wgMLST analysis. Fifty one of the total of 83 isolates represented single STs from the farms and the remaining 32 isolates were from the successive slaughter batches reared simultaneously on the farms. They had similar STs and PFGE types within successive batches. These 32 isolates were therefore excluded in the descriptive statistics analysis to avoid overestimating ST frequencies, as described by Llarena et al. (2015).

In addition to these, 20 *C. jejuni* isolates (from a total of 50 samples) from recreational swimming beaches (12 on lakes and one on a river) were collected from June to August 2012. Seven of the isolates were collected from 11 EU beaches, each sampled once a month (EU directive: 2006/7/EY, Ministry of Social affairs and Health: 711/2014) and located in the same three districts where the human isolates were collected. In addition, 13 isolates originated from samples collected approximately twice a month from a lake and a river located in the Helsinki metropolitan area (used as controls). Samples (100 ml or subsequent volumes of 100 ml and 1.5 l, stored at +4 °C prior to analysis) were concentrated using 0.45 μm pore size membrane filters (Millipore, Billerica, MA, USA), enriched in 100 ml of Bolton enrichment broth (Oxoid Ltd., Basingstoke, Hampshire, UK) including 5% of defibrinated horse blood and incubated microaerobically at +37 °C for 48 h. Ten μl was streaked onto mCCDA plates (Oxoid Ltd.) and incubated microaerobically at 41.5 ± 0.5 °C for 24–48 h. Typical colonies were stored in nutrient broth (85% Nutrient broth, 15% glyserol) at −70 °C. DNA was extracted using the Wizard genomic DNA purification kit (Promega, Mannheim, Germany) and species (*C. jejuni*) was confirmed using multiplex PCR (Kovanen et al., 2014).

2.2. Whole-genome sequencing

Draft genome sequences were determined using Illumina HiSeq sequencing technology (Nextera library, 100 cycles, paired-end library, >40× coverage). NGS library preparation, enrichment, sequencing and sequence analysis were performed by the Institute for Molecular Medicine Finland (FIMM Technology Centre, University of Helsinki, Finland). Reads were assembled using SPAdes 3.2.1 (Bankevich et al., 2012) using default settings.

2.3. MLST and wgMLST

MLST types were assigned using the *Campylobacter* MLST database (http://pubmlst.org/campylobacter/). Assembled contigs of the whole-genome sequences were further analysed applying a gene-by-gene approach using Genome profiler (GeP) (Zhang et al., 2015). The draft genomes of four isolates (three human and one water isolate) were too fragmented (> 100 contigs) and were excluded from further wgMLST analysis. The Split Decomposition networks, representing allelic distance matrix of the shared loci of the isolates, were constructed for each ST using SplitsTree 4 (Huson and Bryant, 2006) and edited using CorelDRAW X6 (Corel Corporation, Ottawa, Ontario, Canada). To increase resolution, isolates forming clusters within the same ST were re-analysed using GeP. To identify single nucleotide polymorphisms (SNPs), alignments of loci having multiple allele types were manually inspected. Variations in homopolymeric tract lengths were not considered as SNPs (Bayliss et al., 2012), even though they were registered