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Enhanced microbiological surveillance reveals that temporal case clusters contribute to the high rates of campylobacteriosis in a model agroecosystem

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ABSTRACT

Infections by pathogenic *Campylobacter* species were determined in diarrheic (n = 2,217) and non-diarrheic control (n = 104) people in Southwestern Alberta (SWA), Canada over a 1-year period using specialized and conventional isolation, and direct PCR. Overall, 9.9% of diarrheic individuals were positive for *C. jejuni* (9.1%), *C. upsaliensis* (0.6%), and *C. coli* (0.5%). No *C. lari* was detected. Four diarrheic individuals were co-infected with *C. jejuni* and *C. coli*, and four different individuals were co-infected with *C. jejuni* and *C. upsaliensis*. Two control individuals were positive for *C. jejuni*. Approximately 50% of stools containing *C. jejuni* and/or *C. coli* were deemed negative by conventional isolation. Direct PCR for *C. jejuni* was less effective than culture-based detection. Most *C. jejuni* infections occurred in people living in the urban centers, but the prevalence of the bacterium was lower in females than males living in urban locations, and both males and females living in rural locations. Although *C. jejuni* was detected throughout the year, a trend for higher infection rates was observed in the late spring to early fall with a peak in August. Forty-six *C. jejuni* subtype clusters were identified, including 44 temporal case clusters attributed to 28 subtype groupings. The majority of infections (70.3%) were linked to subtypes associated with beef cattle. We conclude that many occurrences of pathogenic *Campylobacter* species were not detected by the conventional laboratory methodology, and temporal case clusters of *C. jejuni* subtypes associated with cattle contribute to the high rates of campylobacteriosis in SWA.

1. Introduction

Southwestern Alberta (SWA), Canada, is a large geographical area in Western Canada with a population of approximately 181,000 people (2016 census). The region is serviced by a single public diagnostic facility located at the Chinook Regional Hospital (CRH) in Lethbridge. All stools submitted to the CRH by physicians are subjected to a panel of conventional culture-based methods for recognized pathogenic bacteria, including *Campylobacter* species. The prevalence of campylobacteriosis incited by *C. jejuni*, and to a lesser extent *C. coli*, within the region is substantially higher than both the provincial and Canadian national averages (Alberta Government, 2004; Inglis et al., 2011). Of the thirty-eight characterized species of *Campylobacter* that have been identified, *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* are currently recognized to negatively impact human health (http://www.antimicrobe.org/b91.asp); they are collectively referred to as 'pathogenic *Campylobacter* species' hereafter. Of these taxa, *C. lari* is infrequently isolated and *C. upsaliensis* is not identified by current CRH microbiology procedures, and it is unknown whether they are under-represented as incitants of enteritis in the region. To investigate true infection rates by pathogenic *Campylobacter* species in SWA, we previously conducted a 5-month-long pilot study that primarily relied on the use of direct diagnostic PCR (Inglis et al., 2011). Although this study demonstrated that infection rates were substantially higher than indicated by conventional diagnosis, it was limited in both the duration of sample collection and by the number of putative *Campylobacter* isolates recovered.

Campylobacter species, particularly *C. coli* and *C. jejuni*, are highly genetically diverse (Sheppard and Maiden, 2015). Our research has shown that only a subset of *C. jejuni* subtypes circulating in SWA constitute a concern to public health (i.e. clinically-relevant strains; CRS).

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Furthermore, the majority of CRS in SWA are predominantly associated with beef cattle, which suggests that cattle are an important reservoir of pathogenic *C. jejuni* in the region (our unpublished data). This is consistent with the high densities of beef cattle production in SWA (Alberta Government, 2014), and also with the conclusions of a preliminary epidemiological examination that linked one quarter of individuals infected with *C. jejuni* or *C. coli* to close contact with livestock, primarily cattle (Hasselback, 2002). Given the characteristics of SWA (e.g. high rates of enteritis, high densities of livestock, a single and public diagnostic facility, an approximate 40:60 rural:urban distribution of people, a single prominent watershed, and a spatial gradation of human activity from west to east), our team is currently employing a regional One Health approach using SWA as a model agroecosystem in an attempt to elucidate the epidemiology of campylobacteriosis. Towards this goal, we formulated the following hypotheses: (i) infection rates by pathogenic *Campylobacter* species are under-estimated by the conventional isolation method currently used at the CRH; (ii) subtype diversity will be affected by the isolation strategy used (e.g. diversity will be lower for subtypes recovered by enrichment); (iii) specialized isolation relative to PCR-based detection will provide a more accurate representation of true infection rates by *C. jejuni*; (iv) individuals living in rural areas in SWA will be infected at higher rates than urban dwellers; (v) *C. jejuni* subtypes from diarrheic individuals in SWA will be predominately associated with cattle; and (vi) cases of campylobacteriosis in SWA will be sporadic. To test the above hypotheses, the following objectives were established: (i) develop and validate primers for direct detection/quantification of *C. jejuni*; (ii) determine the prevalence of *C. jejuni* in stools using direct PCR; (iii) contrast direct PCR detection with conventional and specialized isolation methods for pathogenic *Campylobacter* species; (iv) contrast detection frequencies in diarrheic humans living in SWA by date, gender, age, and place of habitation (i.e. rural versus urban); (v) subtype all recovered *C. jejuni* isolates and ascertain subtype diversity within individuals and by isolation method; (vi) link *C. jejuni* subtypes recovered from diarrheic people in SWA to non-human reservoirs to glean information on reservoirs and transmission mechanisms; and (vii) collate the temporal dynamics of *C. jejuni* subtypes with epidemiological data to ascertain the degree to which campylobacteriosis is sporadic in SWA.

2. Materials and methods

2.1. Primers for direct detection of Campylobacter jejuni

To ascertain primer specificity, genomic DNA from select type strains of *Campylobacter*, *Arcobacter*, and *Helicobacter* species/subspecies was extracted using an AutoGen 740 robot (AutoGen, Inc., Holliston, MA) according to the manufacturer's protocol for gram negative bacteria. Isolates were subjected to PCR targeting the *ipxA* gene (Klena et al., 2004), the *mapA* gene (Inglis and Kalischuk, 2003), and the *hipO* gene (Inglis et al., 2018). Extracted DNA from an isolate of *C. jejuni* that was determined to be hippurate negative (Nicholson and Patton, 1995) was included. All samples were run in duplicate using a Mastercycler Pro S (Eppendorf, Mississauga, ON), along with positive (i.e. genomic DNA from the type strain of *C. jejuni* subsp. *jejuni*) and negative (i.e. Optima water) controls. Amplicons were visualized in a 2% Tris-Acetate-EDTA agarose gel. The expected size of the amplicon for the *ipxA* primer set is 331 bp, for *mapA* is 589 bp, and for *hipO* is 695 bp.

As the primers targeting the *mapA* gene were not specific to *C. jejuni*, the ability of primers to detect all subtypes of *C. jejuni* (i.e. primer inclusiveness) was evaluated only with the primers targeting *ipxA* and *hipO*. Inclusiveness was evaluated against 91 isolates of *C. jejuni* that represented unique subtypes as determined by comparative genomic fingerprinting (CGF) (Taboada et al., 2012). The isolates were recovered from human beings, bovine, bison, poultry, a wild avian, and water.

Given that the primers targeting the *ipxA* gene were more inclusive than the primers targeting the *hipO* gene, they were selected for their effectiveness at detecting *C. jejuni* in seeded feces (i.e. primer sensitivity). Furthermore, the *ipxA* primers and their predicted amplicon were confirmed to be appropriate for quantitative (q)PCR. Swine was used as monogastric mammalian model. Freshly excreted porcine feces that was determined to be free of *C. jejuni* by culture and PCR was stored at −20 °C until used. No antibiotic was administered to the pigs. The strategy as described by Webb et al. was used (Webb et al., 2016). Briefly, biomass of *C. jejuni* ATCC 33560 was produced on Karmali Agar (Oxoid Inc., Nepean, ON) (KA) in a low hydrogen microaerobic atmosphere (i.e. 5% O_2, 3% H_2, 10% CO_2, and 82% N_2) at 37 °C for 48 h. In all instances, microaerobic gases were purchased from Praxair Canada Inc. (Lethbridge, AB), and dispensed into anaerobic 3.5 l jars (Oxoid Inc.) using the gas evacuation and input valves in concert; the jar was flushed two times with the microaerobic gas. Cells were suspended in Columbia broth (Difco, BD Biosciences, Mississauga, ON) (CB) and quantified with a spectrophotometer. The absorbance (A_{600}) was adjusted to 0.5 and corresponded to approximately 2.0×10^9 cells ml^{-1}. The suspension was diluted with CB in a 10-fold dilution series, and densities of cells were estimated by dilution-plating onto KA. To inoculate feces, 1.0 ml from each dilution of *C. jejuni* cells was thoroughly mixed into 10 g of the thawed feces. The control treatment consisted of 10 g of feces mixed with 1.0 ml of sterile CB. Three subsamples (200 ± 20 mg) were removed from the seeded feces and stored at −20 °C for later DNA extraction. Densities of *C. jejuni* cells in the seeded feces were estimated by dilution-plating onto KA containing selective supplement, SR167 (Oxoid Inc.) (KKA). The experiment was conducted on two separate occasions.

A synthetic gene was used as the internal amplification control (IAC), and added to the thawed fecal subsamples before extraction of DNA to verify extraction and PCR efficiency (Webb et al., 2016). DNA was extracted from subsamples using a QIAamp DNA Stool Minikit (Qiagen Inc., Toronto, ON) according to the manufacturer's specifications for pathogen detection. The presence of IAC was determined as described previously (Webb et al., 2016). Quantitative PCR for *C. jejuni* was carried out using the following reagents: 10 µl of 2X Quantitect SYBR Green Master Mix (Qiagen Inc.), 2.0 µl of bovine serum albumin (0.1 µg $µl^{-1}$; Promega, Madison, WI), 1.0 µl of IpxACjejuni (10 µM; Integrated DNA Technologies), 1.0 µl of IpxARKK2m (10 µM; Integrated DNA Technologies, Coralville, IA), 2.0 µl of DNA template, and 4.0 µl of nuclease-free water (Qiagen Inc.). The amplification conditions were one cycle at 95 °C for 15 min, followed by 40 cycles of 15 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C for data acquisition. At the end of amplification, melt curve analysis was conducted. The quantitative PCR data were analyzed using MxPro (version 4.10; Agilent Technologies Inc.). Threshold cycles (Ct values) of samples were compared to a standard curve generated from quantified DNA extracted from *C. jejuni* ATCC 33560.

2.2. Stool collection

All stools from human beings exhibiting signs of enteritis (i.e. diarrhea) and submitted to the CRH over a 1-year period, from April 29th 2008 to April 30th 2009, were processed. Information released to researchers included a coded individual identifier, stool collection date and time, as well as the age, gender, and place of habitation (i.e. based on postal code) of the individual. In addition, stools from a cohort of healthy humans living in SWA were processed at two time periods (October 27 to November 14th 2008, and March 23th to April 09th 2009). Information provided with the stools from healthy subjects included a coded individual identifier, stool collection date and time, age, gender, and whether they live in an urban or rural location. The majority of stool samples from diarrheic humans were suspended in an Enteric Pathogen Transport (EPT) medium at the time of collection and transported to the diagnostic facility at the CRH within 1 to 2 days of

sample collection; the EPT medium used was Cary-Blair medium (Cary and Blair, 1964). Stool samples were processed by conventional methods immediately upon arrival at the CRH. The unused portion of the sample was placed at 4 °C in ambient atmosphere, and an aliquot of the stool was transferred to Agriculture and Agri-Food Canada (AAFC) Lethbridge Research and Development Centre (LeRDC) staff daily. Samples determined to be positive for *Campylobacter* at the CRH by culture were not disclosed to LeRDC personnel until the data collection phase of the study was completed. Stool samples from healthy humans were not placed in an EPT medium. In the majority of cases, samples were transferred to AAFC LeRDC within ≈6 h of excretion; samples collected ≥1 h before submission were kept on ice.

2.3. Conventional detection of Campylobacter species

At the CRH, a standard protocol for isolating *C. jejuni* and *C. coli* was utilized (Inglis et al., 2011) (Fig. S1). Briefly, subsamples of feces were streaked onto Campy CVA Agar (Becton Dickinson, Oakville, ON) (CVA), cultures were incubated at 42 °C in an atmosphere consisting of 10% CO_2, 10% H_2 and 80% N_2, and colonies exhibiting characteristic *Campylobacter* morphology were isolated and tested for their ability to hydrolyze hippurate using the disc method (Dalynn Biologicals, Calgary, AB). Hippurate positive strains were presumptively deemed to be *C. jejuni*, whereas hippurate negative strains were presumptively identified as *C. coli*. All *Campylobacter* isolates were frozen in 30% glycerol at −70 °C at the CRH until transferred to LeRDC for further characterization.

2.4. Specialized isolation of Campylobacter species

Campylobacter species were isolated from stool samples at AAFC LeRDC by direct plating, membrane filtration, enrichment, and immunobead capture (i.e. referred to as specialized isolation hereafter) (Fig. S1). A high hydrogen microaerobic atmosphere consisting of 5% O_2, 30% H_2, 10% CO_2, and 55% N_2 was used for all specialized isolations.

For direct plating, a fecal suspension (1:10) was prepared in CB, 25 µl of the suspension was spread onto KKA, KA containing selective supplement SR0183 (Oxoid Inc.) (KBA), and *Arcobacter* Selection and Isolation Agar (ASIA) (de Boer et al., 1996) in 60-mm-diam Petri dishes in duplicate. Cultures were maintained at 37 °C in the microaerobic atmosphere. Cultures were examined after 48 h and 144 h, and where applicable, biomass from each of two colonies per colony morphology, that were characteristic of *Campylobacter* were streaked onto KA, and maintained microaerobically at 37 °C. When necessary, cells from colonies were examined for size, shape, and characteristic motility for *Campylobacter*-like bacteria using phase-contrast microscopy.

For membrane filtration, a slurry of feces was spread on a 4.7-cm-diam membrane (0.45 µm; EMD Millipore, Etobicoke, ON) placed on a Columbia Agar (CA) with 10% sheep blood (CBA). If necessary (i.e. for non-diarrheic samples), a fecal slurry was prepared in CB. The slurry was allowed to filter passively for 1 h in the microaerobic atmosphere at 37 °C, at which the point the membrane was carefully removed and discarded, and the CBA culture was again placed at 37 °C in the microaerobic atmosphere. Cultures were examined at 48 h and 144 h, and biomass from two presumptive *Campylobacter* colonies per morphology was streaked onto CBA as described for direct plating.

For enrichment, all cultures were maintained in a microaerobic atmosphere. The following four enrichment broths were used: (i) Bolton Broth (Oxoid, Inc.) with Bolton selective supplement, SR0183 (BBB); (ii) Johnson Murano Broth (JMB) (Johnson and Murano, 1999); (iii) *Arcobacter* Selection and Isolation Broth (ASIB) (de Boer et al., 1996); and (iv) Bolton Broth with 5% laked horse blood, Amphotericin B (10 mg L^{-1}), and Trimethoprim (5.0 mg l^{-1}) (BAT). A 25 µl aliquot of the fecal slurry in CB was added to 5.0 ml of each enrichment broth in 100 x 16 mm culture tubes. Tubes containing BBB were incubated for 3 h at 30 °C, followed by 21 h at 37 °C; 10 µl of the enrichment broth was then removed and streaked in duplicate onto KKA and KBA and maintained at 37 °C. Tubes containing JMB and ASIB were incubated at 30 °C and 37 °C, and 10 µl of the enrichment broth from the tubes at 24 and 48 h was removed and streaked in duplicate onto Johnson Murano Agar (JMA) (Johnson and Murano, 1999) and ASIA, respectively. Tubes containing BAT were incubated at 37 °C for 48 h, and then processed by membrane filtration onto CBA as described above. Cultures on solid media were maintained in the microaerobic atmosphere at the same temperature as the enrichment culture, examined after 48 h and 96 h, and biomass from two presumptive *Campylobacter* colonies per morphology were streaked onto KA and incubated at the same temperature as was used for isolation.

For isolation using magnetic immunobeads, beads were generated from Dynabeads M-280 Tosylactivated (Thermo Fisher Scientific, Toronto, ON) following the manufacturer's protocol. The beads were coated with *Campylobacter* anti-goat antibody (BacTrace® cat #01-92-93, Mandel Scientific, Guelph, ON) and stored at 4 °C until use. Immunobeads (5 µL) were added to 1 ml of a 1:10 slurry of feces in CB in a 2 ml tube, and the culture was rocked using a Labquake shaker (Barnstead/Thermolyne, Dubuque, IW) for 20 min at room temperature. The beads were concentrated with a magnet (Invitrogen Thermo Fisher Scientific, Toronto, ON) and the liquid removed. The beads were then washed four times with 1 ml of 0.01 M phosphate buffered saline (PBS; pH 7.2), resuspended in 50 µl of PBS, and 25 µl streaked onto KA supplemented with vancomycin (0.02 mg mL^{-1}) and cyclohexamide (0.05 mg mL^{-1}) (KVC), and CA containing 5% sheep blood and supplemented with the same concentration of vancomycin and cyclohexamide (BVC). Cultures were incubated in a microaerobic atmosphere at 37 °C for 48 h and 96 h.

All presumptive *Campylobacter*-like isolates were streaked for purity, biomass transferred to CB containing 40% glycerol, and cultures maintained frozen at −80 °C until identified. For each stool sample, a total of four enrichment tubes, one immunobead tube, and eighteen Petri dishes containing agar media were used to isolate *Campylobacter* species at AAFC LeRDC.

2.5. Identification of presumptive Campylobacter species

Genomic DNA of isolates was extracted using an AutoGen 740 robot (Holliston, MA) according to the manufacturer's protocol. All isolates were identified using genus-specific PCR targeting the 16S rRNA gene (Linton et al., 1996), *C. jejuni*-specific PCR targeting the *hipO* gene (see above) and *ipxA* gene (Klena et al., 2004), and species-specific primers for *C. coli*, *C. lari*, and *C. upsaliensis* targeting the 16S rRNA gene (Linton et al., 1996). Where required, the near complete 16S rRNA and *cpn*60 genes were sequenced, and sequences were compared to those in GenBank (National Center for Biotechnology Information) using BlastN.

2.6. Direct PCR detection and quantification of Campylobacter jejuni

DNA was extracted from 200 ± 20 mg of stool using the QIAamp DNA Stool Mini Kit, protocol for pathogen detection (Qiagen, Inc.) (Fig. S1). Prior to extraction, the IAC was added to each subsample, and DNA (2 µl) from each extracted sample was subjected to quantitative PCR for the IAC and *C. jejuni* as above. For subsamples that were PCR positive for *C. jejuni*, total bacterial DNA was also quantified using the HDA1 and HDA2 degenerate primers targeting the 16S rRNA gene (Abnous et al., 2009).

2.7. Subtyping of Campylobacter jejuni isolates

All *C. jejuni* isolates were subtyped using the CGF method (Taboada et al., 2012). Briefly, multiplex end-point PCR was performed using primers that had been previously designed and validated, and amplicons analyzed using capillary electrophoresis to generate a 40-digit

binary fingerprint representing the presence/absence of each accessory gene for each *C. jejuni* isolate.

2.8. Data analysis

Urban dwellers were defined as people living in Coaldale (population of 7,493 in 2011), Lethbridge (population of 83,517 in 2011), or Taber (population of 8,104 in 2011). Other locations were designated as rural (i.e. rural areas or towns with a population ≤3,743). Comparisons of counts among treatments were analyzed using the Genmod procedure of SAS (Statistical Analysis Software, Cary, NC); where warranted, zero counts were adjusted to 0.5. Temporal infection rates and infections by age class were analyzed using the Genmod procedure of SAS with a log-linear model (i.e. Poisson distribution using a log offset); zero counts were removed from the analyses. In the event of a significant main effect, the least square means test was used to compare means (e.g. age classes). To model temporal infection trends, the Dynamic Fit Wizard feature of SigmaPlot (Systat Software, Inc., San Jose, CA) with the Gaussian, 4 Parameter option was applied.

One representative isolate per subtype was arbitrarily selected from each stool sample based on CGF profile consensus, and isolates were assigned subtype cluster identifiers based on a 95% similarity CGF profile using a simple matching similarity matrix and unweighted pair group method with arithmetic mean clustering in BioNumerics (version 6.6, Applied Maths, Austin USA). Likely reservoirs of *C. jejuni* isolates that infected people in SWA (i.e. subtype clusters) were determined using the Canadian *Campylobacter* CGF database (C3GFdb), which includes > 25,000 *C. jejuni* isolates that have been subtyped by CGF including ≈10,000 isolates from SWA. The C3GFdb currently contains data for 4,791 *C. jejuni* isolates from human beings, 4,357 isolates from water, 6,056 isolates from cattle, 7,214 isolates from poultry, and 3,457 isolates from other sources (e.g. swine, other miscellaneous livestock, wild birds, wild mammals, and miscellaneous environmental matrices). The CGF profiles of *C. jejuni* isolates recovered in the current study were compared to CGF profiles within the C3GFdb with metadata (e.g. source) to ascertain the primary hosts with which a CGF subtype is associated in SWA and elsewhere in Canada. We also compared stool sample collection dates and location with *C. jejuni* isolate subtype clustering to identify temporal case clusters of campylobacteriosis in SWA between 2008 and 2009; case clusters were defined as the presence of the same *C. jejuni* subtype cluster in two or more clinical cases within a two week rolling window. Subtype clusters were used to examine *C. jejuni* genetic diversity and population structure in order to identify linkages between specific epidemiological factors (e.g. patient age, sex, etc.), individual temporal case clusters, and method of isolation. Shannon's index of diversity for *C. jejuni* isolates, and Hutcheson's *t*-test was applied to test significance of observed differences in relation to methods of isolation. Pairwise PermANOVA analysis was performed to test the significance of differences in population structure using Primer 7 (version 7.0.11, Quest Research Limited, Auckland NZ). PermANOVA parameters consisted of 9999 iterations of main test and pair-wise unrestricted permutations of raw data with partial sums of squares.

3. Results

3.1. Inclusivity, specificity, and sensitivity of primers for direct detection of *Campylobacter jejuni*

The *ipxA* primers generated an amplicon for both the *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* type stains, as well as for the hippurate negative *C. jejuni* strain. The *hipO* primers generated a strong amplicon for the type strain of *C. jejuni* subsp. *jejuni*, which is hippurate positive (Table S1). However, the *hipO* primers did not generate an amplicon for the type strain of *C. jejuni* subsp. *doylei*, nor for a hippurate negative *C. jejuni* strain. The *hipO* and *ipxA* primers did not generate an amplicon for any of the non-*C. jejuni* type strains of *Arcobacter*, *Campylobacter*, or *Helicobacter* evaluated. The *mapA* primers generated a strong amplicon for all three *C. jejuni* strains, but also generated weak amplicons for all non-*C. jejuni* strains. As a result the *mapA* primers were not evaluated further. The inclusiveness of the *ipxA* and *hipO* primers was evaluated against 91 genetically distinct strains of *C. jejuni* as determined by CGF (Table S2). The *ipxA* primers generated an amplicon for 94.5% of the strains evaluated, whereas the *hipO* primers generated an amplicon for 87.9% of the strains. The minimum level of detection of *C. jejuni* in feces seeded with the bacterium by qPCR targeting the *ipxA* gene was between 5.0×10^2 and 5.0×10^3 CFU g^{-1} (Fig. S2). No amplification was observed for unseeded porcine feces.

3.2. Demographic characteristics of diarrheic and healthy control individuals

Two thousand, seven hundred and nine samples from 2,264 individuals were submitted to the CRH and processed for enteric bacterial pathogens using conventional diagnosis from April 29, 2008 to April 30, 2009 (i.e. diarrheic individuals). Forty seven of the diarrheic individuals submitting samples to the CRH did not live in SWA, and were removed from the study. Unless indicated otherwise, all results presented hereafter are for individuals who lived in the study region (n = 2,217). Three hundred and twenty six diarrheic individuals (14.7%) submitted multiple samples (2.4 ± 0.6 samples on average; the maximum was five stools from two individuals). The ages of diarrheic individuals submitting samples ranged from 1 month- to 102 years-of-age (mean = 43.2 ± 27.7 years-of-age). Nine hundred, and thirty one (42.1%) of the diarrheic samples submitted were from males, and 1,283 (57.9%) samples were from females; three individuals did not specify their gender. One thousand, three hundred and thirty five (60.4%) and 877 (39.7%) of the individuals submitting samples lived in urban and rural locations, respectively. The number of diarrheic stools submitted per week averaged 50.2 ± 8.6 (range 35 to 67). The average time from stool collection to processing of diarrheic stools for pathogenic *Campylobacter* species by AAFC was 1.9 ± 1.2 days.

One hundred and four healthy control individuals provided informed consent, and submitted stool samples that were processed. One hundred and fifty nine stools from control individuals were submitted at two periods, from October 27th to November 14th 2008, and from March 23th to April 09th 2009. The ages of control individuals submitting samples ranged from 21 to 78 years-of-age (average of 45.7 ± 12.7 years-of-age). Fifty-three (51.0%) were male, and 51 (49.0%) were female, and 94 (90.4%) lived in urban centres, and 10 (9.6%) lived in rural locations.

3.3. Campylobacter isolates recovered by specialized isolation

In total, 17,208 enrichment and 86,040 agar cultures for primary isolation of *Campylobacter* species were processed by AAFC LeRDC staff over the study period. Nine hundred and eighty one isolates of *C. jejuni* (92.0%), 51 isolates of *C. coli* (4.8%), and 34 isolates of *C. upsaliensis* (3.2%), and no isolates of *C. lari* were isolated from diarrheic individuals who lived in the study region using specialized isolation (1,066 isolates in total). An additional four *C. jejuni* isolates were recovered from two diarrheic individuals who did not live in the study area, and one *C. jejuni* isolate was recovered from each of two control individuals.

Campylobacter coli, *C. jejuni*, and *C. upsaliensis* were isolated using direct plating, enrichment, and membrane filtration (Table 1). *Campylobacter jejuni* alone was isolated using magnetic immunobeads coated with *C. jejuni*-specific antibodies. For *C. coli*, most isolates were recovered by enrichment (51.0%) and by direct plating (29.4%); direct plating on KBS and enrichment in BAT followed by membrane filtration provided the highest recovery with the lowest degree of bacterial and fungal contamination. For *C. jejuni*, most isolates were recovered by

Table 1
Number and prevalence (%) of *Campylobacter coli*, *C. jejuni*, and *C. upsaliensis* isolates by isolation method and medium.

Method / media[a]	*C. coli*		*C. jejuni*		*C. upsaliensis*	
Direct plating	15	(29.4%)	316	(32.2%)	8	(23.5%)
ASIA	0	(0.0%)	2	(0.2%)	3	(8.8%)
KBA	15	(29.4%)	252	(25.7%)	5	(14.7%)
KKA	0	(0.0%)	62	(6.3%)	0	(0.0%)
Membrane filtration	10	(19.6%)	254	(25.9%)	15	(44.1%)
CBA	10	(19.6%)	254	(25.9%)	15	(44.1%)
Enrichment	26	(51.0%)	384	(39.1%)	11	(32.4%)
ASIB-ASIA	0	(0.0%)	10	(1.0%)	6	(17.7%)
BAT-MF	16	(31.4%)	15	(1.5%)	0	(0.0%)
BBB-KBA	7	(13.7%)	140	(14.3%)	3	(8.8%)
BBB-KKA	0	(0.0%)	49	(5.0%)	0	(0.0%)
JMB-JMA	3	(5.9%)	170	(17.3%)	2	(5.9%)
Immunobeads	0	(0.0%)	27	(2.8%)	0	(0.0%)
BVC	0	(0.0%)	10	(1.0%)	0	(0.0%)
KVC	0	(0.0%)	17	(1.7%)	0	(0.0%)
Total isolates	51		981		34	

[a] ASIA, *Arcobacter* Selection and Isolation Agar; ASIB, *Arcobacter* Selection and Isolation Broth; BAT, Bolton broth with Amphotericin B and Trimethprim; BBB, Bolton Broth with Bolton supplement; BVC, Columbia Blood Agar with Vancomycin and Cyclohexamide; CBA, Columbia Blood Agar; JMA, Johnson Murano Agar; JMB, Johnson Murano Broth; KBA, Karmali Agar with Bolton supplement; KKA, Karmali agar with Karmali supplement; KVC, Karmali Agar with Vancomycin and Cyclohexamide; and MF, membrane filtration. See text for additional information.

direct plating (32.2%), by enrichment (39.1%), and by membrane filtration onto CBA (25.9%); direct plating on KBS, enrichment in BB followed by plating of the enrichment broth on KBS, enrichment in JBA following by plating of the enrichment broth on JMA, and membrane filtration onto CBS were the most successful methods in general. For *C. upsaliensis*, isolation by membrane filtration onto CBA was best (44.1%). For culture positive stools, the number of isolates recovered per stool averaged 3.9 ± 5.2 (range of one to fourteen) for *C. coli*, 5.0 ± 4.8 (range of one to twenty) for *C. jejuni*, and 2.4 ± 2.3 (range of one to eight) for *C. upsaliensis*. More than one isolate was recovered from the majority of stools (61.4%) by specialized isolation; a single isolate was recovered from 38.6% of *Campylobacter* positive stools (Fig. S3).

3.4. Campylobacter infections not detected by conventional isolation

One (0.04%), 99 (4.5%), and none (0.0%) of the stools submitted by diarrheic individuals living in the study region were deemed positive for *C. coli*, *C. jejuni*, and *C. upsaliensis* by conventional isolation, respectively (Table 2). Substantially more individuals were positive ($P < 0.050$) for all three *Campylobacter* species by specialized isolation; 11 (0.5%), 182 (8.2%), and 14 (0.6%) of the stools submitted by diarrheic individuals were deemed positive for *C. coli*, *C. jejuni*, and *C. upsaliensis* using specialized isolation methods, respectively.

3.5. Detection of Campylobacter jejuni by direct PCR

Only 75 (3.4%) diarrheic individuals were positive for *C. jejuni* by direct taxon-specific PCR. The copy number of *C. jejuni* in diarrheic stools that were *C. jejuni* PCR positive averaged 2.96 ± 1.65 log copies mg^{-1} (range of 0–4.23 log copies mg^{-1}), and comprised 4.2% of the total bacteria present in feces by qPCR on average. A comparison of detection by specialized isolation relative to direct PCR indicated that a majority of stools that were *C. jejuni* culture positive were PCR negative (n = 129; 61.1%), 67 stools were both *C. jejuni* culture positive and PCR positive (31.8%), and 15 stools (7.1%) were *C. jejuni* culture negative and PCR positive. For the 15 stools that were end-point PCR positive but culture negative, quantities of *C. jejuni* in feces were low as determined by qPCR (i.e. a Ct value was not obtained). There was a weak but significant correlation ($r = 0.42$; $P < 0.001$) between number of isolates recovered by specialized isolation and densities of *C. jejuni* in the stool by qPCR. Overall, 111 (51.2%), 21 (9.7%), and 135 (62.2%) of *C. jejuni* positive stools were not detected by conventional isolation, specialized isolation, and direct PCR, respectively.

3.6. Prevalence of diarrheic individuals infected by Campylobacter jejuni

Two hundred and two diarrheic individuals who submitted stools to the CRH and lived in the study region were deemed positive for *C. jejuni* (9.1%) by direct PCR and/or by isolation over the 1-year duration of the study (Table 2). No isolates of *C. lari* were recovered. Eleven (0.5%) and 14 (0.6%) individuals were positive for *C. coli* and *C. upsaliensis*, respectively. Four individuals were co-infected with *C. jejuni* and *C. coli*, and four different individuals were co-infected with *C. jejuni* and *C. upsaliensis*. Overall, 219 of the 2217 diarrheic individuals were positive for pathogenic *Campylobacter* species (i.e. *C. coli*, *C. upsaliensis*, and/or *C. jejuni*) (9.9%). In total, two control individuals were positive for *C. jejuni* (1.9%), and no control individuals were positive for either *C. coli* or *C. upsaliensis*. The prevalence of infection was lower in control relative to diarrheic individuals for the three *Campylobacter* species collectively ($P < 0.021$) and for *C. jejuni* ($P = 0.029$), but not for *C. coli* ($P = 0.983$) or *C. upsaliensis* ($P = 0.850$).

3.7. Campylobacteriosis rates in individuals as a function of age, gender, and place of habitation

Infections by *C. jejuni* differed ($P = 0.012$) among age groups (Fig. 1). Younger and middle-aged diarrheic individuals were more commonly infected by *C. jejuni* than were older individuals. The average and median age of individuals infected by *C. jejuni* was 37.4 and 32.5 years, respectively. There was no effect ($P \geq 0.694$) of age on infection by either *C. coli* or *C. upsaliensis*. There was no difference ($P = 0.063$) in the prevalence of infection by *C. jejuni* between females (n = 104; 8.1%) and males (97; 10.4%). The majority of *C. jejuni* infection events were in people living in the urban centres of Coaldale, Lethbridge, and Taber (n = 112) compared to rural locations (n = 89) (Fig. 2). However, when adjusted for differential stool submission rates,

Table 2
Number of positive individuals and prevalence of infection (%) by pathogenic *Campylobacter* species in diarrheic (n = 2,217) and control (n = 104) individuals.

Group / method	*C. coli*		*C. jejuni*		*C. upsaliensis*		Total	
Diarrheic individuals	11	(0.5%) a[a]	202	(9.1%) a	14	(0.6%) a	219	(9.9%) a
Conventional isolation	1	(0.04%) b	99	(4.5%) b	0	(0.0%) b	–	
Specialized isolation	11	(0.5%) a	182	(8.2%) a	14	(0.6%) a	–	
PCR detection	–		75	(3.4%) b	–		–	
Control individuals	0	(0.0%) ab	2	(1.9%) b	0	(0.0%) ab	2	(1.9%) a
Conventional isolation	–		–		–		–	
Specialized isolation	0	(0.0%) ab	2	(1.9%) b	0	(0.0%) ab	–	
PCR detection	–		0	(0.0%) b	–		–	

[a] Means not followed by the same letter within individual columns differ ($P \leq 0.029$).

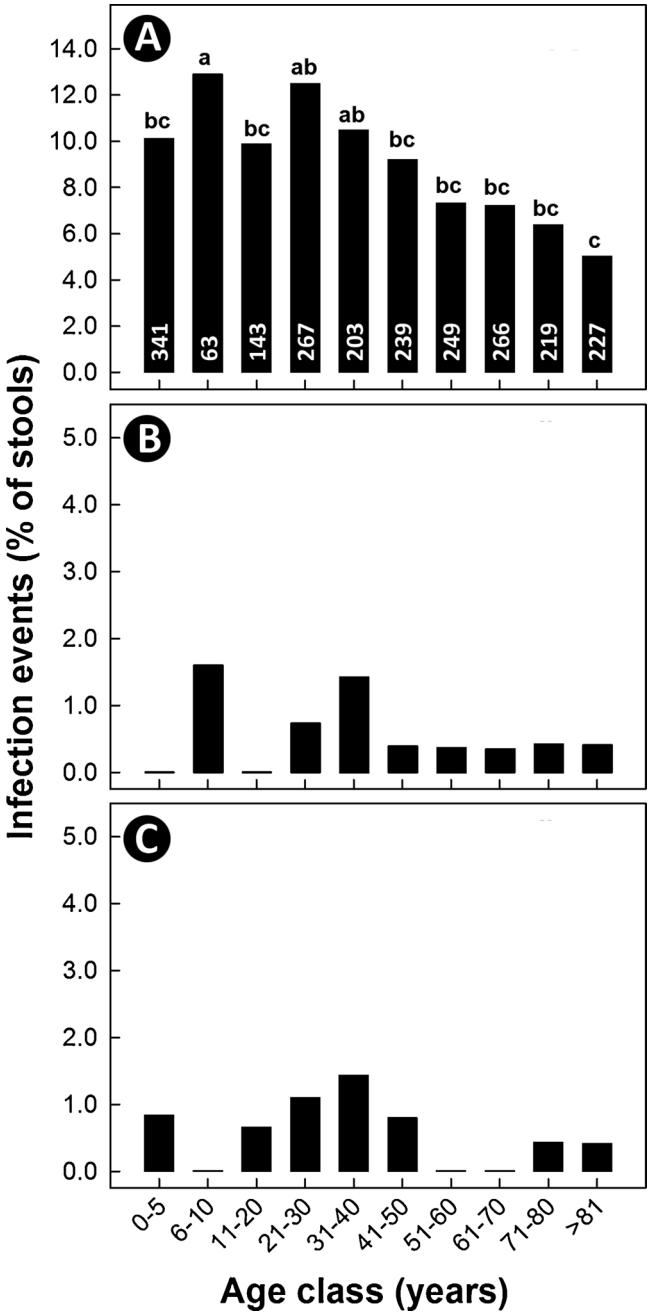

Fig. 1. Prevalence (% of stools) of diarrheic individuals living in Southwestern Alberta that were infected by *Campylobacter* species by age. (A) *Campylobacter jejuni*; (B) *C. coli*; and (C) *C. upsaliensis*. Age classes for *C. jejuni* infections not indicated with the same letter differ ($P < 0.050$) as determined using the Genmod procedure of SAS (see the materials and methods section for additional information). White numbers within histogram bars indicate the total number of individuals examined for each age class. The mean age of infected individuals was 37.3, 46.8, and 32.7 for *C. jejuni*, *C. coli*, and *C. upsaliensis*, respectively.

there was no difference ($P = 0.144$) in *C. jejuni* infection between people living in urban (8.3%) and rural (10.1%) locations. There were too few infections by *C. coli* and *C. upsaliensis* to examine infection trends based on place of habitation or gender. The prevalence of infection by *C. jejuni* (adjusted for differential submission rates) in females living in urban locations ($n = 52$; 6.6%) was less ($P \leq 0.050$) than males living in urban centers ($n = 58$; 10.6%), and both males ($n = 38$, 10.0%) and females living in rural locations ($n = 51$; 10.3%) (Table 3). An examination of infection rates as a function of age class showed that women from 21 to 60 years-of-age (i.e. working age) living in urban centres were infected by *C. jejuni* at rates equivalent to females of the same age living in rural locations, and males living in both urban and rural locations (Fig. 3).

3.8. Temporal rates of campylobacteriosis

Infections by *C. jejuni* occurred throughout the 1-year study period (Fig. S4). Although there were no differences in *C. jejuni* infections over time ($P = 0.566$), a trend for high rates of campylobacteriosis occurred in the late spring to early fall with a peak in mid- to late-August. There was no discernable temporal pattern of infection by *C. coli* ($P = 0.530$) and *C. upsaliensis* ($P = 0.962$).

3.9. Campylobacter jejuni subtype analysis

A total of 1,058 *C. jejuni* isolates collected from 176 stools from 171 individuals were subtyped by CGF, with 185 representative isolates selected for further analysis. Overall subtype diversity of isolated *C. jejuni* was high ($H = 3.23 \pm 0.17$), and isolates formed 46 CGF subtype clusters at a 95% profile similarity, 28 of which contained isolates from two or more clinical cases (Fig. 4). The five most commonly detected subtype clusters (i.e. 'primary clusters' 169.1, 269.4, 44.3, 853.10 and 735.5) accounted for 49.2% of clinical cases of campylobacteriosis in SWA during the sample period. A comparison of the greater than 25,000 isolates within the C3GFdb indicated that all of the primary clusters containing isolates from diarrheic individuals were associated with cattle as the dominant source of non-human isolates; the same trend was found among subtypes observed for 74.1% of all representative *C. jejuni* isolates.

No subtype diversity among *C. jejuni* isolates was observed for the majority of people from which more than one isolate was recovered. However, nine people (5.3%) submitted stools each containing *C. jejuni* belonging to two different subtype clusters, and five patients (2.9%) submitted two stools that contained *C. jejuni* from a different subtype cluster.

There was no difference in subtype diversity for *C. jejuni* isolated using direct plating ($H = 3.10 \pm 0.21$), enrichment ($H = 3.03 \pm 0.22$), and membrane filtration ($H = 3.01 \pm 0.21$). However, 12 (27.3%) subtype clusters were only detected using one of the isolation methods. Too few isolates were obtained from the magnetic immunobead isolation method for statistical comparison.

3.10. Temporal case clusters of Campylobacter jejuni subtypes

A total of 44 temporal case clusters attributed to 23 *C. jejuni* CGF subtype clusters were detected from May 2008 to April 2009, the majority of which occurred between May 2008 and September 2008 (Fig. 5). The subtype clusters, 44.3, 169.1, 269.4, 735.5, and 853.10 were linked to the greatest number of distinct temporal case clusters ($n = 5$, 6, 3, 3, and 4, respectively); the remaining subtype clusters were linked to at most two temporal case clusters. In some instances, there was an obvious connection between the temporal case clusters and other epidemiological variables such as place of habitation. For example, temporal case clusters were observed in individuals who lived in the same small towns for case cluster 1, 6, 22, 29, and 33, or even the same household in Lethbridge (i.e. case cluster 12) (Fig. 5; grey arrows). In other instances, a connection between individuals within the same temporal cluster was enigmatic; that is, infected individuals lived in different locations within SWA.

4. Discussion

Southwestern Alberta is an area in Canada with a rate of campylobacteriosis incited by *C. jejuni* and *C. coli* (59.7 cases $100\,K^{-1}$) that is substantially higher than the provincial (28.5 cases $100\,K^{-1}$) and

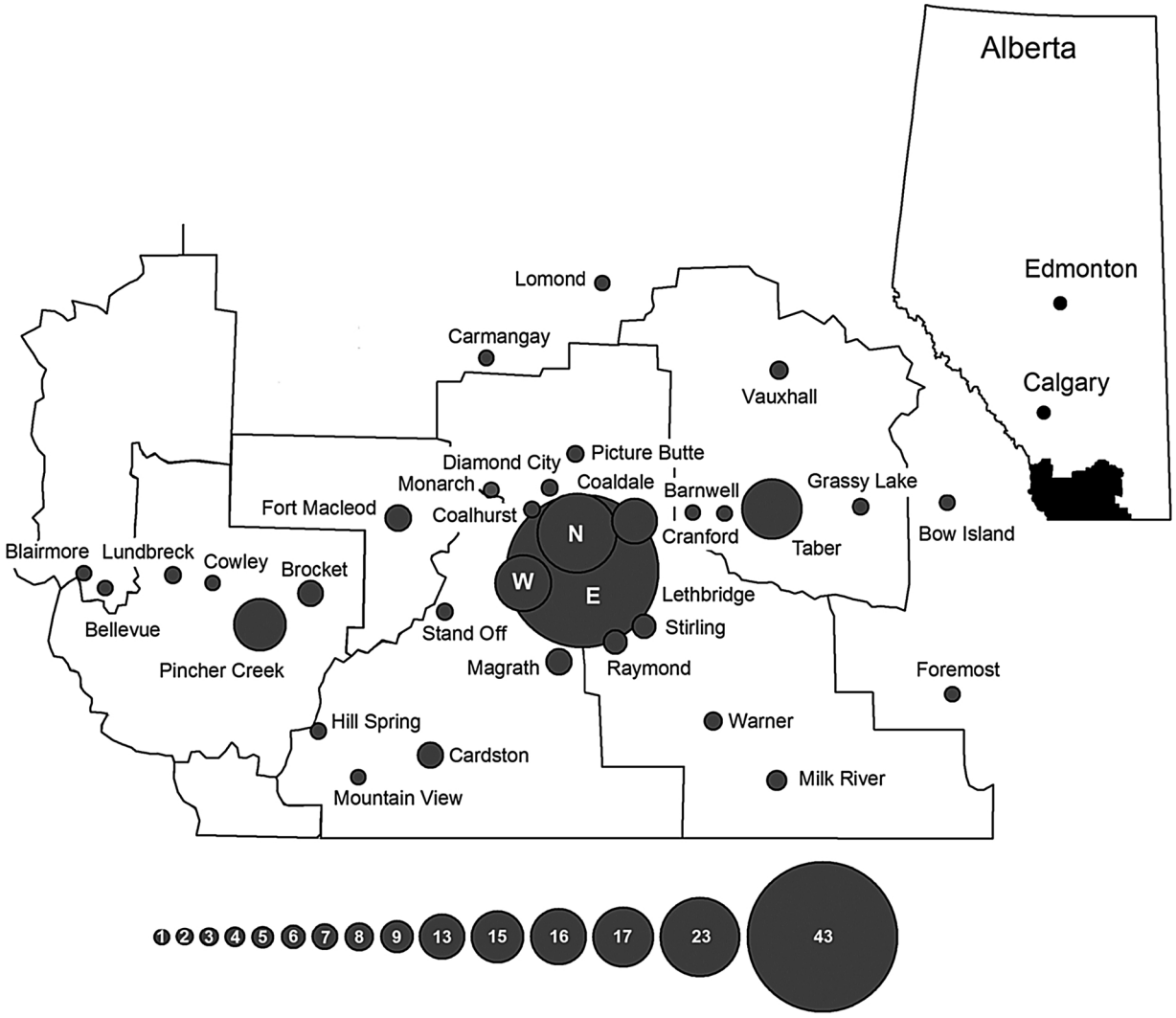

Fig. 2. Number of individuals infected by *Campylobacter jejuni* in Southwestern Alberta over a 1-year period based on location of habitation. The size of the circle is proportional to the number of infected individuals. For Lethbridge, W, N, and E refer to West, North, and East Lethbridge, respectively.

Table 3
Total number of individuals, mean age, and number of positive individuals, and prevalence of infection (%) by *Campylobacter jejuni* in diarrheic people by habitation location and gender.

Location	Gender	Total	Mean age	No of infected individuals	Prevalence of infection
Rural	Female	496	44.7	51	(10.3%) a[a]
Rural	Male	381	38.0	38	(10.0%) a
Urban	Female	784	48.0	52	(6.6%) b
Urban	Male	548	39.1	58	(10.6%) b

[a] Prevalence values not followed by the same letter differ ($P < 0.050$).

national (30.0 cases $100\,K^{-1}$) averages (Alberta Government, 2004; Inglis et al., 2011; Public Health Agency of Canada, 2018). In Alberta, diagnosis of campylobacteriosis is primarily based on isolation using a semi-selective medium. For the current study the method applied for routine detection of *Campylobacter* species involved a single medium (CVA), a single incubation temperature (42 °C), and cultures maintained in an anaerobic atmosphere (10% CO_2, 10% H_2 and 80% N_2). Using this method detection was restricted to *C. jejuni* (n = 99 individuals; 4.5%) and *C. coli* (n = 1 individual; 0.04%), and no isolates of *C. upsaliensis* or *C. lari* were recovered. It is now accepted that rates of campylobacteriosis are under estimated in most jurisdictions, which is primarily attributed to under reporting (i.e. individuals who do not seek medical assistance, a causal agent is not identified, or positive laboratory test results are not reported to surveillance organizations) (MacDougall et al., 2008). To ascertain the extent to which detection rates could be increased, we applied an array of isolation methods (i.e. 'specialized isolation' including direct plating, enrichment, membrane filtration, and immunobeads) with a high hydrogen microaerobic atmosphere and an incubation temperature of primarily 37 °C. We chose to use an incubation temperature of 37 °C and a high hydrogen microaerobic atmosphere based on the work conducted by Lastovica and Le Roux, which indicated that elevated temperatures and low hydrogen in the atmosphere adversely affect isolation of certain *Campylobacter* species, including some strains of *C. jejuni* (Lastovica and Le Roux, 2003). We observed that detection rates were much higher as determined by specialized isolation for both *C. jejuni* (n = 182 individuals; 8.2%) and *C. coli* (n = 11 individuals; 0.5%). Thus, 83 (45.6%) and 10 (90.9%) individuals that were infected by *C. jejuni* and *C. coli* during the 1-year study period were not detected by conventional isolation, respectively. However, no one isolation method was found to be superior and the increased success that we experienced isolating *C. jejuni* and *C. coli* was attributed to the large number of methods applied. Our findings using an array of isolation methods determined a 1.9 times increase in the number of culture positive individuals for *C. jejuni* and *C. coli*, thus indicating that campylobacteriosis infection rates incited by

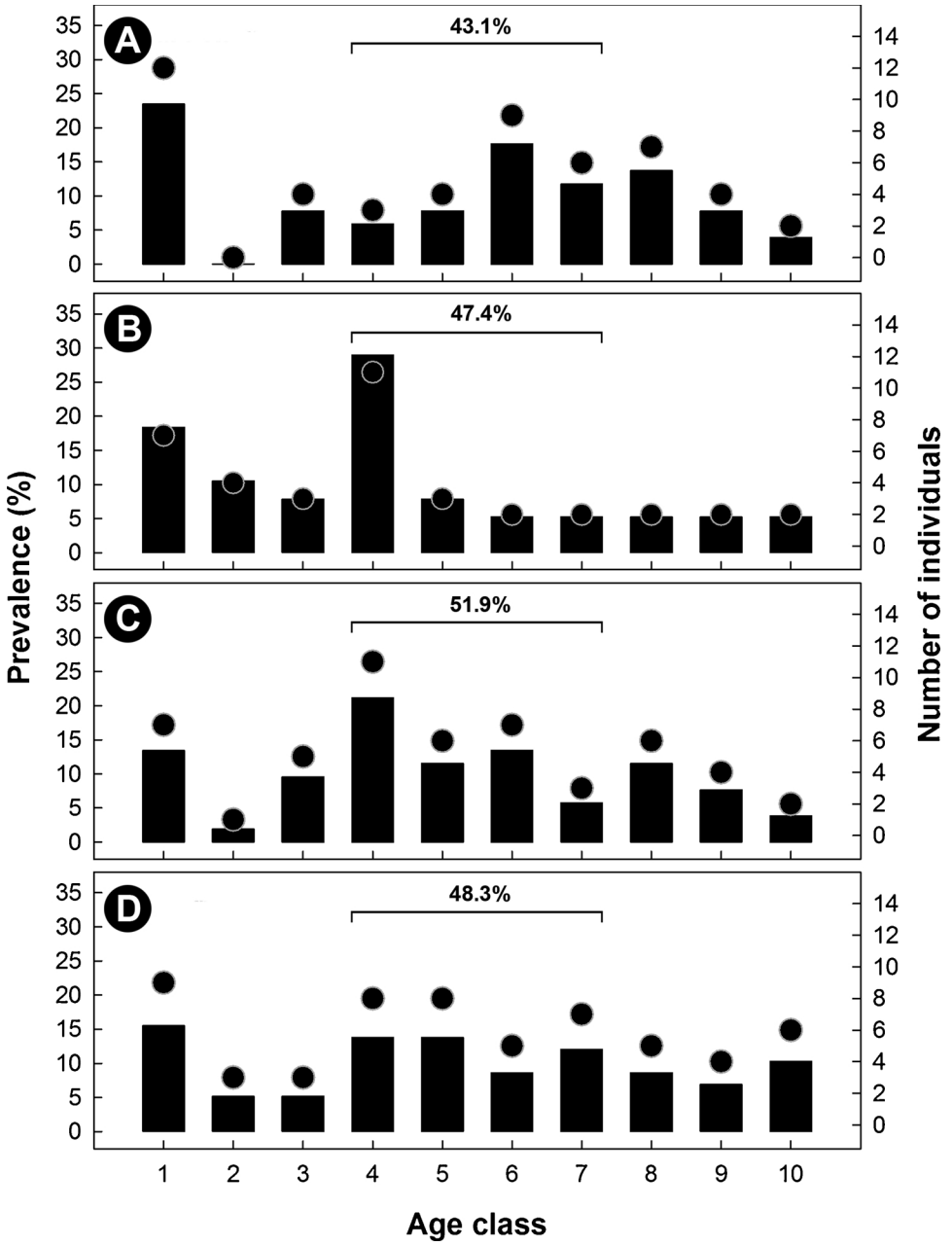

Fig. 3. Prevalence (%) and numbers of *Campylobacter jejuni* infections by age class for females living in rural locations (A), males living in rural locations (B), females living in an urban center (C), and males living in an urban center in Southwestern Alberta (D). Age class are ≤ 5 years (age class 1), 6–10 years (age class 2), 11–20 years (age class 3), 21–30 years (age class 4), 31–40 years (age class 5), 41–50 years (age class 6), 51–60 years (age class 7), 61–70 years (age class 8), 71–80 years (age class 9), and ≥ 81 years of age (age class 10). Horizontal lines with associated prevalence values represent individuals of working age (i.e. 21 to 60 years) infected by *C. jejuni*. Prevalence is indicated with histogram bars, and numbers of individuals are indicated with circle markers.

these two taxa based on culture-based detection in SWA are at least 115 cases $100\,K^{-1}$. This rate is comparable to other locations in the world that report high rates of campylobacteriosis (Kaakoush et al., 2015).

A plethora of published methods claim to optimally isolate *Campylobacter* species (Oyarzabal and Carrillo, 2017), and we chose to apply a variety of strategies and methods in the current study. Membrane filtration has been proposed as a method to increase detection rates for *Campylobacter* species, particularly for fastidious taxa such as *C. upsaliensis* (Lastovica and le Roux, 2000, 2001, 2003); a primary advantage of this method is that it eliminates the need for selective bacterial agents, which can inhibit growth of some strains of *Campylobacter* species. Membrane filtration can be used as a direct isolation method, or after non-specific enrichment as was applied in the current study. We observed that only 19.6%, 25.9%, and 44.1% of the isolates of *C. coli*, *C. jejuni*, and *C. upsaliensis* recovered, respectively, were recovered by direct membrane filtration. That 23.5% and 32.4% of the isolates of *C. upsaliensis* recovered were isolated by direct plating and enrichment, respectively, indicates that some subtypes of this bacterium are not inhibited by selective agents in semi-selective isolation media. The efficacy of membrane filtration requires that a sufficient cell density of actively motile cells is present. Although we processed samples as quickly as possible (i.e. within 1 day of arrival at the CRH), the mean time from collection to processing was ≈2 days, which may not be not be fast enough for optimal isolation by membrane filtration. Furthermore, stool samples were routinely placed in transport buffer, which may have influenced the efficacy of the method. We also applied a magnetic immunobead method to isolate *C. jejuni*, but the method was not effective. Immunobeads have been marketed as a method to facilitate the isolation of *C. jejuni* from microbiologically-complex substrates (Che et al., 2001; Yu et al., 2001). In the current study, we generated magnetic immunobeads using *C. jejuni*-specific polyclonal antibodies. Although the beads were found to be specific for *C. jejuni* relative to other *Campylobacter* species, detection rates using this method were very low, and contamination of cultures with non-*Campylobacter* bacteria was commonly encountered despite repeated washing of the beads. It is noteworthy that the isolation medium

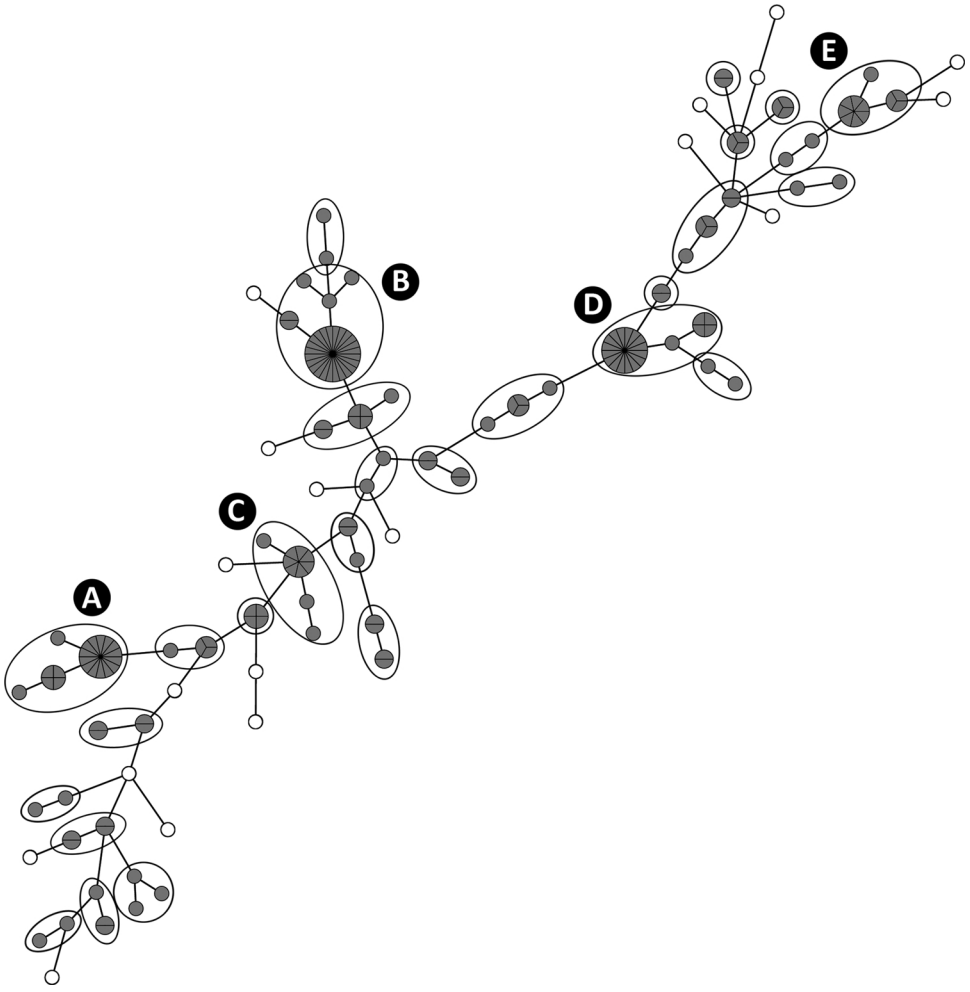

Fig. 4. Minimum spanning tree of *Campylobacter jejuni* isolates recovered from people with clinical cases of campylobacteriosis in Southwestern Alberta over a 1-year period. Comparative Genomic Fingerprinting (CGF) subtype clusters were calculated using the simple matching coefficient and UPGMA clustering in BioNumerics (version 6.6, Applied Maths), and connecting lines in the tree represent two or fewer mismatched loci (95% similarity) between respective subtypes. Grey circles within ellipsoids represent CGF subtype clusters that contain isolates from two or more individuals. Open circles indicate CGF subtype clusters composed of a single isolate. Circles with letters denote primary CGF subtypes. (A) 44.3 (n = 20 individuals). (B) 169.1 (n = 29 individuals). (C) 269.4 (n = 10 individuals). (D) 735.5 (n = 21 individuals). (E) 853.10 (n = 11 individuals).

included vancomycin, which has been implicated in inhibiting the growth of some *Campylobacter* strains (Oyarzabal and Carrillo, 2017). Our findings indicate that no one isolation method is comprehensive and that it is necessary to use a combination of methods to avoid false negative results.

Given the high rates of infection by *C. jejuni* based on culture, we applied direct PCR to detect and quantify DNA of the bacterium in stools. We examined three primer sets for specificity, inclusiveness, and sensitivity. The primer set targeting the *ipxA* (Klena et al., 2004) and *hipO* (Inglis et al., 2018) genes were more specific than the primer set targeting the *mapA* gene (Inglis and Kalischuk, 2003); however, the *ipxA* primer set was more inclusive than the *hipO* primer set, and with a

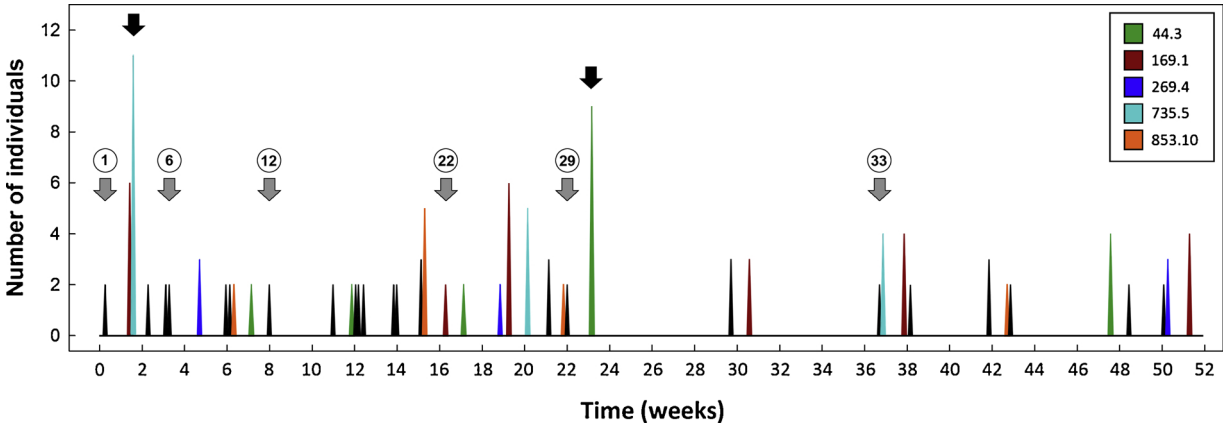

Fig. 5. Temporal case clusters (n = 44) of campylobacteriosis incited by *Campylobacter jejuni* subtypes in people living in Southwestern Alberta over a 1-year period. Temporal case clusters were defined as the occurrence of *C. jejuni* belonging to the same subtype cluster in stool samples of two or more people within 14 days of each other. Five prominent *C. jejuni* subtypes (i.e. 44.3, 169.1, 269.4, 735.5, and 853.10) are indicated with color peaks, whereas non-prominent subtypes are indicated with black peaks. Black arrows indicate two prominent case clusters (n ≥ 9 individuals in each). Grey arrows with corresponding numbers indicate case clusters for which there was a connection to epidemiological variables such as place of habitation. The total number of individuals linked to temporal case clusters of campylobacteriosis incited by *C. jejuni* was 133 (71.9%).

detection limit of 5.0×10^2 and 5.0×10^3 CFU g^{-1} of feces. Although the primers were very sensitive, the minimum detection limit of 500 to 5000 cells is typical for stools as a result of a dilution effect during DNA extraction (Inglis et al., 2011; Inglis and Kalischuk, 2003; Webb et al., 2016). Our results showed that direct non-nested PCR detection of *C. jejuni* DNA was substantially less effective for detecting the bacterium than were culture-based methods collectively; 3.4% (n = 75 individuals) of the stools were positive by direct PCR, compared to 8.2% (n = 182 individuals) by culture. In a previous study, we used direct PCR to detect *C. jejuni* and other species of *Campylobacter* in a subset of submitted stools from diarrheic individuals submitted to the CRH over a 5-month period (i.e. spring to fall) in SWA (Inglis et al., 2011). In this study, we observed that 29.2% of 442 stool samples from diarrheic individuals were PCR positive for *C. jejuni* compared to 3.4% for control individuals (n = 58). In comparison, only 12.2% of 979 diarrheic stool samples were PCR or culture positive for *C. jejuni* during the same period of the year in the current study. We also previously determined that 5.2%, 0.7%, and 0% of diarrheic stools collected over the 5-month period were PCR positive for *C. coli*, *C. upsaliensis*, and *C. lari*, respectively (Inglis et al., 2011), which is higher for infection rates observed for *C. coli* (0.5%), and the same or similar to rates observed for *C. upsaliensis* (0.7%), and *C. lari* (0%) in the current study at the same time of the year. Notably, in our previous study we used nested or semi-nested PCR to increase sensitivity and specificity of primers, and PCR-based methods detected substantially more positive individual than conventional or specialized isolation (Inglis et al., 2011). Enteric bacteria are not uniformly distributed in stools (Inglis et al., 2012), and shedding of *C. jejuni* in feces is periodic (Inglis et al., 2004). That we observed a small number of stools that were PCR-positive for *C. jejuni* DNA (15 individuals) but culture negative is consistent with the high sensitivity of the primers used coupled with probability of sufficient template DNA being present, albeit at low concentrations in some samples for which the bacterium was present. Guarding against both false negative and false positive results using direct diagnostic PCR for enteric pathogens is an important consideration. We used an IAC to protect against a false negative finding due to PCR inhibition (Webb et al., 2016), but evidence indicated that the low densities of *C. jejuni* in some stools were below the detection limit of the PCR assay utilized. While nested PCR increases sensitivity and specificity, it is not practical in diagnostic facilities as a result of increased labor and potential for contamination with amplified DNA from the primary reaction (Hu, 2016). We conclude that the use of non-nested, inclusive, and specific primers was not an effective diagnostic tool for detecting *C. jejuni* in stools from diarrheic individuals.

We observed that two control individuals were culture positive for *C. jejuni* (1.9%). Both individuals were healthy, lived in an urban centre, and had not been diagnosed with clinical enteritis in the 12 months preceding the collection of the stool sample. *Campylobacter jejuni* can be readily isolated from asymptomatic people in some developing countries, particularly in children (Figueroa et al., 1989; Lee et al., 2013; Mason et al., 2013). The bacterium has also been isolated from a low number of control individuals in Europe; for example, 0.5% (n = 3) of 665 control individuals were positive for *C. jejuni* in the Netherlands (i.e. compared to 1.1% of diarrheic individuals), and 0.6% (n = 14) of 2,264 control individuals were positive for the bacterium in England. One possibility is that the strains infecting asymptomatic people do not incite enteritis. Another possibility is that such people possess adaptive immunity to the strain they are infected with, and neglecting to account for this may lead to under estimation of exposure and infection (Havelaar et al., 2009). In the current study, stools from control individuals were not collected and processed throughout the study period; rather they were collected at two time points (i.e. October-November and March-April). It is unknown whether the restricted temporal sampling of healthy control individuals may have resulted in an underestimation of infection rates in this cohort, particular as the second collection date was not during a peak infection period. Furthermore, samples from healthy control individuals were treated somewhat differently from those collected for diarrheic individuals (i.e. they were not placed in an EPT medium, and in some instances were held on ice for a short period of time until processed). However, stools from control individuals were typically processed within 6 h of excretion, and it is unlikely that the differences in methodology adversely affected the viability of *Campylobacter* species within stools from control individuals.

The study region in SWA represents a relatively large geographical region with a gradation of human activity from west to east. The Rocky Mountains form the western border of the region, and an ecozone transition from montane cordillera, to tallgrass prairie, to shortgrass prairie exists from west to east. The study region contains a single prominent watershed, an approximate 40:60 rural:urban human population distribution, and a high density of livestock, including a predominance of beef cattle (≈1,166 K), with approximately 51% of the cattle in confined feeding operations (CFOs), primarily concentrated in the region surrounding Lethbridge and Taber (i.e. 76.2% of all cattle in CFOs), the two main urban centers (Alberta Government, 2014). Similarly to other locales in temperate climates, we observed a peak of infection in the spring and summer (Public Health Agency of Canada, 2009; Public Health Ontario, 2015; San Joaquin and Welch, 1984). However, we also observed relatively high and consistent infection rates by *C. jejuni* over the late summer, and fall and winter months in SWA, which was somewhat higher but within what has been typically observed in other temperate locations (Meldrum et al., 2005; Wei et al., 2015). We observed that younger people were more commonly infected by *C. jejuni* in SWA, which is consistent with trends reported nationally (Public Health Agency of Canada, 2018). Although the majority of individuals infected by *C. jejuni* lived in Lethbridge, the primary urban centre in the study region, after adjustment for differential submission rates, there was no difference in rates of infection between males living in urban centers (10.6%), and both females (10.3%) and males (10.0%) living in rural locations. Somewhat surprisingly, we observed a much lower infection rate in females living in urban centres (6.6%). Agriculture, including livestock agriculture is an important contributor to the economy of SWA, and one possibility for the lower rates that we observed in females living in urban centers is that males living in urban centres, and both females and males living in rural locales have greater contact with livestock (i.e. via occupational activities), and therefore have a higher risk of exposure to *C. jejuni*. Consistent with this possibility, a preliminary epidemiological examination in SWA indicated that occupational contact was a significant risk for campylobacteriosis (Hasselback, 2002). In support of this conclusion, our research in SWA has shown that the majority of beef cattle in CFOs shed *C. jejuni* in their feces, including CRS of the bacterium (Inglis and Kalischuk, 2003; Inglis et al., 2003, 2004; Inglis et al., 2005, 2006; Webb et al., 2018). Examination of the ages of infected females living in urban centers relative to females living in rural locations, and with one exception (i.e. rural males in the 21–30 age class), males living in urban and rural locations, did not reveal conspicuous trends, including for individuals of working age. The reason why males cohort living in rural areas within the 21–30 age class exhibited higher rates of campylobacteriosis is speculative, but may be linked to type and degree of occupational contact, possibly coupled with the fastidiousness of personal hygiene (e.g. hand washing), which is relatively lax in this age group (Szilagyi et al., 2013). The degree to which occupational contact with animals and animal products impacts campylobacteriosis in SWA warrants a more detailed examination.

A primary reason that we emphasized the isolation of *C. jejuni* in the current study was to examine the population dynamics of the bacterium in an epidemiological context. We utilized the high-throughput and high-resolution CGF method to subtype 1,058 *C. jejuni* isolates recovered from diarrheic humans, and obtained good subtype profiles for 1,050 of these isolates. A total of eight isolates (0.8%) were excluded from further consideration due our inability to obtain valid CGF

profiles; exclusion of a subset of isolates is common for all subtyping methods (Karenlampi et al., 2007; Ragimbeau et al., 2008). We observed that the genetic diversity among the *C. jejuni* isolates recovered was high (n = 46 clusters), which coincides with findings from previous studies (Levesque et al., 2008; Sheppard et al., 2009; Wilson et al., 2008). It is noteworthy that we observed twelve clusters not in the C3GFdb; there are currently ≈4,800 subtypes (≈2,500 subtype clusters at the 95% level of resolution) within the database.

The isolation method applied has the potential to bias strain diversity, yet few studies have examined the effects of isolation methods utilizing different media, culturing techniques, and incubation temperatures on subtype diversity (Williams et al., 2012). We observed that subtype diversity remained high regardless of the culturing technique used, and the presence and abundance of subtypes did not change significantly among methods. This result is consistent with the conclusion from our isolation-based detection findings (i.e. no one method is superior, and more than one method is needed). Further, we observed that a total of twelve *C. jejuni* subtype clusters were only identified using single and varied methods of isolation. It is noteworthy that our project was not ideal to ascertain selection bias as a function of isolation method, as we recovered and genotyped a relatively small number of isolates from each method. Furthermore, it is currently thought that cases of campylobacteriosis in human beings are primarily caused by a single *C. jejuni* subtype (Richardson et al., 2001). Our previous experience suggests that the effectiveness of isolation methods varies greatly based on the type of matrix (i.e. stool, surface water, meat products), and it is possible that the proportion of *C. jejuni* subtypes isolated by each method from different matrices may also be affected.

Although we observed that the majority of individuals were infected by a single strain of *C. jejuni*, we did observe some instances where a diarrheic individual was infected with more than one subtype. Co-infection of humans with more than one *C. jejuni* strain has been documented in 5–10% of sporadic cases (Richardson et al., 2001), and approximately one-half of cases of campylobacteriosis linked to outbreaks in the United Kingdom (Forbes et al., 2009; Frost et al., 2002). In most diagnostic clinics in Canada, including in SWA, a single isolate is recovered, which precludes an examination of strain diversity within afflicted individuals. Furthermore, strain isolation bias as a function of the method applied, coupled with predictions of numbers of isolates that need to be recovered to detect different strains are considerations to elucidate the importance of co-infections in campylobacteriosis.

A comparison of the *C. jejuni* subtypes recovered from diarrheic humans in SWA in the current study, with the subtype and metadata of isolates associated with non-humans sources within the C3GFdb indicated that the majority of these clusters were associated with cattle as the dominant non-human host source. Nearly 50% of the isolates recovered from diarrheic humans in SWA were assigned to one of five subtype clusters (i.e. 44.3, 169.1, 269.4, 735.5, and 853.10). CGF subtype clusters 169.1 and 269.4 roughly corresponded to multilocus sequence typing (MLST) clonal complex ST-21 (i.e. sequence types 982 and 8, respectively), cluster 735.5 corresponds to MLST clonal complex ST-42 (sequence type 42), cluster 44.3 equates to MLST sequence type 922 (which has yet to be assigned a broader clonal complex), and cluster 853.10 does not correspond to any current sequence type. In previous studies, ST-21 has been commonly detected in a variety of environmental sources and host animals (Dearlove et al., 2016). However, ST-42 is strongly associated with cattle (French et al., 2005; Sheppard et al., 2014), and sequence type 922 has only been detected in notable numbers during a study of *C. jejuni* associated with dairy cattle (Jay-Russell et al., 2013). As indicated above, SWA is a region with a high density of beef cattle (i.e. 1.2 million head during the study period). Our recent research has shown that not all strains associated with non-human sources within SWA are observed in diarrheic humans. Thus, a number of *C. jejuni* subtypes circulating in SWA appear to be of reduced significance to public health, and this emphasizes the need to define CRS of *C. jejuni* to identify important reservoirs and transmission mechanisms, ideally within model agroecosystems with high rates of campylobacteriosis such as SWA. Furthermore, the CGF method (Taboada et al., 2012) allowed us to examine a relatively large number of isolates in the current study to elucidate the attribution of *C. jejuni* responsible for human infections to their sources (Ravel et al., 2017). Although chickens are thought to be the primary reservoir of CRS throughout the world (Thibodeau et al., 2015), there are locations where this does not appear to be true. For example, in Finland cattle appear to be as important as a source of CRS as poultry (de Haan et al., 2010). Although the mechanisms of transmission are enigmatic, the evidence obtained in the current and other recent studies (Webb et al., 2018) implicating cattle as an important reservoir of CRS, is consistent with the conclusions of an epidemiological risk assessment conducted in SWA that implicated contact with cattle as a primary risk for campylobacteriosis in the region (Hasselback, 2002).

Although outbreaks of campylobacteriosis caused by *C. jejuni* occur (primarily due to consumption of raw water or milk), most infections by the bacterium are thought to be sporadic (i.e. endemic and not linked to a common source), with the vast majority of sporadic cases of campylobacteriosis attributed to animals farmed for meat and poultry (Wilson et al., 2008). By examining subtypes infecting people in SWA in a two week rolling window, we observed that temporal case clusters frequently occurred, and temporal case clusters were responsible for a large proportion of cases of campylobacteriosis in SWA. A total of 44 temporal case clusters were detected over the 1-year study period. The most commonly detected CGF subtype clusters (subtype clusters 44.3, 169.1, 269.4, 735.5, and 853.10) were those with the greatest number of attributed temporal case clusters (i.e. 5, 6, 3, 3, and 4, respectively). The majority of temporal case clusters in SWA were detected during summer and autumn, which coincides with prevalence of infections for individual cases and with those of previous studies (Karenlampi et al., 2007; Nylen et al., 2002). A total of 23 different *C. jejuni* subtype clusters were linked with at least one temporal case cluster. Considering the genetic diversity of *C. jejuni* (Sheppard et al., 2009), it is not surprising that the CGF cluster responsible for individual temporal case clusters also varied greatly. However, the primary clusters 44.3, 169.1, 269.4, 735.5, and 853.10 were responsible for nearly half of all temporal case clusters. In a recent re-examination of 245 *C. jejuni* isolates from domestically infected people in Denmark (i.e. by whole genome sequence analysis), 20% (49 individuals) were linked to temporal and/or geographical clusters (Joensen et al., 2018). Although we observed a greater degree of infections that were linked to temporal clusters (n = 133; 72%), our findings are in agreement with Joensen et al. who concluded that temporal and spatial case clusters of infections incited by *C. jejuni* are more common than previously assumed (Joensen et al., 2018). In the current study, we were unable to link temporal case clusters to a specific age, sex, or place of habitation. However, it is expected that re-examination of infection events by *C. jejuni* subtypes in a temporal and spatial context, in concert with reportable disease data, and supplemented with whole-genome sequence data will further illuminate the molecular epidemiology of campylobacteriosis in SWA. Furthermore, our application of a model agroecosystem approach (with the C3GFdb as a foundational tool) is anticipated to maximize our epidemiological examination of *C. jejuni* subtypes in SWA to identify reservoirs and transmission mechanisms.

Ethics approval

Prior to commencing research involving human subjects, scientific and ethics approval was obtained from the Regional Ethics Committee of the former Chinook Health Region (#2007-07), and the University of Lethbridge Human Subject Research Committee (#715).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijmm.2019.04.003.

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