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Permalink
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Journal
Applied and Environmental Microbiology, 82(24)

ISSN
0099-2240

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Publication Date
2016-12-15

DOI
10.1128/aem.01746-16

Peer reviewed

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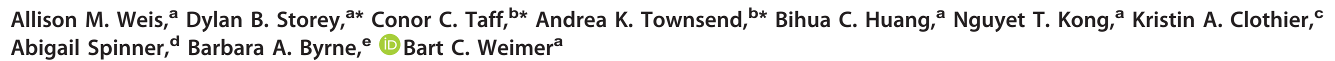
Genomic Comparison of *Campylobacter* spp. and Their Potential for Zoonotic Transmission between Birds, Primates, and Livestock



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ABSTRACT

***Campylobacter* is the leading cause of human gastroenteritis worldwide. Wild birds, including American crows, are abundant in urban, suburban, and agricultural settings and are likely zoonotic vectors of *Campylobacter*. Their proximity to humans and livestock increases the potential spreading of *Campylobacter* via crows between the environment, livestock, and humans. However, no studies have definitively demonstrated that crows are a vector for pathogenic *Campylobacter*. We used genomics to evaluate the zoonotic and pathogenic potential of *Campylobacter* from crows to other animals with 184 isolates obtained from crows, chickens, cows, sheep, goats, humans, and nonhuman primates. Whole-genome analysis uncovered two distinct clades of *Campylobacter jejuni* genotypes; the first contained genotypes found only in crows, while a second genotype contained "generalist" genomes that were isolated from multiple host species, including isolates implicated in human disease, primate gastroenteritis, and livestock abortion. Two major β-lactamase genes were observed frequently in these genomes (*oxa-184*, 55%, and *oxa-61*, 29%), where *oxa-184* was associated only with crows and *oxa-61* was associated with generalists. Mutations in *gyrA*, indicative of fluoroquinolone resistance, were observed in 14% of the isolates. Tetracycline resistance (*tetO*) was present in 22% of the isolates, yet it occurred in 91% of the abortion isolates. Virulence genes were distributed throughout the genomes; however, *cdtC* alleles recapitulated the crow-only and generalist clades. A specific *cdtC* allele was associated with abortion in livestock and was concomitant with *tetO*. These findings indicate that crows harboring a generalist *C. jejuni* genotype may act as a vector for the zoonotic transmission of *Campylobacter*.**

IMPORTANCE

This study examined the link between public health and the genomic variation of *Campylobacter* in relation to disease in humans, primates, and livestock. Use of large-scale whole-genome sequencing enabled population-level assessment to find new genes that are linked to livestock disease. With 184 *Campylobacter* genomes, we assessed virulence traits, antibiotic resistance susceptibility, and the potential for zoonotic transfer to observe that there is a "generalist" genotype that may move between host species.

Campylobacter is a motile Gram-negative spiral bacterium that causes gastroenteritis in humans and other animals (1, 2). In livestock, *Campylobacter* may cause abortion in addition to gastroenteritis (3). It is one of the most common foodborne zoonotic pathogens worldwide and is often transmitted via the fecal-oral route through the consumption of contaminated food or water (1, 2). In the United States, campylobacteriosis is estimated to affect more than 1.3 million people each year, with symptoms including fever, abdominal cramping, and bloody diarrhea (1, 4, 5). Internationally, campylobacteriosis is a significant public health burden; the incidence in developed nations is estimated to be 4.4 to 9.3 per 1,000 people yearly and is a substantial cause of morbidity in developing nations (6). In rare and severe cases, infection can lead to chronic autoimmune disorders, such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (7, 8). Outbreaks in the United States are largely attributed to contaminated poultry and water and are commonly associated with unpasteurized milk (1, 4, 5, 9). Despite many efforts to contain and abate *Campylobacter jejuni* outbreaks, national and international reduction goals remain unmet and the number of new cases continues to increase yearly (6, 10–12).

Recent research has focused on uncovering the initial sources of human infection. Birds are considered to be a primary host of *Campylobacter*, which is a commensal organism in a broad range of wild bird populations, including black-headed gulls (*Chroicocephalus ridibundus*), Sandhill cranes (*Grus canadensis*), European starlings (*Sturnus vulgaris*), and American crows (*Corvus brachyrhynchos*) (13–15). *C. jejuni* isolates from these birds have been implicated in human disease (11, 16, 17). Other studies indicate that some *Campylobacter* isolates found in wild birds may not be pathogenic to humans (14, 18–23). These conflicting reports indicate that studies using higher resolution molecular methods

Received 10 June 2016 **Accepted** 30 September 2016

Accepted manuscript posted online 7 October 2016

Citation Weis AM, Storey DB, Taff CC, Townsend AK, Huang BC, Kong NT, Clothier KA, Spinner A, Byrne BA, Weimer BC. 2016. Genomic comparison of *Campylobacter* spp. and their potential for zoonotic transmission between birds, primates, and livestock. Appl Environ Microbiol 82:7165–7175. doi:10.1128/AEM.01746-16.

Editor: C. A. Elkins, FDA Center for Food Safety and Applied Nutrition

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01746-16.

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may provide insights to more precisely assess *Campylobacter* isolates from wild birds so as to gauge their importance to food safety and public health.

To date, most studies have used 16S rRNA, *fla* typing, or multilocus sequence typing (MLST) to characterize isolates and examine zoonotic transmission. Unfortunately, the resolutions of these methods have limited the conclusions that can be drawn. While 16S sequencing has been used historically to assess microbial diversity, it underestimates the genome variation within a species (24, 25). The MLST method, which uses five to seven genes to build a classification system, is more robust than 16S sequencing and has been used in many studies (26–28). To date, however, the application of taxonomically conserved housekeeping genes, while phylogenetically useful, does not include biologically informative genes, such as virulence factors (29), thereby limiting conclusions about potential pathogenicity. Recent studies have demonstrated the importance of whole-genome sequencing (WGS) in understanding *C. jejuni* pathogenicity and transmission (18, 21, 23, 30); however, to date, studies of *Campylobacter* in wild birds have not taken advantage of WGS approaches to define the genotypes that are associated with zoonotic transmission and virulence.

The American crow is a North American passerine that forages in a variety of settings, including dumps, feedlots, pastures, and urban areas. As such, American crows have the potential to transfer pathogens from human waste or infected animal manure to human food, waterways, and livestock, potentially acting as both a reservoir and a transport host (22). Previous studies indicate that *Campylobacter* prevalence is high in crow species around the world. Corvids from Japan, New Zealand, Malaysia, and Tanzania harbor *Campylobacter* with prevalences ranging from 34% to 43% (31–35). Likewise, 67% of free-ranging American crows in the Sacramento Valley, California, tested positive for *C. jejuni* (22). Crow social behavior may contribute to high infection rates in crows, and foraging patterns may amplify the risk of zoonotic transmission in specific areas, such as feedlots, where many species intermingle (36). The sequencing of 16S rRNA revealed that many of those isolates were similar to *C. jejuni* strains that were isolated from human clinical samples (22).

We used WGS to compare the genomes of 100 *Campylobacter* species isolates (97 *C. jejuni* isolates) that were collected from crows between 2012 and 2014 in the Sacramento Valley, California, with 60 *Campylobacter* species isolates from nonhuman primates, chickens, sheep, cows, and goats from the same geographic region. We examined evidence for genetic traits that are indicative of pathogenicity and zoonotic potential from published *Campylobacter* species whole-genome sequences for a total of 184 genomes.

MATERIALS AND METHODS

Isolates sequenced. In addition to the 100 *Campylobacter* species isolates from crows (22), isolates were collected from rhesus macaques (*Macaca mulatta*) between 2014 and 2015 from the California National Primate Center (CNPRC), an open-air facility housing ~5,000 macaques (47 isolates); from cases of abortion in bovine, ovine, and caprine fetuses (9 isolates); and from chickens collected by the UC Davis Veterinary Medicine Teaching Hospital (VMTH) between 2014 and 2015. The genomes of isolates collected during this study were compared to genomes in NCBI GenBank, which were isolated mainly from humans, chickens, and an ovine abortion case.

Crow and primate sampling. Sample collection of *Campylobacter* was done as described previously (22). Briefly, fecal or cloacal swab samples were collected from American crows (hereafter referred to as "crows") in Yolo County, California, between May 2012 and June 2014 and isolated using Amies clear gel collection and transport swabs (Remel BactiSwab; Thermo Fisher Scientific, Waltham, MA). Individual transport swabs were stored on ice (4 to 7°C) for 2 to 6 h prior to culture. Bacteria were isolated and identified at the VMTH as described previously (22) and in the paragraph below. All crow sampling was done using protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis (IACUC 16897). Primate sampling was done using fecal swabbing at the CNPRC during routine primate health care on primates for diarrhea/gastroenteritis in accordance with the approved protocols with the same methods and reagents as those used at the VMTH (described above and below), and *Campylobacter* isolates were given to this study from the CNPRC after bacterial isolation.

***Campylobacter* testing.** *Campylobacter* culture was performed from fecal samples inoculated onto 5% sheep blood agar (SBA) containing cefoperazone, vancomycin, and amphotericin B and from aborted fetus samples on 5% SBA containing amphotericin B and novobiocin (Campy CVA; Hardy Diagnostics, Santa Maria, CA, USA). Plates were incubated at 37°C in microaerobic conditions (CampyGen; Oxoid Limited, Hampshire, United Kingdom) for 4 to 6 days, as described by Weis et al. (22). Bacterial colonies were Gram stained and subcultured onto 5% SBA (Hardy Diagnostics) for further characterization. Microaerobic isolates with a characteristic appearance on the culture medium and Gram stain (small, curved Gram-negative rods) that were catalase positive were identified as *Campylobacter* spp. and as *C. jejuni* if they hydrolyzed hippurate (Dalynn Biologicals Inc., Calgary, Canada). Additionally, each isolate was evaluated for susceptibility to nalidixic acid and cephalothin by using 30-μg antibiotic disks for further species confirmation (BD Biosciences, New Jersey, USA) (22).

Genomic analysis. These genomes were part of the 100K Pathogen Genome Project using previously published methods (37–45).

DNA extraction, library preparation, and next-generation sequencing. High-molecular-weight genomic DNA (gDNA) was isolated from bacterial colonies grown on 5% SBA plates (UC Davis Vet Med Biological Services) at 37°C in microaerophilic conditions (as described above). DNA was extracted using whole-genome isolation kits (Qiagen, Valencia, CA, USA) with previously published modifications (46). The bacterial cells were processed using the protocols of the 100K Pathogen Genome Project (UC Davis, Weimer laboratory) as previously described (46–48). Briefly, bacteria were lysed with an enzyme preparation, vortexed, and processed according to the manufacturer's recommendations to obtain purified gDNA (Qiagen). Genomic DNA purity and integrity were assessed on the Agilent 2200 TapeStation with the genomic DNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA) as previously described (38, 39, 42). Genomic DNA ratios that were greater than 1.8 for $A_{260/280}$ and $A_{260/230}$ were used for library construction.

Isolated gDNA was sheared using the Covaris E220 with the 96 microTube plate (Covaris, Inc., Woburn, MA, USA) (37). The fragmented DNA size was determined with the Agilent Bioanalyzer 2100 high-sensitivity DNA kit (Agilent Technologies) to confirm the normal size distribution around a 300-bp peak. Libraries were constructed using the KAPA HTP library preparation kit (KK8234, KR0426 [v3.13]; Kapa Biosystems, Wilmington, MA, USA) with dual surface plasmon resonance imaging (SPRI) size selection (40). Libraries were constructed using the Agilent Bravo option B (Agilent Technologies). Libraries were indexed using Bioo Scientific NEXTflex-96 DNA barcodes v13.05 (Bioo Scientific Corp., Austin, Texas, USA) and Integrated DNA Technologies Weimer 384 TS-LT DNA barcodes. Library quantification was done using the KAPA library quantification kit (KK4824; Kapa Biosystems) to ensure the final library concentration prior to normalization and pooling for sequencing (40). Sequencing was performed with BGI@UCDavis (BGI@UCDavis, Sacramento, CA, USA) using the Illumina HiSeq 2000 platform with PE100

(Illumina Inc., San Diego, CA, USA) or the Illumina HiSeq 3000 platform with PE150 at the UC Davis Genome Center (Davis, CA, USA) (43, 45).

Sequence assembly, annotation, and whole-genome analyses. Assembly of paired-end reads was done with ABySS 1.5.2 using the following parameters: Kmer length = 64 (49). Annotations were done with the Prokka pipeline using the following parameters: –force –addgenes –compliant –genus *Campylobacter* –usegenus –rfam (50). Genomic distances were determined using the genome-to-genome distance calculator (GGDC), an *in silico* DNA-DNA hybridization (isDDH) technique, using the webserver at http://ggdc.dsmz.de/distcalc2.php as published previously (51, 52) and implemented locally as PanCake (53). The DNA-DNA hybridization (DDH) model "formula 2" was used as is recommended for draft genomes. Distance matrices were translated into the Newick tree format with Trex webserver software using the neighbor-joining method (54, 55), and distance matrixes were clustered and visualized using the R statistical programming language (56). Single-gene (16S rRNA genes and *cdtC*) analyses were performed by extracting the sequences from each genome and aligning them using MUSCLE through Geneious (v6.1.8) to align sequences and generate phylogenetic trees (57, 58). Trees were edited using Dendroscope 3.0 (59). SplitsTree4 was used to compute trees and splits using the equal angle method with the "use weights" and "run convex hull" parameters (60).

Genome alignments were done using Mauve under progressiveMauve (61, 62). Contigs were reordered using the "reorder contigs" option in Mauve under the default parameters using *C. jejuni* subsp. *jejuni* NCTC 11168 as the reference genome, and then reordered genomes were aligned to each other using progressiveMauve. This publication made use of the *Campylobacter* MLST database (http://pubmlst.org/campylobacter) for *in silico* MLST (29).

Genomic assessment of virulence factors and antibiotic resistance genes. Genomes were compared against a database (63, 64) consisting of virulence factor genes from six published *Campylobacter* genomes (*Campylobacter fetus* 82-40, *C. jejuni* 81-176, *C. jejuni* 81116, *C. jejuni* RM 11168, *C. jejuni* RM1221, and *C. jejuni* subsp. *doylei* 269.97) and plasmid *C. jejuni* 81-176 pVir. All published *Campylobacter* virulence factor genes were examined for each isolate to determine genotype variation; subsequently, five specific features associated with virulence and infection were selected for comparison and occurrence. These targets included (i) *cdtA*, *cdtB*, and *cdtC*, which code for cytolethal distending toxin (CDT), the main toxin in *Campylobacter* (65–67); (ii) putative type IV secretion system (T4SS) *virB* genes, associated with invasion (68); (iii) secreted invasion proteins CiaB and FlaC (69–71); and (iv) adherence genes *jlpA*, *porA*, *pebA*, and *cadF* (63, 72, 73). Virulence factor proteins were defined using the Pathogenic Bacterial Virulence Factor database (63, 64) using USEARCH (74) and by hand using previously published genes (75–78) in Geneious using Prokka annotations.

Antibiotic resistance genes were analyzed in every genome using the Resistance Gene Identifier software and the Comprehensive Antibiotic Resistance database (CARD) (79). Using these tools, *Campylobacter* genomes were assessed for the presence of known antibiotic resistance genes (75–78). Specifically, we examined each genome for five antibiotic resistance genes or operons that are relevant to *Campylobacter* antibiotic resistance: (i) the multidrug-resistant efflux complex CmeABC and its regulatory gene *cmeR* (76, 77); (ii) the MacAB efflux locus, which confers resistance to macrolides (79); (iii) the *tetO* locus, a ribosomal protection protein that confers resistance to tetracycline and its derivatives (75); (iv) the *oxa-184* and *oxa-61* genes, which are members of the class D β-lactamase family and confer resistance to β-lactam antibiotics (78); and (v) specific point mutations in the *gyrA* gene known to mediate resistance to fluoroquinolones in *Campylobacter*.

Accession number(s). All raw genome sequences generated in this study are available in the NCBI SRA as part of the 100K Pathogen Genome Project under BioProject accession number PRJNA186441. Accession numbers are listed in Table S1 in the supplemental material.

RESULTS

Assembly and annotation. Each of the 160 sequenced *Campylobacter* genomes were assembled and annotated using ABySS and Prokka with the same settings and conditions (49, 50). The genomes of all three species ranged from 1,491,293 to 2,006,566 bp (the smallest being *Campylobacter lari*), with an average of 1,772,774 bp assembled in an average of 55 contigs per genome. They contained an average of 1,799 coding DNA sequences (CDS), 40 tRNAs per genome, and one transfer-messenger RNA (tmRNA). For a full list of the genome structural details, see Table S1 in the supplemental material as well as the SRA accession numbers.

Each *Campylobacter* genome was examined for 16S rRNA genes sequence variation and MLST pattern, including the sequence type and clonal complex. However, the discriminative resolution in many cases was poor and inconclusive, particularly between some *Campylobacter coli* and *C. jejuni* 16S rRNA gene sequences (see Fig. S1 in the supplemental material). Although indiscriminant in some sequences, this analysis did confirm the observations of Weis et al. (22), who observed a "crow-only" host clade.

MLST was considered the gold standard for genotyping an isolate in the pregenomics era; however, this method relies on the perfect match of an isolate from within the already existing database (29). If the sequence is not contained in the database, no assignment can be made and other methods are needed to determine the relationship. In this study, 100 *C. jejuni* and *C. coli* genomes matched known MLST patterns. Unfortunately, 60 genomes had no match in the database of commonly used housekeeping genes (see Table S2 in the supplemental material). Consequently, further comparisons were done to determine specific similarities using the entire genome sequence and genome distances.

Whole-genome analysis. Genome distance calculations resolved individual species into distinct groups of *C. jejuni*, *C. coli*, and *C. lari* (Fig. 1; see also Fig. S2 in the supplemental material). A phylogenetic tree constructed from the genome distances placed 83% (72/87) of the *C. jejuni* isolates from crow origin into a separate clade from those isolates obtained from primates or humans, while 17% (15/87) of the isolates from crows clustered closely with human, primate, and sheep *C. jejuni* (Fig. 1). This finding demonstrated that multiple genotypes exist within each bacterial species, and in the case of *C. jejuni*, it also resolved host origin, which may be evidence for host species adaptation. These observations confirm that the genome sequence has sufficient analytical resolution to link host range with genotype as observed previously in environmental samples using *Vibrio* spp. (80).

To further examine that link between genotype and host source, we used genome distance measurements to define two major groups within *C. jejuni*: (i) a crow-only cluster and (ii) a distinct cluster that contained isolates from many host species that we defined to be "generalists," a concept similar to what other groups have previously defined for this organism (81, 82). The generalist group contains phylogenetically similar organisms obtained from seven different host species (Fig. 1). Several crow isolates were interspersed among the generalist *C. jejuni* clade. Notably, the genome distance of two crow isolates that clustered closely with human isolate *C. jejuni* ICDCCJ07001, an isolate known to cause GBS, suggests that crows may carry organisms that are re-

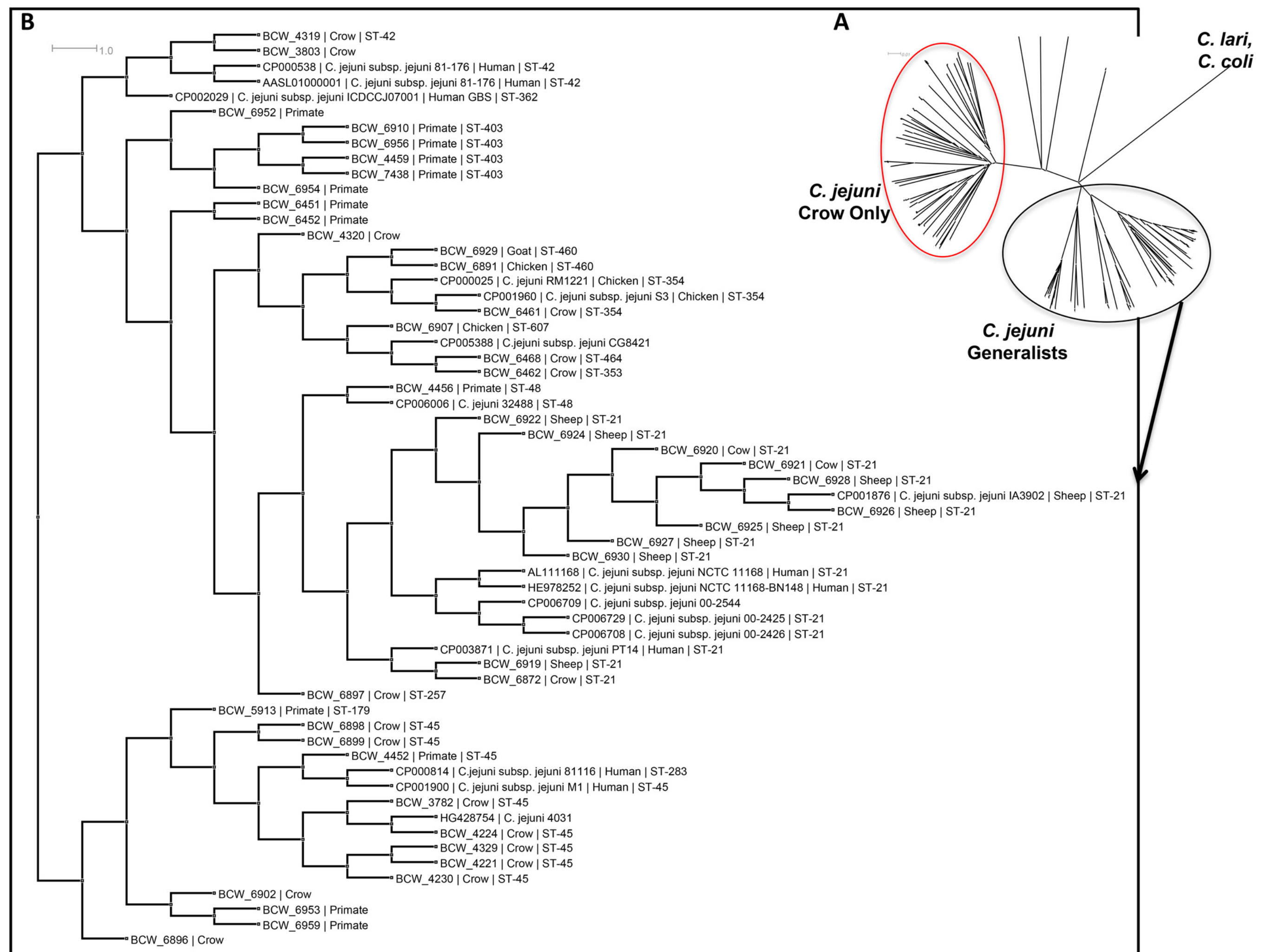

FIG 1 Genome distance calculations from draft genomes show two main groups within *C. jejuni*: a crow-only clade and a generalist clade (A). The generalist clade subset (B) shows *C. jejuni* isolates from a mixture of crow, primate, sheep, cow, goat, and human (GenBank) hosts. Tips are labeled with accession number (GenBank), strain name, source (when available), MLST (when available). Scale bar indicates genomic distance.

sponsible for this disease in humans. Similar relationships were observed for isolates that caused human gastroenteritis (i.e., *C. jejuni* M1) and isolates associated with abortion in livestock. Generalist genotypes that were linked closely to the three disease presentations were distinctly different from crow-only genotypes, warranting further examination of genomes to determine the specific genes and genotypes that were associated with the disease phenotype across the host range.

After careful investigation of the DDH analyses between the crow-only and generalist groups, it is possible that the isolates in the crow-only clade are potentially a novel third subspecies of *C. jejuni*. For instance, comparing two separate isolates from crows, BCW_3810 from the crow-only clade and BCW_6872 from the generalist clade, the DDH estimate is 74% (71% to 76.8%) where >70% is the same species. This indicates that the crow-only and the generalist isolates are both *C. jejuni*; however, the DDH estimate to belong to the same subspecies is 79%, leaving the crow-only isolate as a separate subspecies from the generalist *C. jejuni* subsp. *jejuni* isolate. Therefore, using this whole-genome analysis method, it is possible that these isolates are a separate subspecies.

Genome structure. Genome synteny was examined in the 160 genomes sequenced in this study, with an additional 24 genomes (184 total comparisons) from the NCBI SRA that spanned host sources and disease phenotypes. This analysis found that the collection of genomes did not contain any major structural differences (e.g., inversions or translocations). As expected, structural alignments between isolates that were placed closely together using the genome distance analysis also aligned the closest structurally (see Fig. S4 in the supplemental material). Between the crow-only and the generalist clades, the genomes were more diverse between clades than within each clade, indicating that the major differences between each clade were at the level of allelic variation.

Whole-genome antibiotic resistance genotypes. All *Campylobacter* genomes examined, regardless of clade, species, or host origin, contained the *cmeABC* efflux complex (data not shown), while 79.4% (127/160) contained the *cmeR* regulatory gene (Fig. 2) and all contained the *macAB* efflux loci (data not shown). The *tetO* locus, however, was found primarily in *C. jejuni* isolates from crows and ruminant abortion cases. In crow-derived *C. jejuni*, 23.7% (23/97) of genomes contain *tetO*, 65.2% (15/23) from the

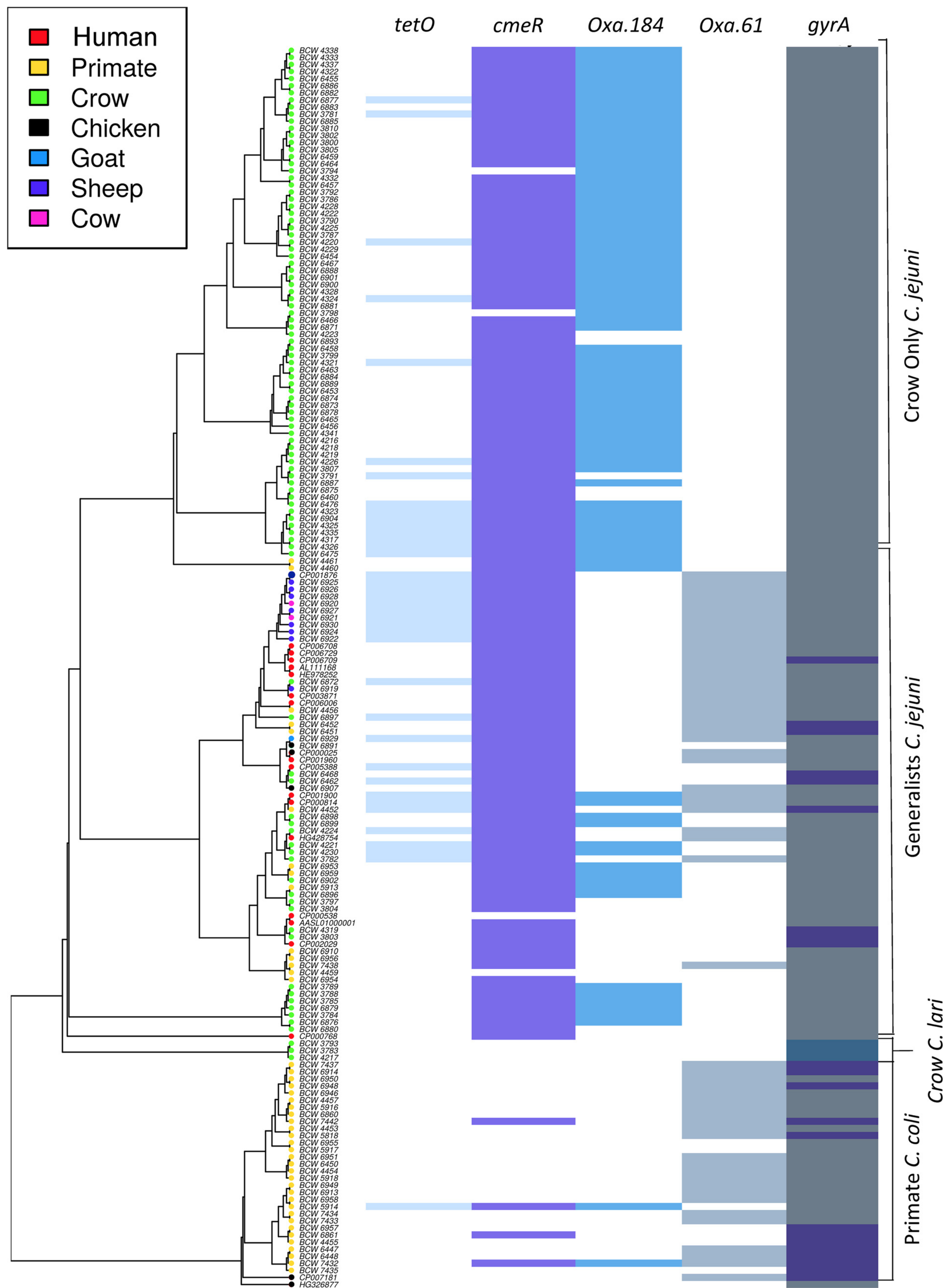

FIG 2 Genomic distance and antibiotic resistance genes. Dendrogram built from genomic distance calculations shows host in colored dots (key). The top arm of the dendrogram shows the *C. jejuni* isolates, and the bottom arm of the dendrogram shows the primate *C. coli* isolates. Tetracycline resistance gene *tetO* can be found predominantly in crow and sheep (abortive) isolates, and *oxa-184* is found more often in crow populations whereas *oxa-61* is found more often in the sheep and primate populations. Point mutations in *gyrA* are represented by purple, indicating Thr-Ile 86 mutation, and blue, indicating Thr-Val 86 (found only in *C. lari*). Gray indicates the presence of *gyrA* but no fluoroquinolone resistance-related SNP.

crow-only clade and 34.7% (8/23) from the generalist clade. Two *C. jejuni* isolates from primates in the generalist clade contained *tetO*. Almost all (91%, 10/11) abortion isolates contained *tetO* (Fig. 2). These results signify that *tetO* is widespread in abortion isolates (91% of the population was positive), is potentially linked to the disease phenotype, and may be useful in differentiating host specificity.

Out of the total genomes sequenced in this study, 84% of isolates contained class D β-lactamase genes. Of those, 55% (88/160) of the genomes contained the *oxa-184* gene and 29.4% (47/160) contained the *oxa-61* gene. Almost all *C. jejuni* isolates from crows contained *oxa-184* (except for 5 generalists), whereas the *C. jejuni* isolates in the generalist clade typically contained *oxa-61*. The *C. coli* isolates from primates contained the *oxa-61* loci (Fig. 2; see also Fig. S3 in the supplemental material). The two prominent β-lactamase genes (*oxa-61* and *oxa-184*) were differentially present within the genomes and were indicative of host source.

Genomes were further examined for the presence of specific point mutations in *gyrA* known to mediate resistance to fluoroquinolones. Of the *Campylobacter* isolates from nonhuman primates, 32% contained a mutation that would result in a Thr-Ile 86 mutation in the GyrA protein, whereas only 4% of *Campylobacter* isolates from crows contained this mutation. Importantly, all *Campylobacter* isolates from crows with the *gyrA* mutation were from the generalist clade; none were identified in the crow-only clade (Fig. 2; see also Fig. S3 in the supplemental material). No other known substitutions in *gyrA* were found in the *C. jejuni* genomes; however, all three *C. lari* isolates from crows contained a Thr-Val 86 mutation as well. These results demonstrated that while *Campylobacter* isolates from crows (i.e., wildlife) contain fewer antibiotic resistance genes than *Campylobacter* isolates from agriculture-associated sources and captive animals, the presence of fluoroquinolone resistance genes in a small number of *C. jejuni* isolates from crows (all generalists) may be indicative of zoonotic transmission.

Whole-genome virulence loci analysis. Virulence genes were specifically examined in conjunction with each *Campylobacter* isolate's host species (Table 1). Nearly all genomes (95%) contained one or more cytolethal distending toxin gene (*cdtA*, *cdtB*, and *cdtC*). All genomes contained genes associated with invasion (*ciaB* and *flaC*) and adherence genes (*jlpA*, *porA*, *pebA*, and *cadF*).

Genes involved in the type IV secretion system (T4SS) were identified much less frequently in these genomes than the other virulence genes that were studied. *virB4* was present in 31% of genomes, while the remaining *vir* genes were found in only 8% to 21% of the evaluated genomes. Most T4SS genes were found in tandem with other T4SS genes in one genome (Table 1). Nearly all genomes were potentially pathogenic because of the presence of known virulence loci in all clades, suggesting that all 160 genomes are potentially pathogenic in humans.

Cytolethal distending toxin. Since all loci were present, genomes were further investigated for specific point mutations within each virulence gene as a possible differentiation metric for virulence by host species. To evaluate the hypothesis that adherence molecules in *Campylobacter* may provide the critical difference between zoonotic and nonzoonotic species and can inform us of the origin and functional differences between the generalist and crow-only groupings, the membrane-binding protein CdtC that facilitates adhesion to the host cell and promotes cell entry of CdtB (65–67) was specifically identified and examined as a candidate for this hypothesis. Alignments of *cdtC* from each genome revealed single nucleotide polymorphism (SNP) variants that would lead to protein coding changes segregated by *Campylobacter* species. Within *C. jejuni*, *cdtC* allelic variation exactly recapitulated clades defined by whole-genome distance calculation into crow-only or generalist *C. jejuni* (Fig. 3). Differentiation of this gene into host-specific clades indicated that significant phenotypic differences can be explained by small changes in a single gene.

TABLE 1 Occurrence of common virulence factors in 160 *Campylobacter* isolates sequenced in this study

Toxin type/function and gene	Frequency (%)
CDT toxin	
cdtA	96
cdtB	95
cdtC	97
Invasion	
ciaB	100
flaC	100
Adherence	
cadF	100
jlpA	100
porA	100
pebA	100
Type IV secretion system	
virB1	12
virB2	17
virB3	13
virB4	31
virB6	21
virB8	21
virB9	8
virB10	20
virB11	12

Analysis of CdtC protein alignment found specific amino acid changes separating the protein sequences into four main groups, i.e., *C. coli*, *C. lari*, and two groups with *C. jejuni* (generalist and crow only) (Fig. 3; see also Fig. S5 in the supplemental material). The CdtC alignment, when assessed for SNPs, revealed that the crow-only CdtC alleles were nearly identical. The generalist clade contained seven alleles of *cdtC* that coded for amino acid changes (Fig. 3A). All *C. coli* and *C. lari cdtC* alleles were identical within each bacterial species and were more similar to each other than to *C. jejuni*. This finding indicates that within *C. jejuni*, *cdtC* may be under evolutionary pressure in relation to host species colonization and transmission. This suggests that the allelic variation of *cdtC* may be a fundamental factor in disease after transmission and may impact zoonotic and disease phenotypes. We further hypothesized that a combination of adherence specialization and antibiotic selection may provide clues to zoonotic potential and disease potential.

Abortion isolates. To test this hypothesis, we examined genomes from isolates that caused abortion in multiple livestock species. Previous studies demonstrate that abortive *C. jejuni* isolates are associated with the occurrence of *tetO* (83). We observed that 91% (10/11 isolates) of abortion cases contained *tetO* in the

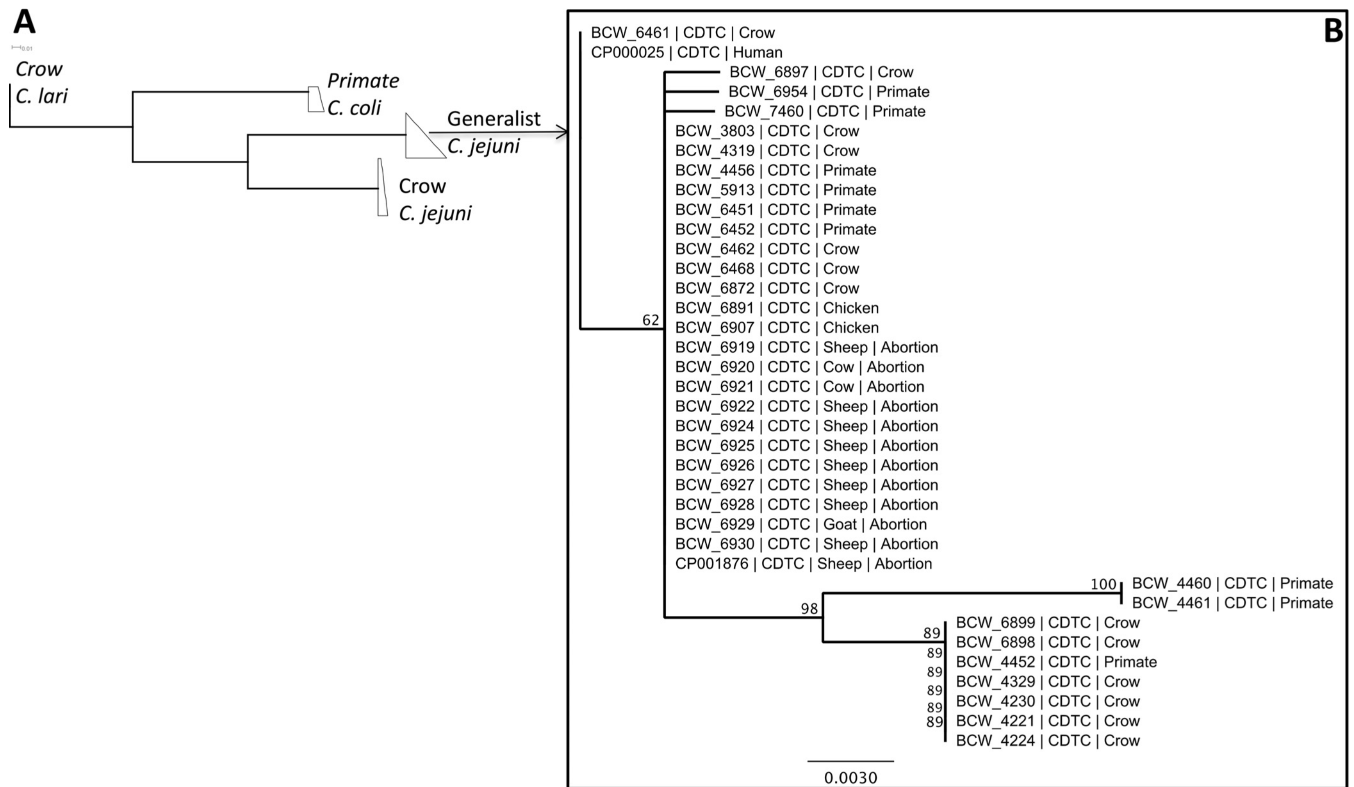

FIG 3 Alignment of CdtC sequences replicates the whole-genome phylogenetic tree and correctly splits species and clades. The *cdtC* nucleotide sequence was extracted from every genome, aligned, and built into a phylogenetic tree (A). Replicating the full genome tree, *cdtC* drives the separation between *C. lari*, *C. coli*, and between the *C. jejuni* generalists and the *C. jejuni* crow-only host grouping. Within the generalists, CdtC sequences from the abortion isolates are identical to those from select chickens, crows, and primates (B). Numbers represent bootstrapping, and the scale bar is at the bottom.

genome of the *C. jejuni* isolate. Only one abortion-associated *C. jejuni* isolate (BCW_6919) did not contain *tetO* (Fig. 2). When CdtC was assessed using the abortion isolates, the resultant phylogenetic tree was very similar to the genomic distance tree, and the CdtC variant was identical in all abortion isolates and genomic near-neighbors (Fig. 3B). Genomic distance calculations of the abortion isolates reveal several instances where *C. jejuni* isolates from crows, chickens, humans, and primates cluster with those that caused abortion (Fig. 4). Taken together, these data indicate that CdtC and TetO may play a more important role in disease than previously thought. This may have broad implications for pathogenicity, zoonotic transmission, and disease phenotype as highlighted by the findings for livestock abortion.

DISCUSSION

This study describes the first large-scale (184 isolates) genomic comparison of *Campylobacter* isolates collected from wild birds, livestock, nonhuman primates, and humans. Whole-genome sequencing evaluation identified distinct clades within *Campylobacter* spp., particularly *C. jejuni*, and provided a much more robust vehicle for isolate comparison than 16S rRNA genes or MLST approaches, as additional molecular resolution was needed to uncover new discoveries for allelic variation and disease. While evidence of major structural alterations in genomes was not detected, gene-level differences were identified that may contribute to host adaptation and pathogenic potential. Since this tool clearly defined bacterial species, we hypothesized that this approach may be predictive of host source, especially when coupled with the metadata and genomic markers that link transmission capability, such as those in the generalist clade.

Nearly all (95%) of the isolates from crows, primates, and livestock contained loci associated with invasion and virulence. Although the exact mechanistic details of how *C. jejuni* invades human host cells and causes disease are still unclear, several virulence pathways have been implicated in *C. jejuni* pathogenesis. CDT is a holotoxin composed of three proteins, CdtA, CdtB, and CdtC, and has been shown to disrupt the host cell cycle, leading to cell arrest and even host cell death (65–67, 84). The flagellar apparatus of *C. jejuni* consists of many proteins, including filament proteins FlaA and FlaB, and has been shown to act in motility of the bacteria and as a putative secretion system, secreting Cia (*Campylobacter* invasion antigen) proteins and FlaC into the host epithelial cells (69–71, 84, 85). Type IV secretion system proteins typically encoded on plasmids (pVir) have been shown previously to contribute to virulence although their function is still unknown (68). The *C. jejuni* isolates in this study contained many of these virulence loci and can be characterized as potential pathogens by their genomes. Whether those virulence genes are expressed still needs to be determined. Phylogenetic analysis based on one protein sequence in particular (*cdtC*) recapitulated the full genome phylogenetic tree, which may indicate a role in each isolate's host predilection and zoonotic potential.

Our results show that several genomes from crows (17%; 15/87) displayed high similarity to sequences of isolates implicated in

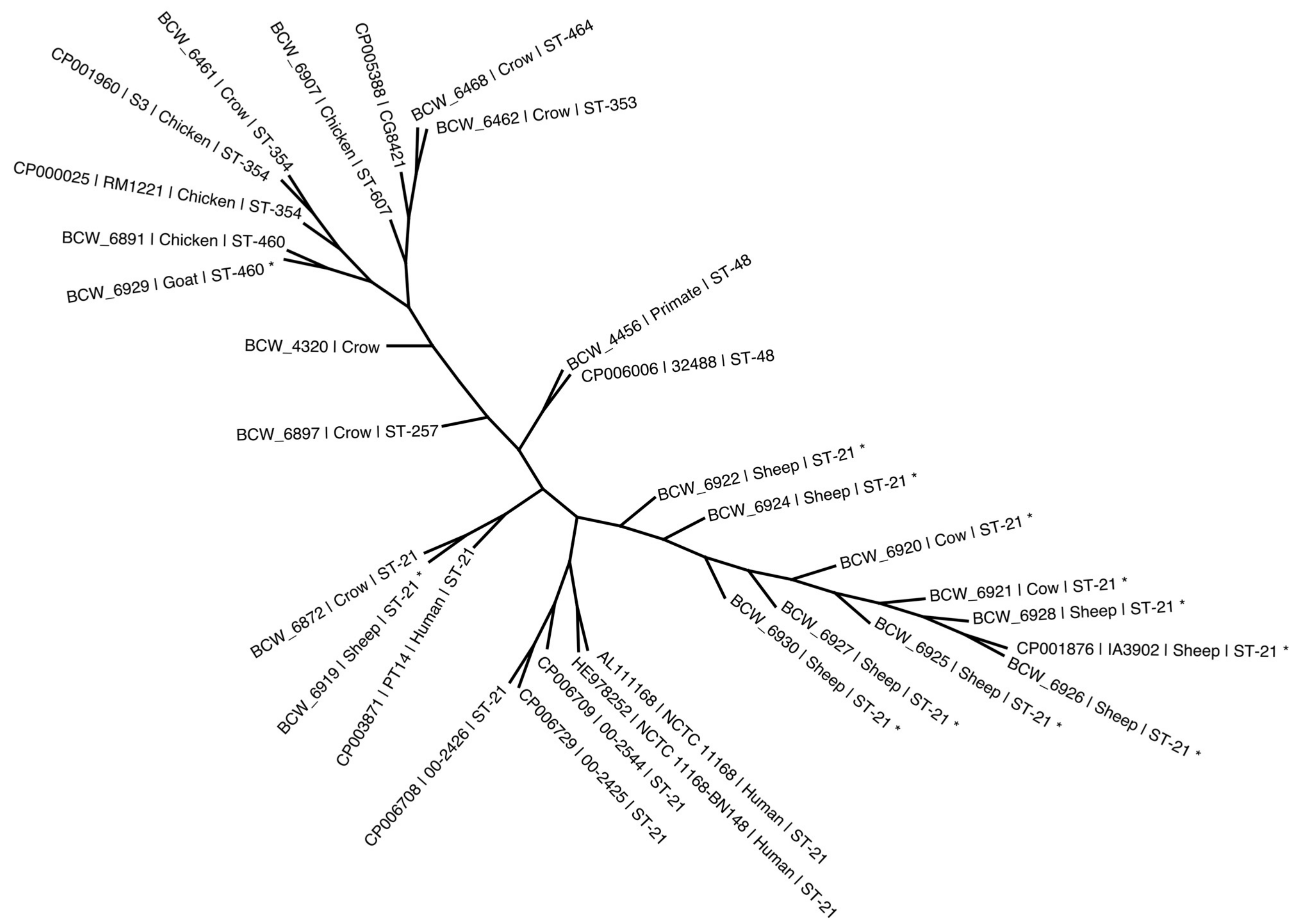

FIG 4 Genomic distance dendrogram of *C. jejuni* isolates implicated in causing abortion compared with GenBank *C. jejuni* from humans and with crow and primate isolates from this study. This dendrogram reveals greater than assumed genomic distance between those isolates with the same MLST and those thought to be clonal and shows two novel isolates from sheep and goats that associate more closely with a crow isolate and a chicken isolate from the Sacramento Valley. Tips are labeled as strain identifier, isolate source, and MLST (when available). Abortive *C. jejuni* isolates are indicated by stars.

human disease, suggesting that these isolates are potential pathogens of public health importance and zoonotic transfer. The *C. jejuni* isolates from crows fell into two major clades: 83% (72/87) made up a crow-specific clade of *C. jejuni*, whereas 17% (15/87) was associated with other hosts and made up part of the generalist clade.

The *Campylobacter* isolates collected from macaques that were evaluated in this study were split between *C. coli* and *C. jejuni*. The *C. coli* isolates from primates all clustered together by genomic distance calculations and were very similar to one another (all 30 *C. coli* isolates were in the same *C. coli* clade). Most (81%; 13/16) of the primate *C. jejuni* isolates and all of the livestock isolates were members of the generalist clade. *Campylobacter* is widely accepted to be a genomically diverse group. However, we observed that the crow-only clade was very genomically similar, whereas the generalist clade contained four major clades that contain 60 different genotypes that are associated with multiple host sources. It is likely that these are the genomes that represent high diversity and likely high transmissibility.

Zhao et al. (86) recently found that the *Campylobacter* genotype, using WGS, is predictive of antibiotic resistance phenotypes using 114 isolates of *Campylobacter* (*C. jejuni* and *C. coli*) and 18 antibiotic resistance genes. They found between 95% and 100% correlation to phenotype. The findings of Zhao et al. provide additional evidence to verify that the antibiotic resistance genes and SNPs observed in this study may be predictive of phenotype from crow isolates. With this hypothesis, we examined all of the isolates from crows to find that they had either genes or SNPs associated with antibiotic resistance genes, and 85% of isolates contained class D β-lactamase genes. Specifically, 80% contained *oxa-184* whereas 5% contained *oxa-61*. All five of the *C. jejuni* isolates from crows that contained *oxa-61* were part of the generalist clade, whereas the crow-only clade only contained *oxa-184*. Over 23% of *C. jejuni* isolates from crows contained *tetO*, 4.5% of *C. jejuni* isolates from crows contained a *gyrA* SNP, and one isolate had both *tetO* and the *gyrA* SNP. From the generalist clade of *C. jejuni* isolates from crows, 53.3% (8/15) were positive for *tetO* and all four crow *C. jejuni* isolates with the *gyrA* SNP were from the generalist clade (26.6%; 4/15). Humans infected with *C. jejuni* are frequently treated with fluoroquinolones (e.g., ciprofloxacin) (87, 88). Because of this, food animals (post 2005) are no longer treated with fluoroquinolones in the United States (89). While the

point mutations conferring resistance to fluoroquinolones were absent in chicken and livestock isolates, mutations were frequently identified in primate isolates (36.7% [11/30] of *C. coli* isolates and 25% [4/16] of *C. jejuni* isolates). Similarities between the generalists (32% of *C. jejuni* isolates) from crow, sympatric macaque, and sheep isolates suggest that transmission between avian, primate, and ungulate hosts can occur. The prevalence of antibiotic resistance genes in isolates from a wide host range may pose a public health risk from this agent.

These results add to recent studies exploring the importance of wild birds in disseminating *Campylobacter* throughout a large geographic area (13–15). Here, we have developed a method using genomics, antibiotic resistance markers, and specific virulence factors to detect isolates of pathogenic and zoonotic potential. Isolates in the generalist category contain clinically important antibiotic resistance genes and, through potential dissemination of *Campylobacter* isolates via crows, represent a risk to human and animal health and should be regarded as a public health threat. Further, we show that using the whole genome is more accurate and informative for phylogenetics, virulence, and antibiotic resistance assessment than all other previous forms of analysis.

In conclusion, this study demonstrated the power of population-scale WGS with *Campylobacter* isolates from wild bird populations, captive nonhuman primates, isolates from human infections, and livestock abortion isolates. This approach provided a robust and powerful method to determine genome-scale pathogenicity and the potential for zoonotic exchange in ecological settings to link disease and host range, where previously used molecular tools lacked the necessary resolution. Here, we showed that whole-genome distance calculations classified each species accurately. Further, *C. jejuni* isolates from crows segregated into two main clades, that of a crow-only clade not associated with disease and a generalist clade, which contained genomes from multiple hosts many of which were associated with disease (gastroenteritis, GBS, or abortion). Antibiotic resistance genes such as β-lactamases also segregated along the genome distance clades. This was also observed for *cdtC*, again, recapitulating the genome distance groupings. Remarkably, the isolates from livestock that caused abortion all contained the identical *cdtC* allele, implicating that this specific *cdtC* allele was unique to this livestock disease. Interestingly, *tetO* was found with all but one of the abortion cases, suggesting that *cdtC* and *tetO* may be coevolving to produce a generalist genome that is zoonotic and abortion linked. Use of WGS and population genomics provided a high-resolution view of *Campylobacter* genomes that was linked to host range, diseases, and possible zoonotic transmission genotypes.

ACKNOWLEDGMENTS

We would like to acknowledge Karen Pacheco and JoAnn Yee at the VMTH for their help with isolation and identification of crow *Campylobacter* isolates and Abigail Spinner at the CNPRC for primate isolates. We thank the 100K Pathogen Genome Project team. We also thank Walter Boyce and Woutrina Smith for collaborations and discussions.

Studies were funded in part by the University of California, Davis to Andrea K. Townsend, by the Agriculture and Food Research Initiative under competitive grant no. 2014-67012-21622 from the USDA National Institute of Food and Agriculture to Conor C. Taff, and by the 100K Pathogen Genome Project under FDA grant no. 5U01FD003572-04.

FUNDING INFORMATION

This work, including the efforts of Andrea K. Townsend, was funded by UDSA, Agriculture and Food Research Initiative Competitive Grant (2014-67012-21622). This work, including the efforts of Bart C. Weimer, was funded by FDA/CFSAN (5U01FD003572-04).

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