Wide but variable distribution of a hypervirulent Campylobacter jejuni clone in beef and dairy cattle in the United States

Yizhi Tang
*Iowa State University, ytag@iastate.edu*

Richard J. Meinersmann
*U.S., Department of Agriculture*

Orhan Sahin
*Iowa State University, osahin@iastate.edu*

Zuowei Wu
*Iowa State University, wuzw@iastate.edu*

Lei Dai
*Iowa State University, ldai@iastate.edu*

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Abstract
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Disciplines
Large or Food Animal and Equine Medicine | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments

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Authors
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Departments of Veterinary Microbiology and Preventive Medicine, and Veterinary Diagnostic and Production Animal Medicine, Ames, IA, USA; Russell Research Center, USDA Agricultural Research Service, BEAR-RU, Athens, GA; National Wildlife Research Center, USDA APHIS, Fort Collins, CO; Food Animal Health Research Program, The Ohio State University, Wooster, OH.

Running Title: Prevalence of Campylobacter jejuni clone SA in cattle

#To whom correspondence should be addressed:
Qijing Zhang
Department of Veterinary Microbiology and Preventive Medicine
Iowa State University
Ames, Iowa 50010, USA
zhang123@iastate.edu
Abstract

Campylobacter jejuni clone SA is the major cause of sheep abortion and contributes significantly to foodborne illnesses in the United States. Clone SA is hypervirulent because of its distinct ability to produce systemic infection and its predominant role in clinical sheep abortion. Despite the importance of clone SA, little is known about its distribution and epidemiological features in cattle. Here, we describe a prospective study on C. jejuni clone SA prevalence in 35 feedlots in 5 different states in the U.S. and a retrospective analysis of clone SA in C. jejuni isolates collected by National Animal Health Monitoring System (NAHMS) Dairy Studies 2002, 2007 and 2014. In feedlot cattle feces, the overall prevalence of Campylobacter was 72.2%, 82.1% of which were C. jejuni. Clone SA accounted for 5.8% of the total C. jejuni isolates, but its prevalence varied by feedlot and state. Interestingly, starlings on the feedlots harbored C. jejuni in feces including clone SA, suggesting it may play a role in the transmission of Campylobacter. In dairy cattle, the overall prevalence of clone SA was 7.2%, but a significant decrease in the prevalence was observed from 2002 to 2014. Whole genome sequence analysis of the dairy clone SA isolates revealed that it was genetically stable over the years and most of the isolates carried the tetracycline resistance gene tet(O) in the chromosome. These findings indicate clone SA is widely distributed in both beef and dairy cattle, and provide new insights into the molecular epidemiology of clone SA in ruminants.
Importance  

C. jejuni clone SA is a major cause of small ruminant abortion and an emerging threat to food safety because of its association with foodborne outbreaks. Cattle appears to serve as a major reservoir for this pathogenic organism, but there is a major gap in our knowledge about the epidemiology of clone SA in beef and dairy cattle. By taking advantage of surveillance studies conducted on a national scale, this manuscript describes wide but variable distribution of clone SA in feedlot cattle and dairy cows in the United States. Additionally, the work revealed important genomic features of clone SA isolates from cattle. These findings provide critically needed information for the development of pre-harvest interventions to control the transmission of this zoonotic pathogen. Control of C. jejuni clone SA will benefit both animal health and public health as it is a zoonotic pathogen causing disease in both ruminants and humans.

Introduction  

Campylobacter jejuni is a major zoonotic bacterial pathogen and primarily causes foodborne enteritis in humans (1, 2). The organism is widely distributed across a broad range of animal species including livestock, poultry, and wildlife, and is transmitted to humans mainly via consumption of contaminated food, water and milk (2). As reported by the Centers for Disease Control and Prevention (CDC)’s FoodNet surveillance program in 2016, Campylobacter ranked second (12.97 per 100,000 population) among the causes of laboratory-confirmed bacterial food-borne illnesses in the United States (3). Poultry, especially market-age broiler chickens, are frequently colonized by C. jejuni,
resulting in carcass contamination in processing plants (4, 5). Consequently, poultry meat is considered a major source of infection for human campylobacteriosis.

In addition to poultry, cattle also serve as an important reservoir for Campylobacter. Bovine Campylobacter contributes significantly to both outbreak and sporadic cases of campylobacteriosis in humans (6, 7). Cattle Campylobacter can be transmitted to humans via multiple transmission routes including direct contact (e.g. petting zoo and occupational exposure), consumption of unpasteurized milk (and associated dairy products), and environmental contamination (water, produce, etc.) (8-10). Molecular typing of C. jejuni isolates using multilocus sequence typing (MLST) attributed approximately 40% of sporadic human cases to cattle sources in the United Kingdom (11). The contribution of bovine Campylobacter to outbreaks of human campylobacteriosis is even more prominent because Campylobacter from cattle feces frequently contaminates raw milk (9, 12-14). Ruminant Campylobacter may also contaminate water supplies via agricultural runoff, leading to large waterborne outbreaks (8). Of note, red meat is infrequently contaminated by Campylobacter (15) and does not appear to play a major role in the transmission of Campylobacter to humans. Additionally, ruminants are an integral part of Campylobacter ecology and may serve as a source of Campylobacter transmission to the environment and other farm animals, such as poultry. Thus, poultry and cattle are the two most important animal reservoirs for this zoonotic pathogen.

Campylobacter is highly prevalent in both beef and dairy cattle in the U.S. and worldwide (10, 16-20). In cattle, Campylobacter is mainly carried in the intestinal tract and less frequently can be isolated from the rumen, gall bladder, and bile (11, 21).
predominant *Campylobacter* species isolated from cattle is *C. jejuni*, followed by *C. coli* (10, 22-25). Isolation rates vary with country, herd size and type, age of animals, season, and confinement levels (10, 25). In the U.S., several nationwide surveillance studies of cattle (NAHMS Dairy 1996, 2002, 2007, and Feedlot’99) indicated that fecal carriage rates ranged from 15-50% and the majority of the tested operations (herds/farms/feedlots) were positive for *Campylobacter* (17, 24, 26). Several other studies conducted in different states in the U.S. also revealed a similar range of prevalence (between 20-60% at the fecal sample level) of *Campylobacter* in feedlot cattle and dairy cattle (18, 22, 23, 25, 27).

Although *Campylobacter* mainly colonizes in the gastrointestinal tract in animals, it may translocate across the intestinal epithelial barrier, leading to systemic infection, such as bacteremia and abortion in small ruminants and occasionally in humans (28). Indeed, *Campylobacter* infection is one of the most prevalent causes of ovine abortion in the United States and worldwide, with an overall abortion rate of 5% to 50% (average, 23.2%) in affected flocks (29). Historically, *C. fetus* subsp. *fetus* was the major cause of *Campylobacter*-associated ovine abortion. However, studies conducted during late 1980s and early 1990s in the United States revealed progressive increase in isolation of *C. jejuni* from aborted sheep placentas (30, 31). Recently, our studies demonstrated that a single hypervirulent tetracycline-resistant *C. jejuni* clone (named clone SA) has emerged as the predominant cause of *Campylobacter*-associated ovine abortions and is responsible for > 90% of the clinical abortion cases in the United States (29, 32, 33). The hypervirulence of Clone SA is related to its ability to translocate across the intestinal epithelium, producing systemic infection and clinical abortion (33). Additionally, clone SA was also associated...
with bovine and goat abortion cases in the United States (29, 34). Importantly, *C. jejuni* clone SA has been implicated in a number of cases of foodborne illnesses, both outbreaks and sporadic cases, in the United States (34). These findings clearly indicated that *C. jejuni* clone SA is an important pathogen for both animal health and food safety in the United States and suggest that cattle may serve as a major reservoir for its zoonotic transmission.

Despite the obvious significance of *C. jejuni* clone SA to ruminant health and food safety, little information is available about its distribution in beef and dairy cattle, which represents an important knowledge gap in our understanding of the overall epidemiology and this particular zoonotic risk. To close this knowledge gap and facilitate the control of *C. jejuni* clone SA, we conducted a before-after controlled impact (BACI) study, with repeated sampling of 35 feedlots located in various geographical regions on two different occasions. Additionally, we analyzed the *Campylobacter* isolates in the collections of NAHMS (National Animal Health Monitoring System) Dairy 2002, 2007 and 2014 studies (17, 35). The purposes of this work were to: 1) investigate the overall prevalence of *Campylobacter* in feedlot cattle and evaluate the effect of starling control intervention on the occurrence and spread of *Campylobacter* in feedlot operations, and 2) determine the occurrence and distribution of *C. jejuni* clone SA in feedlot and dairy cattle.

**Materials and Methods**

**Sample collection and bacterial isolation**

In the prospective BACI study, a total of 3,184 cattle fecal samples were collected from 35 different feedlot herds located in Iowa, Texas, Colorado, Missouri, and Kansas on two different occasions during December 2012 to March 2013. Collection of cattle fecal
samples followed the methods described previously (36). A sample was collected from a fecal pat only after a cow was observed defecating. Freshly voided fecal pats were scraped with a sterile cotton-tipped swab, and the swab was immediately placed in 10 ml glass tubes containing *Campylobacter* Thioglycollate Broth (CAMPY-THIO). All cattle fecal samples were shipped priority overnight to the testing laboratory. All samples received the next day of collection were accepted and processed to culture *Campylobacter* as described in a previous study (37). Of note, the fecal samples were collected during a European starling intervention program taking place on the farms (38).

The intervention program was designed to examine the role of invasive European starlings in the spread of antibiotic resistant bacteria in in feedlots. During the intervention, Wildlife Services biologists baited starlings using a 2% solution of DRC-1339 (3-chloro-p-toluidine hydrochloride) on treated corn chop. Technical DRC-1339 powder was mixed with water to create a 2% solution. Treated corn chop was soaked in the 2% solution and screen dried. The bait was applied at a concentration of 1:10 treated to untreated corn chop. All DRC-1339 applications were implemented in accordance with label requirements “Compound DRC-1339 Concentrate – Feedlots” (EPA Registration 56228-10). In order to determine the effect of this control program on *Campylobacter* prevalence, approximately one-half of the samples was obtained before the intervention, while the other half was obtained after the intervention.

In addition to cattle fecal specimens, we collected 150 starlings from 7 feedlots (from which post-intervention cattle samples were also tested concurrently) within cattle pens and pen lanes during February and March of 2013. All starlings were collected with shotguns and no birds were collected outside the feedlots. Starling collections followed
the methods conforming to agency policy as stated in United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Service Directive 2.505 and were approved by the National Wildlife Research Center’s (NWRC) Internal Animal Care and Use Committee (NWRC protocol, QA-1919). All specimens were individually bagged in sterile Whirl-Paks® and stored in coolers until shipping. European starlings were shipped to the United States Department of Agriculture, National Wildlife Research Center (NWRC) in Fort Collins, Colorado, USA. All samples received the next day of collection were accepted and processed. All European starling dissections occurred at the NWRC and were conducted using published methods (39). Starling lower gastrointestinal tracts (GI, duodenum to the cloaca) were removed and placed in sterile Whirl-Paks®. To reduce the risk of cross-contamination, we cleaned the starling carcasses, scissors, scalpels, and lab stations with 70% ethanol before the removal of each starling GI tract. Lab mats and gloves were replaced after processing each starling. The starling GI samples were macerated for 120 sec at 200 rpm using a Stomacher 80 paddle blender (Seward Laboratory Systems, Bohemia, NY, USA). Fecal material from the macerated starling GI tracts was squeezed by hand to one corner of the bag and an aliquot was extracted using sterile cotton swabs, making sure to completely saturate the tip of the swab. In the laboratory, 1 mL of the transport media containing a fecal swab was added into a tube containing 9 mL of Campylobacter enrichment broth, which was then incubated at 42 °C for 48 h under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂). The enrichment medium was Mueller–Hinton (MH) broth supplemented with Campylobacter-specific selective agents (SR084E and SR117E; Oxoid). From the enrichment culture, an
inoculum of 100 μL was streaked onto an MH agar plate containing the same supplements, which was further incubated for 48 h at 42 °C under microaerobic conditions. A single *Campylobacter*-like colony from each sample was subpassaged onto a plain MH agar plate and the pure culture was stored in glycerol stocks at -80 °C until further use.

To determine the distribution of *C. jejuni* clone SA in dairy cattle feces, retrospective collections of *Campylobacter* isolates from NAHMS Dairy 2002, 2007, and 2014 studies (17, 35, 40) were screened for clone SA. Respectively, 205, 627, and 576 *C. jejuni* isolates from the three studies were screened for putative clone SA using a specific PCR (see below). Further confirmation of the putative clone SA isolates was performed via whole genome sequence analysis (WGS).

**DNA extraction and PCR identification**

DNA was extracted from *Campylobacter* colonies using the single-cell lysis buffer (41) and was used as template for PCR reactions. In order to detect and/or differentiate *C. jejuni*, *C. coli*, and *C. jejuni* clone SA, three sets of previously published primers were used. The first primer pair (CCCJ-F: 5’-AAT CTA ATG GCT TAA CCA TTA-3’; CCCJ-R: 5’-GTA ACT AGT TTA GTA TTC CGG-3’), targeting 16S rRNA, was designed to co-identify *C. jejuni* and *C. coli* (42). The second primer pair (mapA-F: 5’-GAG TGC TTG TGC AAC TAA AC-3’; mapA-R: 5’-ATA GCA TCT TGA GTT GCT CC-3’) was specific for *C. jejuni* (43). The third PCR primer pair (CJSA_1356F: 5’-TCC CAT TTG GA-3’; CJSA_1356R: 5’-CAG AAC CTG GCC ACA AAC TT-3’) was used for identification of putative *C. jejuni* clone SA as described previously (44). *C. jejuni* IA3902, a clinical isolate of clone SA, was used as positive controls for the PCR, whereas...
reactions with no DNA template were used as negative controls. Each PCR amplification was carried out in a 25-μl volume containing 16 μL of distilled water, 2.0 μL of template DNA, 10 pmol of each primer, and 5 μl of GoTaq (Promega) green master mix following the cycling conditions described previously (42-44).

**Pulsed-field gel electrophoresis (PFGE)**

PFGE analysis of *C. jejuni* isolates was performed using *KpnI* following the PulseNet protocol (Centers for Disease Control and Prevention; CDC) with minor modifications (29). Briefly, fresh cultures of *Campylobacter* were embedded in 1% Seakem Gold agarose (Fisher Scientific, Fair Lawn, NJ) and lysed with proteinase K for 1 h at 55 °C in a water bath shaker. The gel plugs were digested with *KpnI* for 4 h at 37 °C. Digested plugs were embedded into 1% agarose and separated by electrophoresis in 0.5 × TBE buffer (Promega) at 14 °C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA). Gels were stained with ethidium bromide for 30 min and then photographed by using ChemiImager™ 5500 (Alpha Innotech, CA, USA). The PFGE patterns were analyzed by the GelCompare II v.6.5 software program (Applied Maths, Kortrijk, Belgium) using Dice similarity coefficient and unweighted-pair group method with arithmetic averages (UPGMA) with 0.5% optimization and 1.5% position tolerance.

*C. jejuni* IA3902 was used as a control for identification of *C. jejuni* clone SA isolates. Lambda DNA ladder (Bio-Rad) was used as the molecular size marker.

**Multi Locus Sequence Typing**

To confirm the PFGE results, multilocus sequence typing (MLST), originally developed by Dingle et al. (45), was performed on eleven representative *C. jejuni* isolates (ten from cattle, one from starlings) from the prospective study on feedlots. Of the eleven isolates...
chosen, 4 (3 from cattle and one from starlings) had indistinguishable PFGE profiles from that of the positive control \((C. \textit{jejuni} \text{ IA3902})\), 4 had minor differences in PFGE patterns, and 3 showed totally different PFGE profiles as compared to IA3902. The seven housekeeping genes from these 11 \textit{C. jejuni} isolates were amplified and sequenced using the primer sets described at the \textit{C. jejuni} MLST website (http://pubmlst.org/campylobacter/), which was developed by Keith Jolley and Man-Suen Chan at the University of Oxford (46). Allelic numbers were assigned to the isolates by performing BLAST searches for the assembled sequences using the single-locus query function, whereas sequence types were assigned using the allelic profile query function in the MLST database. Sequences that were identical to existing alleles in the MLST database were assigned the corresponding allele numbers. Novel allele profiles \((n = 5)\) were assigned new sequence types (STs) within the MLST database.

**Whole genome sequence analysis (WGS)**

The putative clone SA isolates identified by PCR screening from the retrospective NAHMS dairy studies were subject to WGS. Total DNA was extracted from each isolate using the Wizard Genomic DNA Purification kit (Promega) and then used for WGS. The library was constructed using the NEXT Ultra DNA Library Prep kit (New England Biolabs) and 250 bp paired-end reads were obtained using an Illumina Hiseq2500 (Bionova 42 Biotech Co.). A draft assembly of the sequences of each genome was generated using the de novo short-read assembler Velvet (47) and Velvet Optimiser (http://bioinformatics.net.au/software.velvetoptimiser.shtml). Draft genome sequences were aligned and the core-genome phylogenetic tree was constructed using the SNPs by Parsnp in the Harvest package (48), while the pan-genome phylogenetic tree was
constructed using binary accessory nucleotide data by Panseq (49). The phylogenetic tree was visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree). MLST typing of these isolates was performed using the sequences of the 7 housekeeping genes according to the scheme from PubMLST (https://pubmlst.org/campylobacter/).

**Statistical analysis**

A chi-square ($\chi^2$) test was used to compare the prevalence of *Campylobacter* before and after starling intervention as well as the prevalence of clone SA in different states for the feedlot cattle and in different year for the dairy cattle. The prevalence of *Campylobacter* was taken as the response, the intervention, the state and the year were taken as the factors, respectively. The independence between the factors and the response was tested by SPSS (version 17.0; SPSS Inc., Chicago, IL, USA). $P$ values less than 0.05 were considered significant.

**Results**

**Overall prevalence of *Campylobacter* in feedlot cattle.**

In total, 2,298 (72.1%) out of 3,184 fecal samples from feedlots were positive with *Campylobacter*. The overall prevalence rates of *Campylobacter* were 69.2% (554/800), 71.9% (414/576), 70.0% (210/300), 78.2% (593/758), and 70.3% (527/750) in Iowa, Texas, Missouri, Colorado, and Kansas, respectively. The *Campylobacter* prevalence rates among the states were not statistically different ($p > 0.05$). Of the 2,298 *Campylobacter* isolates, 1,886 (82.1%) isolates were determined to be *C. jejuni* by PCR. In each of the states, 487 (87.9%), 367 (88.6%), 191 (91.0%), 438 (73.9%), and 403 (76.5%) were identified as *C. jejuni*, respectively (Table 1), indicating that *C. jejuni* was the predominant *Campylobacter* species isolated from cattle feces.
Effect of starling intervention on *Campylobacter* prevalence in feedlot cattle.

Delineation of the prevalence data by pre- and post-starling control intervention is shown in Table 2. The intervention program did not appear to significantly affect the overall prevalence of *Campylobacter* \((p = 0.10)\) and *C. jejuni* \((p = 0.29)\) in the feedlot cattle.

Before intervention, the overall prevalence of *Campylobacter* (69.1%; 1044/1510 samples tested) and the relative prevalence of *C. jejuni* (79.3%; 828/1044) were comparable to those observed post-intervention (74.9% and 84.4%, respectively). Also, analysis of data by each state separately indicated no significant differences between pre- and post-intervention in the prevalence rates for overall *Campylobacter* and *C. jejuni* (Table 2).

Prevalence of *C. jejuni* clone SA in feedlot cattle.

Initial screening of the *C. jejuni* isolates for putative clone SA using PCR revealed that 8.7% (164/1886) of the isolates were positive with the PCR. As this PCR test is not 100% specific for clone SA, PFGE was performed to confirm their identity as clone SA. Of the 164 isolates initially identified by the PCR as putative clone SA, 110 (67.1%) had patterns that matched to the known subtypes of clone SA: I and II (Fig. 1a), which is in accordance with our previously published results (29). Additionally, MLST was performed on a subset of these isolates in both PFGE subtypes, which identified all of them as ST-8 genotype, which confirmed the PFGE typing result. MLST was also performed on seven isolates with non-clone SA PFGE patterns, two of which with one band difference from clone SA were also identified as ST-8 genotype, while the remaining five isolates were identified with sequence types different from ST-8, including ST-2876, ST-93, ST-239, and ST-14.
Based on the genotyping results, a relative prevalence of 5.8% (110 out of 1886 \textit{C. jejuni} isolates) and an absolute prevalence of 3.5% (110 out of 3184 total samples tested) were calculated for clone SA occurrence in the feedlot cattle surveyed in this study. The absolute prevalence rates of clone SA varied by state: 1.8% (14/758) in Colorado, 2.0% (16/800) in Iowa, 3.1% (23/750) in Kansas, 5.0% (15/300) in Missouri, and 7.3% (42/576) in Texas (Table 1). The \( \chi^2 \) test revealed that at least one state is significantly different from the rest \((p < 0.0001)\). The relative prevalence rate of clone SA also varied by states: Iowa 2.9% (16/554), Texas 10.1% (42/414), Missouri 7.1% (15/210), Colorado 2.4% (14/593), and Kansas 4.4% (23/527) \((p < 0.0001)\). However, starling intervention did not affect the prevalence of clone SA on the surveyed farms (Table 2). Clone SA strains were isolated from at least half of the feedlots surveyed in each state, in the range of 1 to 28 isolates per feedlot (result not shown). Although PFGE was performed only on putative clone SA isolates identified by PCR, the non-clone SA \textit{C. jejuni} isolates showed diverse PFGE patterns (Fig. 1), suggesting the overall genetic diversity of \textit{C. jejuni} isolates from feedlot cattle. These findings indicate that \textit{C. jejuni} clone SA is widely distributed and constitutes a substantial portion (~ 6%) of the total \textit{C. jejuni} population in feedlot cattle.

**Presence of \textit{Campylobacter} including Clone SA in starlings.**

European Starlings are commonly found on farms, serving as a potential transmission vehicle for \textit{Campylobacter} \((50)\). To investigate whether they may be a source of farm cattle infection of \textit{C. jejuni} clone SA, fecal samples from European starlings present on 7 feedlots were tested for \textit{Campylobacter} occurrence. Of note, the same feedlots were also sampled for cattle feces at or about the same time of starling survey. Of the 150 total
starling fecal samples tested, 51 (34%) were positive for *Campylobacter*, of which 50 (98%) were identified as *C. jejuni* by PCR and the remaining one isolate was of a species other than *C. jejuni* or *C. coli*. Initial screening using PCR identified one of the 50 *C. jejuni* isolates to be a putative clone SA (Table.1). This isolate and additional 14 randomly chosen *C. jejuni* isolates were analyzed by PFGE, which confirmed the putative clone SA isolate identified by PCR had a PFGE pattern indistinguishable from IA 3902 of clone SA (Fig. 1). MLST analysis further identified this starling isolate as ST-8, indicating it was a clone SA isolate. All together, these results indicate that starlings carry diverse *C. jejuni* strains and can serve as a vector for transmission of *Campylobacter* including clone SA within and between farms.

**Prevalence of Clone SA in dairy cattle**

A previous study reported that raw milk was the main source of foodborne illness outbreaks caused by *C. jejuni* clone SA (34), suggesting the presence of clone SA in dairy cattle. Thus, we performed an analysis of the retrospective collections of *Campylobacter* isolates derived from dairy cattle by NAHMS. In 2002, 2007 and 2014, NAHMS conducted national surveillance studies on *Campylobacter* prevalence in dairy cattle (17, 35). In total, 205, 627 and 576 *C. jejuni* isolates collected in 2002, 2007, and 2014, respectively, were available for clone SA screening. Of these *C. jejuni* collections, 11.2% (23/205), 10.5% (66/627) and 6.8% (39/576) were initially identified as putative clone SA by PCR (n= 128 total), respectively. All but three (one from Dairy 2007 and two from Dairy 2014) of the putative clone SA isolates were subjected to WGS analysis. Overall, 16 STs were identified among the genome-sequenced isolates (Table S1). Of the 125 isolates sequenced, 102 (81.6%) were confirmed as clone SA, which gave a relative...
prevalence of 7.2% (102/1408) for clone SA among the C. jejuni isolates from the U.S. dairy cattle. These clone SA isolates included 21 (10.2%) from Dairy 2002, 55 (8.8%) from Dairy 2007, and 26 (3.2%) from Dairy 2014 studies (Table 3). The differences between the earlier time points and the 2014 data were statistically significant ($p < 0.05$).

Of those non-clone SA isolates that were PCR positive and whole genome sequenced, fifteen STs were identified (Table S1), five of which were novel sequence types (i.e., they have not been reported previously). Of the fifteen STs, eleven STs were represented by one isolate each, two STs represented by two isolates, one ST represented by five isolates, and one ST by three isolates (Table S1).

As carrying tetracycline resistant gene $tet(O)$ on chromosome is one of the key features of clone SA isolates from sheep (18), its presence was investigated in the dairy clone SA isolates. Results showed that 81 (79.4%) of the dairy clone SA isolates contained the $tet(O)$ gene either in the chromosome ($n = 68$) or on plasmid pTet ($n = 13$), including 13 (61.9%) isolates from Dairy 2002, 51 (92.7%) isolates from Dairy 2007, and 17 (65.4%) isolates from Dairy 2014. In contrast, of the 23 non-clone SA isolates with whole genome sequenced, 9 isolates harbored a $tet(O)$ in the pTet plasmid, but none of them had $tet(O)$ in the chromosome. The pVir plasmid was also found in some of the NAHMS Dairy 2002 (n = 2), 2007 (n = 1) and 2014 (n = 2) isolates (Table 3).

Previously we have determined the whole genome sequences of clone SA isolates derived from sheep abortion (33). To investigate the genomic relationship between the clinically abortifacient isolates from sheep and the clone SA isolates from dairy cattle feces, maximum-likelihood phylogenetic trees were constructed based on pangenome (Fig. 2a) and core genome of the clone SA isolates (Fig. 2b). The trees were constructed with 170
clone SA isolates, including 72 isolates from sheep abortion collected previously (33) and 98 dairy isolates sequenced in this study (the genomic sequences of 3 dairy isolates were excluded due to poor quality). The 72 ovine isolates represented historical and contemporary isolates of clone SA in the United States over the last two decades, while the 98 bovine isolates were selected from the NAHMS studies (2002 - 2014). In both trees, clone SA isolates from sheep and cattle were intermixed and formed clusters irrespective of their host species, indicating that clone SA isolates were not host specific. There were some discrete clusters in the trees (Fig. 2). The isolates from the same feedlot and collected in the same year tended to be clustered together, but each of the clusters contained isolates from different feedlots. In addition, we didn’t observe any specific evolution patterns from the genomic data over the 12-year time span, suggesting the genome of clone SA was fairly stable.

Discussion

Results from this study revealed high prevalence (72.2%) of Campylobacter spp. in feedlot cattle and the distribution of C. jejuni clone SA in both feedlot cattle and dairy cattle in the U.S. The identification of C. jejuni as the predominant Campylobacter species in cattle is consistent with previous findings reported by others (17, 26). Considering that genetically diverse C. jejuni strains are present in cattle (32), the prevalence of clone SA (5.8% in feedlot cattle and 7.2% in dairy cattle) is substantial, suggesting that clone SA is well adapted in cattle, similar to the situation in sheep (34). Additionally, we found that European starlings on cattle farms carry C. jejuni including clone SA and may serve as a vehicle for the transmission of Campylobacter on farms. Furthermore, WGS analysis of the clone SA isolates collected from dairy cattle during
2002-2014 revealed high genomic stability of the isolates. These findings provide new information on the epidemiology of *C. jejuni* clone SA in both beef and dairy cattle. To our knowledge, this is the first study that documents the distribution of *C. jejuni* clone SA in beef and dairy cattle, and the work has closed a major knowledge gap in understanding the ecology of this zoonotic pathogen in animal reservoirs.

In this study, initial preliminary identification of clone SA was done with a rapid PCR method that targets CJSA_1356, which is one of the variable genes in the capsule locus and is quite specific for clone SA isolates. A previous work has shown the utility of this PCR method for initial screening for clone SA isolates (44). However, this method is not 100% specific for clone SA, which requires further confirmation of the putative clone SA isolates by other methods. For the prospective study on feedlots, we used PFGE and MLST to confirm the identity of clone SA. In the absence of whole genome sequences, PFGE and MLST are considered the gold standards for establishing clonality in *Campylobacter* isolates (34, 51), and their utility in identifying clone SA was further proven by WGS analysis (33). For the retrospective analysis of the dairy isolates from NAHMS studies, WGS was used to confirm the identity of the clone SA isolates initially identified by PCR. The use of multiple approaches ensured the accuracy of detecting clone SA from a large number of samples.

An interesting finding is that the prevalence rate of clone SA varied significantly in feedlots of different states, highest (7.3%) in Texas and lowest (2.4%) in Colorado. Even within a single state, the prevalence varied from farm to farm. For example, the highest prevalence of clone SA was detected with #4 feedlot in Texas, where 22 of 47 isolates tested were identified as clone SA, including the clone SA isolate from a starling. The
exact reasons for the variable prevalence in different feedlots and states are unknown, but it is possible that the variations are related to differences in management practices that influence transmission and persistence of clone SA in cattle feedlots. NAHMS examined *Campylobacter* prevalence in dairy cows by analyzing individual fecal samples in three separate studies: Dairy 2002 (17), Dairy 2007(35), and Dairy 2014. By taking advantage of NAHMS’ collections of *Campylobacter* isolates, we were able to determine the prevalence of clone SA in dairy cattle on a national scale. The availability of isolates from studies conducted in three different years (2002, 2007, and 2014) allowed us to examine the temporal changes in clone SA prevalence over the years. Interestingly, the prevalence of clone SA in 2002 and 2007 was comparable: 10.2% and 8.8%, respectively. However, in 2014, the prevalence decreased to 4.5%, which is significantly different from the previous two studies. What is responsible for the decrease of clone SA in dairy cattle is interesting and remains to be determined in future studies. It was found in this study that 34% of starling fecal samples were *Campylobacter* positive, with *C. jejuni* identified as the predominant *Campylobacter* species. This prevalence rate is within the range of 11.1% - 50.4% previously reported in the United States and outside the United States (38, 50, 52, 53). PCR screening and molecular typing identified one clone SA isolate in the starling samples. Additionally, PFGE analysis of selected starling isolates revealed genetically diverse strains (Fig. 1), consistent with previous findings in starlings (53, 54). Despite the genetic diversity, two isolates (including a clone SA isolate) showed indistinguishable PFGE patterns with the cattle isolates (Fig. 1), suggesting that starlings may play a role in spreading *Campylobacter* on cattle farms. It should be pointed out that PFGE analysis of the cattle isolates was biased...
toward putative clone SA isolates and did not represent the entire genetic profiles of the cattle isolates. Thus, the matching between the cattle and starling isolates might be even higher if more cattle isolates (non-clone SA) were analyzed by PFGE. Regardless, results from this study demonstrated frequent isolation of Campylobacter from European starlings on cattle farms and suggest possible two-way transmission of Campylobacter between the two animal species. Interestingly, starling intervention on farms did not affect the overall prevalence of Campylobacter (Table 2), suggesting starling control alone does not appear to be an effective intervention strategy to reduce cattle fecal shedding of Campylobacter or clone SA in feedlot cattle. This may be due to the fact that multiple interacting factors contribute to the transmission of Campylobacter on cattle farms and control of a single factor has limited impact on its prevalence. Regardless, starlings can be a source for clone SA and can move these isolates between otherwise separate feedlots visited by foraging starlings.

The advance of next-generation sequencing technologies has made it possible to perform high-resolution molecular typing of bacterial isolates. We conducted WGS analysis of the putative clone SA isolates from NAHMS dairy studies, not only for identification of clone SA, but also for understanding evolution of clone SA over the 12-year time period (2002-2014). The WGS analysis confirmed that 102 of the 128 putative clone SA isolates identified by PCR were true clone SA isolates. The genomic data were further used for maximum-likelihood phylogenetic tree construction, which revealed that the clone SA isolates derived from 2002-2014 are genetically stable and a clear pattern of evolution was not detected as indicted by lack of clustering of the isolates by isolation years (Fig 2). Inclusion of sheep clone SA isolates (33) in the phylogenetic analysis also revealed
that the sheep and cattle isolates are mixed in clustering (Fig. 2), suggesting that the
genomic sequences of clone SA isolates are not uniquely associated with host species and
the possibility of inter-species (cattle and sheep) transmission of clone SA. These
genomic features and the identified wide distribution of clone SA in both beef and dairy
cattle suggest that bovine clone SA may serve as an important reservoir for the source of
infection in sheep, where clinical abortion induced by *C. jejuni* clone SA continues to be
a significant burden for sheep producers (34).

Tetracycline resistance is an important feature of *C. jejuni* clone SA isolated from sheep
and acquisition of this resistance trait is likely due to antibiotic selection pressure as
tetracyclines are frequently used for control of sheep abortion on farms in the U.S. (34,
55). The *tet* (*O*) gene is the only tetracycline resistance determinant identified in
*Campylobacter* so far. Although *tet*(*)O*) is typically carried by plasmids, it is
predominantly located in chromosome in clone SA (34). In this study, we found that
79.4% (Table. 3) of the dairy clone SA isolates carried the *tet*(*O*) gene, and in most of the
isolates (68/81) it was located on chromosome. However, the *tet*(*)O*) gene in the non-clone
SA isolates was all carried by a plasmid. These results are consistent with our previous
findings with the sheep *Campylobacter* isolates (55) and further indicate the advantage of
*C. jejuni* clone SA in dealing with the selection pressure from tetracycline antibiotics.
The pVir plasmid was also identified in a small number (5/102) of the clone SA isolates
in this study. This plasmid is not required for abortion induction by clone SA (56) and is
also infrequently present in sheep clone SA isolates (33). Thus, pVir is not unique to
clone SA and its *in vivo* function is still unknown.
In summary, this study revealed detailed molecular and epidemiological features of *C. jejuni* clone SA in beef and dairy cattle, as well as in European starlings present on cattle farms. These findings underscore the importance of cattle and wild birds in the overall ecology of *C. jejuni* clone SA in animal reservoirs and provide critically needed information for development of intervention strategies. For example, the high prevalence of *C. jejuni* clone SA in cattle explains why many of the clone SA-associated foodborne disease outbreaks were attributed to consumption of raw milk (34) and highlights the need to reduce fecal contamination of milk and pasteurize milk before consumption to prevent the transmission of clone SA to humans. Additionally, the variable distribution of clone SA on cattle farms suggest that production practices and/or environmental factors may influence its prevalence and may be managed to control clone SA in cattle. Furthermore, our findings also suggest that control of *Campylobacter*-induced abortion in small ruminants should consider intervening the transmission of clone SA from the cattle reservoir. These findings provide directions for designing future studies to evaluate intervention strategies. Considering the significance of *C. jejuni* clone SA in ruminant health and food safety, reducing its prevalence on cattle farms will benefit both animal health and public health.

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None to declare.

References:


Table 1. Prevalence of *Campylobacter jejuni* and *C. jejuni* clone SA in feces of feedlot cattle and starlings in the United States

<table>
<thead>
<tr>
<th>State</th>
<th>% <em>Campylobacter</em> in cattle (No. isolates/ total No. samples)</th>
<th>Cattle isolates</th>
<th>Bird isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. jejuni (%)^a^</td>
<td>Clone SA (%)^b^</td>
</tr>
<tr>
<td>Iowa</td>
<td>69.2 (554/800)</td>
<td>487 (87.9)</td>
<td>16 (3.3)</td>
</tr>
<tr>
<td>Texas</td>
<td>71.9 (414/576)</td>
<td>367 (88.6)</td>
<td>42 (11.4)</td>
</tr>
<tr>
<td>Missouri</td>
<td>70.0 (210/300)</td>
<td>191 (91.0)</td>
<td>15 (7.9)</td>
</tr>
<tr>
<td>Colorado</td>
<td>78.2 (593/758)</td>
<td>438 (73.9)</td>
<td>14 (3.2)</td>
</tr>
<tr>
<td>Kansas</td>
<td>70.3 (527/750)</td>
<td>403 (76.5)</td>
<td>23 (5.7)</td>
</tr>
<tr>
<td>Total</td>
<td>72.2 (2298/3184)</td>
<td>1886 (82.1)</td>
<td>110 (5.8)</td>
</tr>
</tbody>
</table>

^a^The percentage is the proportion of *C. jejuni* in the number of *Campylobacter* isolates

^b^The percentage is the proportion of clone SA in the number of *C. jejuni* isolates

N/A: starling samples were not available
Table 2. Prevalence of *Campylobacter* isolated from fecal samples of feedlot cattle before and after starling intervention

<table>
<thead>
<tr>
<th>State</th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Sample tested</td>
<td>Positive samples (%)</td>
</tr>
<tr>
<td>Iowa</td>
<td>400</td>
<td>280 (70.0)</td>
</tr>
<tr>
<td>Texas</td>
<td>250</td>
<td>170 (68.0)</td>
</tr>
<tr>
<td>Missouri</td>
<td>150</td>
<td>85 (56.7)</td>
</tr>
<tr>
<td>Colorado</td>
<td>360</td>
<td>274 (76.1)</td>
</tr>
<tr>
<td>Kansas</td>
<td>350</td>
<td>235 (67.1)</td>
</tr>
<tr>
<td>Total</td>
<td>1510</td>
<td>1044 (69.1)</td>
</tr>
</tbody>
</table>
Table 3. Occurrence and characteristics of C. jejuni clone SA isolates in dairy cows

<table>
<thead>
<tr>
<th>Dairy Study*</th>
<th>No. C. jejuni tested</th>
<th>No. (%) clone SA by:</th>
<th>pVir presence</th>
<th>tet (O) location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PCR</td>
<td>WGS</td>
<td>chromosome</td>
</tr>
<tr>
<td>2002</td>
<td>205</td>
<td>23 (11.2)</td>
<td>21 (10.2)</td>
<td>2</td>
</tr>
<tr>
<td>2007</td>
<td>627</td>
<td>66 (10.5)</td>
<td>55 (8.8)</td>
<td>1</td>
</tr>
<tr>
<td>2014</td>
<td>576</td>
<td>39 (6.8)</td>
<td>26 (4.5)</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>1408</td>
<td>128 (9.1)</td>
<td>102 (7.2)</td>
<td>5</td>
</tr>
</tbody>
</table>

*NAHMS national surveillance studies

Fig. 1. Dendrogram showing the PFGE patterns (KpnI) of C. jejuni isolates from feces of feedlot cattle and starlings. The clone SA strains are represented by two closely associated PFGE patterns (I and II), as was the case in sheep clone SA isolates (29).

IA3902 is a known isolate of clone SA and is used as a reference. The isolates’ names are listed on the right of the dendrogram. “★” indicates starling isolates. TX: Texas; CO: Colorado; MO: Missouri; IA: Iowa; and KS: Kansas. FC indicated feedlot cattle, while ST depicts starling. The numbers in the names of the isolates are arbitrary numbers assigned to feedlots and samples. Please note the starling isolates represent the total C. jejuni population isolated from the birds, while the cattle isolates included in the PFGE analysis were preselected for putative clones SA by the PCR.
Fig. 2. Maximum-likelihood phylogenetic tree constructed with the pangenome (a) and core genome (b) differences among 170 *C. jejuni* clone SA isolates from sheep and cattle. The clone SA strains are intermixed between sheep and cattle, and among the isolation years (2002, 2007, and 2014). The isolates are color-coded based on their source hosts and isolation years: red for sheep, blue for NAHMS Dairy 2002, green for NAHMS Dairy 2007, and black for NAHMS dairy cattle 2014.