Genetic diversity and antimicrobial susceptibility of *Campylobacter jejuni* isolates associated with sheep abortion in the United States and Great Britain

Zuowei Wu, a Rachel Sippy, a Orhan Sahin, a# Paul Plummer, a,b Ana Vidal, c Diane Newell, c,d Qijing Zhang a

Departments of Veterinary Microbiology and Preventive Medicine a, and Veterinary Diagnostic and Production Animal Medicine b, Iowa State University, Ames, IA, USA; Animal Health and Veterinary Laboratory Agency, Surrey, RH2 7JB, UK c; Foodborne Zoonoses Consultancy, Wherwell, Andover, SP11 7AW, UK d.

Running Head: Genotypes of abortifacient *Campylobacter* from sheep

#Address correspondence to Dr. Orhan Sahin, osahin@iastate.edu
Campylobacter is a leading cause of ovine abortion worldwide. Historically genetically diverse C. fetus and C. jejuni strains have been implicated in the disease, but since 2003 a highly pathogenic, tetracycline-resistant C. jejuni clone (named clone SA) has become the predominant cause of sheep abortions in the U.S. Whether clone SA was present in earlier U.S. abortion isolates (before 2000) and associated with sheep abortions outside the U.S. are unknown. Here we analyzed 54 C. jejuni isolates collected from U.S. sheep abortions at different time periods and compared them with 42 C. jejuni isolates associated with sheep abortion during 2002-2008 in Great Britain (GB) using MLST, PFGE and array-based CGH. Although clone SA (ST-8) was present in the early-U.S. isolates, it was not as tetracycline-resistant (19% vs. 100%) or predominant (66% vs. 91%) as it was in the late-U.S isolates. In contrast, C. jejuni isolates from GB were genetically diverse comprising 19 STs and lacking ST-8. PFGE and CGH on representative strains further confirmed the population structure of the abortion isolates. Notably, the GB isolates were essentially susceptible to most tested antibiotics including tetracycline, while the late-U.S. isolates were universally resistant to this antibiotic, which could be explained by the common use of tetracyclines for control of sheep abortions in the U.S., but not in GB. These results suggest that the dominance of clone SA in sheep abortion is unique to the U.S. and use of tetracyclines may have facilitated selection of this highly pathogenic clone.
INTRODUCTION

Abortion in ewes causes significant economic losses to sheep producers. 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 (1). Campylobacteriosis is a highly contagious disease in sheep. Once an abortion storm starts, healthy ewes can be exposed to high levels of Campylobacter organisms through contact with the aborted fetus, placenta, and uterine discharges, which may cause up to 50% of ewes to abort when the organisms are newly introduced into a naive flock (2). Although Campylobacter species can be carried in the intestine and gall bladder of healthy sheep without causing clinical diseases (3), some Campylobacter strains can cause systemic infections. In susceptible pregnant ewes, the infection is characterized by bacteremia with subsequent placentitis, fetal infection, and abortion, which usually occurs in the last trimester of pregnancy (1). Pathologically, aborted or stillborn fetuses may have no lesions or they may show subcutaneous serosanguineous edema, liver lesions and or bronchopneumonia (4). Microscopically, placental lesions consist of septal necrosis, leukocyte infiltration, and high numbers of bacteria within chorionic trophoblast cells (4). On culture, high numbers of Campylobacter can be recovered from aborted placentas, fetal stomach contents, and to a lesser extent, from lung and liver of aborted lambs.

Historically, C. fetus subsp. fetus (herein referred to as C. fetus) accounted for the majority of the Campylobacter species associated with ovine abortion worldwide, but recent studies have indicated a clear trend that C. jejuni is increasingly prevalent in the disease in some parts of the world (5-9). In the United States, the species shift (from C. fetus to C. jejuni) in the distribution of Campylobacter isolates causing sheep
abortion occurred during early 1980s, and by late 1980s and early 1990s *C. jejuni* became the predominant species causing sheep abortion (5, 6). This species shift was further confirmed by our recent study, in which 68 (91.8%) of the 74 *Campylobacter* isolates from ovine abortion cases that occurred on different farms located in Iowa, California, Idaho, Oregon, Nevada, and South Dakota during 2003-2007 were identified as *C. jejuni* (10). Most strikingly, genotyping analyses (using PFGE and MLST) of these *C. jejuni* strains indicated that the majority (66 of 71; 93%) belonged to a single genetic clone (named clone SA for sheep abortion). This finding represents a paradigm shift considering the fact that the sheep carry highly heterogeneous *Campylobacter* organisms in its bile and the intestine and that genetically diverse strains of *Campylobacter* were traditionally associated with sheep abortion (7, 11-13). Interestingly all clone SA isolates were found to be resistant to tetracycline, the only class of antibiotics approved for control and prevention of *Campylobacter* abortion in sheep in the United States (10). Moreover, studies, using a pregnant guinea pig model (14), demonstrated that clone SA was highly abortifacient compared to other *C. jejuni* strains indicating the evolution of increased virulence in this clone. Recently, we reported that clone SA has emerged as a zoonotic pathogen causing human gastroenteritis linked mainly to the consumption of raw milk (13). A representative of this clone, *C. jejuni* IA3902, has now been genome sequenced (15).

To date limited information is available on the genetic diversity and antimicrobial susceptibility of *Campylobacter* isolates from ovine abortion in countries other than the United States. The only published exception to this is New Zealand, where *C. fetus* continues to be a major cause of *Campylobacter*-associated abortion in sheep. Nevertheless, in that country *Campylobacter* ovine abortion isolates (both *C. fetus* and *C. jejuni*) from different farms during different lambing seasons were of multiple
genotypes (7, 16, 17) suggesting that, at least in New Zealand, no single 
Campylobacter clone was dominant as a cause of ruminant abortion.

In England and Wales from 1997 to 2008 clinical samples (fetuses or placentas) 
from ruminant abortions (cattle, sheep and goats) submitted to the Veterinary 
Laboratories Agency (now named the Animal Health and Veterinary Laboratories 
Agency (AHVLA), Surrey, England, were routinely cultured for Campylobacter. All 
Campylobacter isolates recovered were speciated and subspeciated where relevant, 
and catalogued. In addition abortion-associated isolates were submitted from Scotland 
for speciation. Thus this constitutes a unique collection of isolates from Great Britain 
(GB) (England, Wales and Scotland) for comparative studies.

The current study was undertaken to determine the genetic diversity and 
antimicrobial susceptibility of representative sheep abortion C. jejuni isolates from the 
GB and the U.S. in an effort to enhance our knowledge on the overall epidemiology 
and ecology of C. jejuni population in ovine abortion worldwide and in particular, to 
determine whether C. jejuni clone SA is present in ruminant abortions outside the U.S. 
This information is critically needed for development and implementation of effective 
control measures in sheep flocks. In addition, C. jejuni isolates from sheep abortions 
that occurred before (pre-2000) the clone SA became epidemic in the U.S. (post-2003) 
were characterized to provide clues on the evolution of clone SA. The results indicate 
significant differences in the geographically and temporally separated populations. 
Further studies suggest that these differences may be associated with evolutionary 
selection pressures exerted by variances in antimicrobial use in veterinary medicine.
MATERIALS AND METHODS

Collection of *C. jejuni* from sheep abortion

In this study, *C. jejuni* isolates (n=54) derived from sheep abortion cases in the U.S. were comprised of two different temporal collections: The first set included isolates (n=21) cultured from ovine abortions that occurred from 1991 to 2000 in California (n=8) and Idaho (n=13) (hereafter referred to as “early-U.S. isolates”). The second set of isolates (n=33) were recovered from sheep abortions from 2008 to 2011 from cases in Colorado (n=2), Iowa (n=27) and North Dakota (n=4) (hereafter referred to as “late-U.S. isolates”). All of the isolates were obtained from respective veterinary diagnostic laboratories processing the submissions. In addition, data of 92 *C. jejuni* isolates from ovine abortions in the U.S. occurring during 2003-2007 (also considered part of the late-U.S. isolates) from a previously published investigation (10) were included for comparison as indicated below.

The AHVLA has a collection of 924 stored *Campylobacter* ruminant abortion isolates from GB from cases from 1997 to 2008. All isolates have been identified to the species/subspecies level using standard biochemical and molecular methods (18). The majority of these isolates (66%) are *C. fetus* subsp. *fetus* but 147 (~16%) are *C. jejuni*. For this study, 42 of these *C. jejuni* isolates were selected as representative of cases of ovine abortion throughout the GB from 2002 to 2008.

MLST and phylogenetic analysis

Multilocus sequence typing (MLST) was performed following the method originally developed for *C. jejuni* by Dingle et al. (19). The primer sets for the amplification and sequencing of the seven housekeeping genes were used and the
PCRs were performed as described on the C. jejuni MLST website (http://pubmlst.org/campylobacter/), developed by Keith Jolley and Man-Suen Chan (20). Allelic numbers were assigned to the isolates by performing BLAST searches for the assembled sequences using the single-locus query function. 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 sequences were assigned new allele numbers and sequence types (STs) by the MLST database.

Clonal genealogy of STs was estimated using a model-based approach of ClonalFrame computer package for determining bacterial microevolution (21). This model calculates clonal relationships with improved accuracy compared with standard phylogenetic inference techniques for recombining bacteria, such as C. jejuni. It distinguishes point mutations from imported chromosomal recombination events, the source of the majority of allelic polymorphisms. The model has been used successfully to distinguish clades within Campylobacter species and can describe the relationships between genotypes (21). Nucleotide sequences of seven-locus STs were concatenated and the program was run with 50,000 burn-in iterations followed by 50,000 data collection iterations. The tree required 75% consensus for inference of relatedness.

**Genetic distance analysis**

Pairwise comparisons of the genetic distances between each of the C. jejuni populations of interest (geographic and temporal) were calculated using the standardized genetic distances (d₁) as described previously (12, 22). When d₁ is 1,
there are no genotypes in common and when $d_1$ is 0, the two bacterial populations
have the same distribution of genotypes. Genetic distance was determined at the level
of ST, clonal complex (CC), and allele (for the allelic level the right-hand side of the
equation was modified to sum the contribution across all seven MLST alleles, which
was then divided by seven). The genetic distance between each pair of groups was
tested for significance in Visual Basic Application under Excel (VBAE) by
comparing this distance with a distribution of 10,000 distances obtained by
randomizing the data without replacement.

**PFGE typing**

Pulsed field gel electrophoresis (PFGE) analysis of the macrorestriction fragment
patterns of genomic DNA using SmaI and KpnI enzymes was performed following
the Centers for Disease Control and Prevention (CDC)’s standardized PulseNet
protocol for *C. jejuni* (23) and as described elsewhere (13). As the main purpose of
performing PFGE was to further confirm the clonal relatedness of isolates having the
same STs, a detailed restriction pattern-based genetic clustering was not carried out.

**Array-based comparative genomic hybridization (CGH)**

70-mer oligo arrays were synthesized by JCVI (http://pfgrc.jcvi.org) or
MYcroarray (http://www.mycroarray.com) according to the complete genome of *C.
jejuni* IA3902 (15). Genomic DNA was extracted with Promega genomic DNA
purification kit (Madison, WI). DNA labeling, hybridization, and washing were
performed following the standard protocol from JCVI or MYcroarray. Genomic DNA
from each isolate (test DNA) was mixed with equal amount of genomic DNA of
IA3902 (reference DNA) and was hybridized onto two replicate slides by a dye swap strategy. Microarray slides were scanned at 532-nm (Cy3) and 635-nm (Cy5) wavelengths using a GenePix 4100A Microarray Scanner (Molecular Devices, Sunnyvale, CA) at a 5 µm resolution. Fluorescence intensities of each spot were extracted using the GenepixPro 7.0 (Molecular Devices). Analysis of the microarray data was conducted as follows: (i) After local background correction, the fluorescence intensity in each wavelength was log2 transformed and normalized using locally weighted linear regression (LOWESS) using statistical software R (http://www.r-project.org); (ii) Genes with the signal intensity ratio (Test/Reference) less than 0.6 in both replicate slides were considered divergent between the test genome and the reference genome. Data were submitted to the GEO database (Accession number to be provided later). Divergent or conserved genes were translated to a binary code (1 for divergent, 0 for conserved) and analyzed with Cluster 3.0 and visualized by TreeView (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm).

Antimicrobial susceptibility testing and PCR screening for tetO

The minimum inhibitory concentrations (MICs) of nine antibiotics were determined using a standard microbroth dilution method as recommended by CLSI and National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) (10, 13). Commercially available Sensititre Campylobacter plates (Trek Diagnostic Systems, Cleveland, Ohio) were used for antimicrobial susceptibility testing. For all plates, C. jejuni ATCC 33560 was used as a quality control strain. The presence of the tetO gene (the main determinant of tetracycline resistance in
C. jejuni) was determined by PCR as described previously (10). In Campylobacter, tetO is usually located on conjugative plasmids (such as pTet) although it can also be inserted in the chromosome (24, 25). To determine the location of tetO (plasmid or chromosome), new primer sets were designed and used in a PCR. The genome sequence of IA3902 (a clone SA isolate) indicates that tetO is located in the chromosome within the CJSA_0191 locus (corresponding to Cj0203 in the genome of NCTC11168). To ascertain whether tetO gene were located in this chromosomal region, primer pairs tetO-F2 (5’-CCT GGC GTA TCT ATA ATG TTG ACT-3’) and CJSA191-R1 (5’-ATA AGC GCC TAA ATA ATCTGGA-3’) were used to generate an amplicon size of approximately 2000bp. In contrast, to determine if tetO was located on pTet plasmid, primer sets tetO-F1 (5’-TAG CCG TAT AGA TAA GGT TCG-3’) and cpp6-R1 (5’-CTG TGC ATA AAA TCA TAG AAT-3’) designed based on the pTet sequence (26) were employed for an amplicon size of about 3500bp.

RESULTS

Genotyping and phylogeny of C. jejuni isolates from sheep abortion

MLST analysis was performed on a total of 188 isolates derived from sheep abortions in the U.S. (n=146) and GB (n=42). Overall 28 sequence types (STs) were identified (Figure 1). Twenty-three of these STs were clustered into 10 clonal complexes (CC). The remaining 5 STs were singletons (STs that could not be assigned to any CC). Three of these singletons were of newly identified STs (4841, 4842, and 4843; all from GB), while the remaining two (ST-2165 from GB and ST-441 from the U.S.) were previously reported. There was only one novel sequence type (ST-5189) within the U.S. strain collection. Fifteen STs (38, 43, 52, 239, 262,
The U.S. sheep abortion isolates were represented by 13 STs and 7 CCs (one ST was singleton as described above; Figure 1). Overall, ST-8 was the predominant sequence type within the U.S. collection, accounting for 87.6% (128 of 146) of the isolates. When the U.S. collection was stratified by time (early-U.S./pre-2000 vs. late-U.S./post-2003), there was a substantial increase in the proportion of ST-8 strains after 2003 (91.2% of 125 total isolates) compared with isolates recovered before 2000 (66.6% of 21 isolates) (Figures 2A and 2B). The early-U.S. strains also included 5 isolates of ST-50 (23.8%), one isolate of ST-21 (4.6%) and one isolate of ST-441 (4.6%), whilst the late-U.S. strains included two isolates of ST-806 (1.6%) and one isolate of each of STs-38, 42, 43, 45, 50, 239, 607, 982 and 5189 (Figures 1, 2A and 2B). Apart from ST-8, there was little overlap in STs between the early- and the late-U.S strains, except the second most predominant ST in the early-U.S. isolates, ST-50, was detected once in 2004 only (Figure 2A and 2B). Table 1 shows the genetic distance between the two temporal collections, which indicates substantial genetic diversity between isolates from the early- and the late-U.S. collections at the ST-level, but this difference was not statistically significant.

The 42 sheep abortion isolates from GB were assigned to 19 STs and 8 CCs (4 STs were singletons) (Figures 1 and 2C). The most common sequence type was ST-227 (19%; 8 isolates), followed by ST-206 (14.2%; 6 isolates), ST-270 (9.5%; 4 isolates), STs-19 and 61 (7.1% each; 3 isolates) and STs-42, 50, 137 and 1517 (4.7% each; 2 isolates). STs-21, 45, 52, 262, 432, 2165, 3153, 4841, 4842, and 4843 were each represented by a single isolate. These 19 STs appeared to occur sporadically during the years 2002-2008 and there were no obvious predominant STs within the
GB collection. The predominant ST within the U.S. collection, ST-8 (clone SA), was not found within the GB strains investigated. However, STs-21, 42, 45 and 50 were present in both the U.S and the GB strain collections (Figures 1 and 2). The observed difference in population structure between the U.S. and the GB _C. jejuni_ isolates from sheep abortion was supported by genetic distance analysis (Table 1).

PFGE confirmed the differences in U.S and GB strain populations observed by MLST. As previously reported (10), all ST-8 isolates from the U.S. sheep abortions were basically represented by two closely related KpnI subtypes and a single Smal type. These PFGE types persisted over the time periods studied (results not shown). As expected, isolates with different STs had clearly distinguishable PFGE restriction profiles. The technique confirmed the genetic heterogeneity among the GB isolates, which were represented by 26 KpnI and 18 Smal restriction patterns (results not shown). Interestingly, strains of some STs (ST-21, ST-42 and ST-45), despite having the same MLST type, had very distinct PFGE patterns in the U.S. compared with GB. In contrast, isolates of ST-50 were indistinguishable by PFGE regardless of the country of origin (results not shown).

**Array-based CGH analysis of _C. jejuni_ isolates from sheep abortion**

Because MLST only considers diversity in 7 genes, a CGH approach was adopted to investigate gene divergence across the whole genome. Seventeen representative strains, with distinct STs and PFGE profiles, were selected. This strain set included 4 early-U.S. isolates, 9 late-U.S. isolates and 4 isolates representing the most common STs within the GB strains (Table 2). Strain IA3902, representing clone SA, was used as the reference genome (GenBank accession: CP001876.1 for the chromosome and
Among all the strains investigated, 258 of the chromosomal genes diverged from those in the reference genome (Figure 3; Supplementary Dataset S1). These divergent genes were found to be functionally enriched in the Clusters of Orthologous Group (COG) categories of “cell wall/membrane biogenesis”, “general function prediction only” and “Not in COGs” (Figure 4). A dendrogram based on the clustering of the divergent genes showed that all of the ST-8 genomes were closely related and had very similar gene content (Figure 3). The U.S. isolate ND9, clustered together with the ST-8 genomes, despite having a different sequence type (ST-239). It is notable that both ST-8 and ST-239 belong to CC-21.

A close examination of the distribution of the variable genes along the genomes revealed that most of them were present in clusters or variable regions (VRs), with the largest cluster comprising 30 consecutive genes (capsule biosynthesis locus; cjsa_1345-cjsa_1375). Overall, 74.8% (193/258) of the variable genes also had divergent neighbors. Moreover, 12 VRs (VR1-12), containing more than 5 consecutive genes, accounted for 58.5% (151/258) of the divergent chromosomal genes (Figure 3; Supplementary data). These VRs encode genes involved in iron transport (VR1, cjsa_0167-cjsa_0171), zinc transport (VR2, cjsa_0238-cjsa_0242), pantothenate and biotin biosynthesis pathway and molybdenum transport (VR3, cjsa_0269-cjsa_0274), altronate hydrolysis and fucose transport (VR4, cjsa_0450-cjsa_0461), phosphate-regulation and iron uptake (VR6, cjsa_0690-cjsa_0714), glutamine binding (VR7, cjsa_0770-cjsa_0774); lipooligosaccharide biosynthesis (VR9, cjsa_1074-cjsa_1087), flagellar modification and O-linked glycosylation (VR10, cjsa_1234-cjsa_1276); capsular biosynthesis (VR11, cjsa_1345-cjsa_1375); DNA restriction/modification(VR12, cjsa_1465-cjsa_1473), and unknown function(VR5,
cjsa_0532-cjsa_0536; VR8, cjsa_0912-cjsa_0916). All of the large VRs, except VR10, were identified to be conserved among the non-ST-8 genomes only. VR10, encoding flagellar modification and O-linked glycosylation locus, was also divergent in strain ID15 (an early U.S. ST-8 isolate) compared with the rest of the ST-8 genomes. Besides the large VRs, small highly chromosomal variable loci were also identified among the tested genomes. Five loci (cjsa_0032-cjsa_0033, encoding a type II restriction-modification enzyme and a putative cytoplasmic protein; cjsa_130, encoding McrBC restriction endonuclease system; cjsa_0192-cjsa_0193, encoding a hypothetical protein and tetO; cjsa_0585-cjsa_0586, encoding hypothetical proteins; cjsa_0982, encoding murG) were highly divergent among more than 50% of the tested genomes. Interestingly, the pVir plasmid (present in IA3902) was absent from all the isolates tested regardless of their sequence type and country of origin (Figure 3). The heterogeneity within the genomes with respect to the pVir carriage was further confirmed by PCR (data not shown).

Antimicrobial susceptibility of the C. jejuni isolates

The MICs at which the growth of 50% and 90% of the C. jejuni isolates from sheep abortions in the G.B. and U.S. (n= 41 and 54, respectively) were inhibited, and the percentages of resistance for each antimicrobial drug are summarized in Table 3. Of 41 G.B. isolates tested, 7 (17.1%) were resistant to nalidixic acid, 4 (9.76%) were resistant to clindamycin, 2 (4.88%) were resistant to tetracycline, and 1 (2.44%) to azithromycin. None of the G.B. isolates showed any resistance to ciprofloxacin, erythromycin, florfenicol, gentamycin, or telithromycin. In comparison, all of the U.S. isolates were susceptible to clindamycin and florfenicol but had low levels of resistance (less than 5%) to azithromycin, ciprofloxacin, gentamycin, nalidixic acid...
and telithromycin (Table 3). In contrast to the G.B. isolates, 68.5% (37 of 54 total) of
the U.S. isolates were resistant to tetracycline, with an MIC₉₀>64 µg/ml. There was
also a dramatic increase in resistance to tetracycline between the early-U.S. and
late-U.S. isolates (19% compared with 100%, respectively) (Table 3). All of the
tetracycline resistant isolates (n=4) within the early-U.S strain collection were of ST-8,
i.e., none of the other STs present in the early-U.S. group (STs-21, 50, and 441) were
resistant to tetracycline (see below).

Characterization of tetracycline resistance in C. jejuni isolates.

By aggregating data from this study with data from a previous study (10), a
collection of tetracycline resistant C. jejuni isolates from sheep abortions was
obtained. This collection included 123 from the U.S. and 2 from G.B. The presence of
the tetracycline-resistance gene, tetO, and its location (chromosome or plasmid) were
determined in 99 of the 123 tetracycline resistant U.S. isolates and both of the G.B.
isolates by PCR (Table 4). The tetO gene was detected in 96% (95/99) of the U.S.
isolates, of which 92.6% (88/95) had it within cjsa_0191 locus in the chromosome
and 7.3% (7/95) carried it on pTet plasmid. All 4 of the tetracycline resistant strains
(all ST-8) from the early-U.S. collection, but only 3 out of the 91 late-U.S strains, had
tetO on pTet (Table 4). Interestingly, one of the G.B. isolates also carried the tetO
gene within the cjsa_0191 locus while the other isolate appeared to have it at an
undetermined chromosomal locus. A detailed description of tetO presence and
location by country of origin, year of isolation and MLST type is presented in Table 4.

DISCUSSION
Bacteria of the genus *Campylobacter* are associated with several disease presentations (2, 27-30). There are 27 species of *Campylobacter*, and the species *C. fetus* is historically recognized as associated with abortion especially in sheep and cattle (1, 6, 31, 32). However, it is known that the thermophilic species *C. jejuni* and *C. coli* can also cause abortion (5, 33). Recently in the U.S. one clone of *C. jejuni* (clone SA) has become predominate in *Campylobacter* isolates recovered from ovine abortions (10). The reason for, and the geographical extent of, this disease shift are as yet unknown. There are few other national collections of *Campylobacter* isolates from ovine abortions, but in GB such isolates have been collected at the AHVLA for over a decade. We have now undertaken a comparison of U.S. and GB strains from similar timelines using the genotypic approaches of PFGE, MLST and array-based CGH. All three methods were used because they have different levels of sensitivity. MLST is the least sensitive method for typing as it is dependent on only seven housekeeping genes (19). PFGE is dependent on point mutations in the enzyme site and is consequently more sensitive, but in *C. jejuni* the plasticity of the genome is renown and minor changes can falsely indicate strain differences (34). Array-based CGH provides the most discriminatory power utilizing all the genomic detail of the bacterium. The use of these three methods sequentially, particularly the array-based GCH (Figure 3), confirmed the genetic identity and clonal nature of the clone SA strains. Moreover, these techniques demonstrated that, unlike in the U.S., where the population of ovine strains has become increasingly genotypically homogeneous (i.e. predominantly Clone SA), the GB strain population remains heterogeneous (Fig 2). Interestingly, the GB strains were even significantly different (p<0.0001 in the genetic distance analysis) from the early-US strains, i.e. before the predominance of clone SA (Table 1). The reason for this is unclear but suggests a historical geographical
separation in the bacterial populations. Whether the differences seen between strains from the U.S. and GB are consistent worldwide has yet to be investigated.

MLST was developed as a technique to determine evolutionary trends. The population structure of *C. jejuni* and its close relative *C. coli* is now well established with nearly 30,000 isolates included in the database (http://pubmlst.org/campylobacter/). A detailed analysis was undertaken of the MLST genotypes of the ovine abortion strains relative to the population distribution of all *C. jejuni* strains. Interestingly the ST types of the ovine abortion strains in the joint UK and early-US collections, is largely representative of the strains from sheep/sheep feces submitted to the MLST *C. jejuni/C. coli* database (n=385 strains as of 27 January 2014) and similar to those described in other ruminant studies in GB (12). This indicates that, until recently, there has been no particular selection of strains with specific virulence properties for ovine abortion and that the organism, which is normally a commensal of the ovine intestinal tract, inherently has all the phenotypic properties enabling it to translocate across the intestinal epithelium and infect the placenta of naive (previously unexposed) sheep causing fetal mortality. The definitive evidence for this would come from a genomic comparison of clone SA and other strains isolated from sheep but unassociated with abortion.

The absence of evidence for specific virulence properties of *C. jejuni* essential for ovine abortion suggests the rapid expansion of clone SA in affected sheep in the U.S. is due to selection pressure independent of the host. The presence of tetracycline resistance in this clone suggests that the veterinary use of this antimicrobial has provided the required selection pressure. This hypothesis is supported by antimicrobial susceptibility analysis of the strain collections, which showed that tetracycline resistance was very low in *C. jejuni* isolates from the GB (<5%) and, to a
lesser extent in the early-U.S. collection (19%), but most recently all of the ovine abortion C. jejuni isolates display resistance to this antibiotic (Table 3). At the same time there has been a general shift in the location of the tetO gene apparently responsible for this resistance, from the plasmid, pTet, to the chromosome (Table 4). Tetracyclines are commonly used in sheep in the U.S., particularly for the control of ovine abortion storms associated with Campylobacter (35). In GB, the use of tetracyclines in livestock is highly restricted (36), though tetracyclines are registered for therapeutic use in sheep and are frequently used for diseases such as foot rot and abortion. C. jejuni is known to rapidly acquire resistance as a result of exposure to tetracycline, so it is not surprising that resistant organisms are common in the feces of sheep in North America (37). It is, therefore, tempting to speculate that antibiotic selection pressure may have encouraged the expansion of tetracycline resistant C. jejuni strains, and subsequently promoted the integration of tetO from the plasmid into the chromosome, facilitating its persistence in the sheep production environment. However, it is unlikely that the veterinary use of tetracycline wholly accounts for the overwhelming predominance of clone SA in the U.S. ovine abortion strains. Clearly, other C. jejuni strains have been associated with ovine abortion in the U.S., and even become tetracycline resistant, but have not displayed the population explosion seen in clone SA. This suggests that some other factors, probably related to sheep husbandry in the U.S., has advantaged strains of ST-8 in the U.S. sheep production environment.

In an attempt to identify any unique characteristics of C. jejuni clone SA, which would account for its predominance, isolate IA3902, has been genome sequenced (15). The genome comprises a ~1.6 Mb chromosome encoding 1613 genes and a ~37 Kb plasmid (pVir) encoding 53 genes. The genome sequence was then used for array-based CGH analysis. The results showed that 16% (258 of 1613) genes were
highly divergent among the strains tested and the variable genes were clustered into 12 large regions (Figure 3). Such patterns of genetic variation are consistent with previous studies in *C. jejuni* (38-41). The VRs were highly conserved among the ST-8 isolates, further confirming the MLST and PFGE results on the high homogeneity of the clone SA strains in the U.S., but the pVir plasmid was not observed in other isolates and so appears to be a unique observation unrelated to the phenotype under consideration.

The divergent genes among the *C. jejuni* isolates from sheep abortion were found to be enriched in the COG functional categories of cell wall/membrane biogenesis, general function prediction and function-unknown proteins (Figure 4). Many of these variable regions involve genes associated lipoooligosaccharide biosynthesis locus (VR9, *cjsa_1074-cjsa_1087*), flagellar modification and O-linked glycosylation locus (VR10, *cjsa_1234-cjsa_1276*), and capsular biosynthesis locus (VR11, *cjsa_1345-cjsa_1375*). Previous studies indicate that the pathogenicity of *C. jejuni* can be significantly influenced by changes in the genomic content of such regions. Variations in the flagellar locus can lead to differences, both in the flagellin protein backbone and post-translational modifications of flagellin (42). Diversity in the capsular locus can result in the presence of many different capsule types (43) and variation in LOS biosynthesis loci can produce different surface lipooligosaccharides (43, 44). It is generally accepted that the LOS, capsule and flagella are important in the *C. jejuni* pathogenic mechanisms of campylobacteriosis (42), but whether differences in these bacterial structures would confer properties, which enabled ovine abortion, is unknown. One possibility is the development of antigenic diversity (43), which could facilitate host immune evasion. Recent studies using multi-omics approaches and a guinea pig abortion model to compare clone SA with *C. jejuni* strain
NCTC11168 also implicate such genetic and phenotypic characteristics in the generation of a highly successful abortifacient C. jejuni strain (15). The genomic sequencing of multiple ovine abortion strains is now being undertaken in an attempt to identify those genes which both confer the phenotypic properties enabling C. jejuni to overcome innate immune responses and cause the systemic maternal infection resulting in fetal abortion, and which have enabled clone SA to outcompete all other C. jejuni strains to become predominant as a cause of ovine abortion in the U.S.

ACKNOWLEDGMENTS

This work was supported by the National Research Initiative Competitive Grants Program from the National Institute of Food and Agriculture at USDA (Grant No. 2010-65110-20419).

We would like to thank personnel at the VDLs in the participating states including Idaho (Dr. Beth Mamer and Greta Anderson), South Dakota, and California (Dr. Kris Clothier) for providing the isolates from sheep abortion cases.
REFERENCES


36. Wimalarathna HM, Richardson JF, Lawson AJ, Elson R, Meldrum R,


40. Parker CT, Quinones B, Miller WG, Horn ST, Mandrell RE. 2006. Comparative genomic analysis of *Campylobacter jejuni* strains reveals diversity due to genomic elements similar to those present in C-jejuni strain RM1221. Journal of Clinical Microbiology 44:4125-4135.


FIGURE LEGENDS

Figure 1. ClonalFrame phylogenetic tree for the 7-gene MLST data set, displaying the clonal relationship among the STs of 188 *C. jejuni* isolates from sheep abortion. The x-axis is time in coalescent units. STs found among the GB collection are marked in blue, those of U.S. are marked in red, and STs found among both collections are marked in green. Singletons (STs that could not be assigned to a CC) are denoted by UA. Number of isolates within each ST is shown in parenthesis.

Figure 2. Genotype composition of *C. jejuni* isolates from sheep abortion as determined by MLST. Distribution of each sequence type (ST) is shown within the early-U.S. collection (2000 and before) of 21 isolates (A), the late-U.S. collection (2003 and beyond) of 125 isolates (B), and the GB collection (2002-2008) of 42 isolates (C). Percentages of ST-8 (clone SA) in the U.S. collections are indicated.

Figure 3. Dendrogram based on CGH analysis of 18 *C. jejuni* isolates from sheep abortion. Divergent or conserved genes were translated to a binary code and analyzed with Cluster/TreeView. For each strain (shown on the top), a black line indicates the divergence of the gene in comparison to the one in IA3902. Although pVir plasmid encodes 53 genes, it is represented here as one unit. The large variable regions (RV1-12) are shown on the left. The conservation rate curve is shown on the right.

Figure 4. Gene enrichment analysis of the divergent genes (identified in CGH) among 18 *C. jejuni* isolates from sheep abortion. The fraction of genes in each COG category is shown on the X axis. COG categories that are significantly enriched (*P* < 0.0001, Z-test) are indicated by an asterisk.
Table 1. Standardized pairwise genetic distances (d1) at the level of CC, ST, and allele between geographic and temporal groups of 188 *C. jejuni* abortion isolates in the U.S. and GB.

<table>
<thead>
<tr>
<th>Comparisona</th>
<th>Genetic distance (d1)</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Comparisona</th>
<th>Genetic distance (d1)</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EUS vs GB</strong></td>
<td></td>
<td></td>
<td><strong>US vs GB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0.8337</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CC</td>
<td>0.8162</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ST</td>
<td>0.928</td>
<td>&lt;0.0001</td>
<td>ST</td>
<td>0.9456</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Allele</td>
<td>0.5025</td>
<td>&lt;0.0001</td>
<td>Allele</td>
<td>0.6701</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>LUS vs GB</strong></td>
<td></td>
<td></td>
<td><strong>EUS vs LUS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0.8095</td>
<td>&lt;0.0001</td>
<td>CC</td>
<td>0.0587</td>
<td>0.409</td>
</tr>
<tr>
<td>ST</td>
<td>0.9759</td>
<td>&lt;0.0001</td>
<td>ST</td>
<td>0.3252</td>
<td>0.0404</td>
</tr>
<tr>
<td>Allele</td>
<td>0.5485</td>
<td>&lt;0.0001</td>
<td>Allele</td>
<td>0.0188</td>
<td>0.0199</td>
</tr>
</tbody>
</table>

<sup>a</sup>EUS, early-U.S. collection; LUS, late-U.S. collection.

<sup>b</sup>p-values are raw; those in bold are significant after Bonferroni correction (n=21).

<sup>c</sup>Statistically significant comparisons are shown in bold.
Table 2. List of *C. jejuni* isolates used in array-based CGH analysis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host/disease</th>
<th>Region</th>
<th>Year</th>
<th>ST/CC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA3902</td>
<td>Sheep Abortion</td>
<td>Iowa</td>
<td>2006</td>
<td>8/21</td>
<td>Tet-resistant; reference</td>
</tr>
<tr>
<td>CA6e</td>
<td>Sheep abortion</td>
<td>California</td>
<td>1991</td>
<td>8/21</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>ID8</td>
<td>Sheep Abortion</td>
<td>Idaho</td>
<td>1993</td>
<td>50/21</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>ID15</td>
<td>Sheep Abortion</td>
<td>Idaho</td>
<td>1993</td>
<td>8/21</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>CA3e</td>
<td>Sheep abortion</td>
<td>California</td>
<td>1999</td>
<td>8/21</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>VDL705</td>
<td>Sheep Abortion</td>
<td>Iowa</td>
<td>2003</td>
<td>8/21</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>VDL35</td>
<td>Sheep Abortion</td>
<td>Iowa</td>
<td>2003</td>
<td>8/21</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>UK18</td>
<td>Sheep abortion</td>
<td>GB</td>
<td>2004</td>
<td>206/206</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>UK29</td>
<td>Sheep Abortion</td>
<td>GB</td>
<td>2005</td>
<td>21/21</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>UK24</td>
<td>Sheep Abortion</td>
<td>GB</td>
<td>2005</td>
<td>45/45</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>VDL3080</td>
<td>Sheep Abortion</td>
<td>Iowa</td>
<td>2005</td>
<td>8/21</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>UK33</td>
<td>Sheep Abortion</td>
<td>GB</td>
<td>2006</td>
<td>227/206</td>
<td>Tet-susceptible</td>
</tr>
<tr>
<td>VDL902</td>
<td>Sheep abortion</td>
<td>Iowa</td>
<td>2008</td>
<td>982/21</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>ND9</td>
<td>Sheep abortion</td>
<td>N. Dakota</td>
<td>2008</td>
<td>239/21</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>1E2B2a</td>
<td>Sheep bile</td>
<td>Iowa</td>
<td>2008</td>
<td>8/21</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>VDL213</td>
<td>Sheep abortion</td>
<td>Iowa</td>
<td>2009</td>
<td>806/21</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>VDL2945</td>
<td>Sheep Abortion</td>
<td>Iowa</td>
<td>2009</td>
<td>45/45</td>
<td>Tet-resistant</td>
</tr>
<tr>
<td>VDL1957</td>
<td>Sheep abortion</td>
<td>Iowa</td>
<td>2010</td>
<td>8/21</td>
<td>Tet-resistant</td>
</tr>
</tbody>
</table>
Table 3. Antimicrobial susceptibility of 54 U.S. and 41 GB *C. jejuni* isolates from sheep abortion

<table>
<thead>
<tr>
<th></th>
<th>GB Isolates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>US Isolates&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Early-US Isolates&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Late-US Isolates&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>Resistance (%)</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.12</td>
<td>0.12</td>
<td>2.44</td>
<td>0.06</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.12</td>
<td>0.25</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.12</td>
<td>0.25</td>
<td>9.76</td>
<td>0.12</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5</td>
<td>1</td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>8</td>
<td>32</td>
<td>17.1</td>
<td>8</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>0.5</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25</td>
<td>2</td>
<td>4.88</td>
<td>64</td>
</tr>
</tbody>
</table>

<sup>a</sup>41 isolates; <sup>b</sup>54 isolates; <sup>c</sup>21 isolates; <sup>d</sup>33 isolates
Table 4: Characteristics of tetracycline resistant (MIC ≥16 µg/ml) *C. jejuni* isolates from sheep abortions in the U.S. (n=99) and GB (n=2) with respect to *tetO* gene

<table>
<thead>
<tr>
<th><em>tetO</em> presence/ location</th>
<th>No. isolate</th>
<th>Country origin</th>
<th>Isolation year</th>
<th>ST</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ / CISA_0191</td>
<td>85</td>
<td>U.S.</td>
<td>2003-2011</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.S.</td>
<td>2009</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.S.</td>
<td>2008</td>
<td>239</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.S.</td>
<td>2008</td>
<td>982</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>GB</td>
<td>2008</td>
<td>4843</td>
<td>UA</td>
</tr>
<tr>
<td>+ / pTet</td>
<td>4</td>
<td>U.S.</td>
<td>1993</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.S.</td>
<td>2010</td>
<td>38</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>U.S.</td>
<td>2004/2009</td>
<td>806</td>
<td>21</td>
</tr>
<tr>
<td>+ / unknown</td>
<td>1</td>
<td>GB</td>
<td>2008</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>- / -</td>
<td>1</td>
<td>U.S.</td>
<td>2005</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.S.</td>
<td>2007</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.S.</td>
<td>2007</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.S.</td>
<td>2008</td>
<td>5189</td>
<td>61</td>
</tr>
</tbody>
</table>

*Based on chromosomal locus tag of IA3902 genome (GenBank# CP001876.1), or pTet plasmid; +: present; −: absent
Figure 1. ClonalFrame phylogenetic tree for the 7-gene MLST data set, displaying the clonal relationship among the STs of 188 *C. jejuni* isolates from sheep abortion. The x-axis is time in coalescent units. STs found among the GB collection are marked in blue, those of U.S. are marked in red, and STs found among both collections are marked in green. Singletons (STs that could not be assigned to a CC) are denoted by UA. Number of isolates within each ST is shown in parenthesis.
Figure 2. Genotype composition of C. jejuni isolates from sheep abortion as determined by MLST. Distribution of each sequence type (ST) is shown within the early-U.S. collection (2000 and before) of 21 isolates (A), the late-U.S. collection (2003 and beyond) of 125 isolates (B), and the GB collection (2002-2008) of 42 isolates (C). Percentages of ST-8 (clone SA) in the U.S. collections are indicated.
**Figure 3.** Dendrogram based on CGH analysis of 18 _C. jejuni_ isolates from sheep abortion. Divergent or conserved genes were translated to a binary code and analyzed with Cluster/TreeView. For each strain (shown on the top), a black line indicates the divergence of the gene in comparison to the one in the reference strain IA3902. Although pVir plasmid encodes 53 genes, it is represented as one unit. The large variable regions (RV1-12) are shown on the left. The conservation rate curve is shown on the right.
Figure 4. Gene enrichment analysis of the divergent genes (identified in CGH) among 18 *C. jejuni* isolates from sheep abortion. The fraction of genes in each COG category is shown on the X-axis. COG categories that are significantly enriched (*P* < 0.0001, Z-test) are indicated by an asterisk.