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Genetic and phenotypic characteristics of *Clostridium* (*Clostridioides*) *difficile* from canine, bovine, and pediatric populations

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Abstract

Objectives: Carriage of *Clostridioides difficile* by different species of animals has led to speculation that animals could represent a reservoir of this pathogen for human infections. The objective of this study was to compare *C. difficile* isolates from humans, dogs, and cattle from a restricted geographic area.

Methods: *C. difficile* isolates from 36 dogs and 15 dairy calves underwent whole genome sequencing, and phenotypic assays assessing growth and virulence were performed. Genomes of

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CRediT authorship contribution statement

L.E. Redding: Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **V. Tu:** Methodology, Investigation, Data curation, Formal analysis, Software, Writing – original draft, Visualization. **A. Abbas:** Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **M. Alvarez:** Methodology, Investigation, Data curation, Formal analysis. **J.P. Zackular:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Supervision. **C. Gu:** Methodology, Data curation, Formal analysis, Software, Visualization. **F.D. Bushman:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing. **D.J. Kelly:** Methodology, Investigation. **D. Barnhart:** Methodology, Investigation. **J.J. Lee:** Methodology, Investigation, Data curation, Formal analysis, Software, Writing – original draft, Visualization. **K.L. Bittinger:** Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing, Visualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2022.102539.

animal-derived isolates were compared to 29 genomes of isolates from a pediatric population as well as 44 reference genomes.

Results: Growth rates and relative cytotoxicity of isolates were significantly higher and lower, respectively, in bovine-derived isolates compared to pediatric- and canine-derived isolates. Analysis of core genes showed clustering by host species, though in a few cases, human strains co-clustered with canine or bovine strains, suggesting possible interspecies transmission. Geographic differences (e.g., farm, litter) were small compared to differences between species. In an analysis of accessory genes, the total number of genes in each genome varied between host species, with 6.7% of functional orthologs differentially present/absent between host species and bovine-derived strains having the lowest number of genes. Canine-derived isolates were most likely to be non-toxigenic and more likely to carry phages. A targeted study of episomes identified in local pediatric strains showed sharing of a methicillin-resistance plasmid with dogs, and historic sharing of a wide range of episomes across hosts. Bovine-derived isolates harbored the widest variety of antibiotic-resistance genes, followed by canine

Conclusions: While *C. difficile* isolates mostly clustered by host species, occasional co-clustering of canine and pediatric-derived isolates suggests the possibility of interspecies transmission. The presence of a pool of resistance genes in animal-derived isolates with the potential to appear in humans given sufficient pressure from antibiotic use warrants concern.

Keywords

Clostridioides difficile; Zoonosis; Phylogenetics; Antimicrobial resistance; Animal reservoir

1. Introduction

Clostridioides difficile is an anaerobic Gram-positive, toxin-producing pathogen that is the leading cause of antibiotic-associated and nosocomial diarrhea in humans and a significant enteric pathogen in many species of animals [1–3]. The genome of *C. difficile* is large and genetically diverse, with low levels of conservation among strains and high levels of plasticity, which is typical of bacteria that can survive in a diverse range of human, animal, and abiotic environments [4].

Increasing numbers of community-acquired *C. difficile* infections [5] among people with no prior exposure to antibiotics or healthcare facilities [6,7] and the recognition that companion animals and livestock can be asymptomatic carriers of *C. difficile* [2,8] have led to speculation that *C. difficile* may be zoonotic. Highly similar or even identical strains of *C. difficile* have been found in people and animals in close proximity with each other: in one study, a dog was found to harbor *C. difficile* with an identical pulse field gel electrophoresis profile as its owner [9], while in other studies, pigs and pig farmers carried isolates with few or no differences between isolates [10,11]. Analyses of large-scale global collection of *C. difficile* isolates from animals and people revealed co-clustering of human- and animal-derived strains [12,13]. However, directionality of transmission has yet to be established, and a common environmental source cannot be ruled out.

If *C. difficile* strains are largely host-adapted with infrequent spillover to humans, it may be that the animal reservoir of *C. difficile* contributes only a small amount to human infection and that human-to-human and nosocomial transmission represents the dominant mode of acquisition of *C. difficile*. However, given that healthcare associated epidemics of *C. difficile* tend to be dominated by single strains of *C. difficile* [14–16], even infrequent spillover events from animal reservoirs could seed outbreaks. Better knowledge of the strains that colonize animal populations and have potential to cause disease in people is needed to understand the potential likelihood of such events. Here, we characterize *C. difficile* isolates from three different host species – humans, dogs, and cattle – from a relatively restricted geographic area and compare their characteristics and relatedness using whole genome sequencing and phenotypic assays assessing growth characteristics and virulence.

2. Methods

2.1. C. difficile isolates

C. difficile strains were isolated from fecal samples from puppies and dairy calves. Canine fecal samples were obtained from pet owners bringing their healthy puppies to the pediatric service of the University of Pennsylvania veterinary hospital or from breeders who collected fecal samples from their puppies. Bovine fecal samples were manually collected from up to five randomly selected healthy dairy calves less than two weeks of age from 23 dairy farms in Pennsylvania, Maryland, and Delaware. Human-derived isolates were from pediatric patients with inflammatory bowel disease and oncology patients from the Children's Hospital of Philadelphia [17]. Collection of samples was approved by the Institutional Animal Care and Use Committee and by the Institutional Review Board of the University of Pennsylvania.

2.2. C. difficile detection and isolation

Anaerobic culture was performed as described previously [18]. Briefly, a 0.5 g pellet of formed fecal sample was mixed with 0.5 ml of 100% ethanol. The mixture remained for 60 min at room temperature before being inoculated on BBL CDSA/Clostridioides difficile selective agar (BD; Sparks, Maryland, USA) and Columbia CNA agar (Remel; Lenexa, KS, USA). Inoculated plates were incubated in BD Gas-Pak™ EZ container systems with BD BBL™ CO2 generators and BD BBL™ Gas Pak™ anaerobic CO2 indicators (Franklin Lakes, NJ) at 36 °C ± 2 °C under anaerobic growth conditions for seven days and checked for growth every other day. Colonies suspected as being *C. difficile* were identified isolated and confirmed by Maldi-TOF MS identification and/or RapID ANA II System (ThermoFisher Scientific, USA).

2.3. Growth rate and cytotoxicity assays and analysis

C. difficile animal isolates were grown in an anaerobic chamber (85% nitrogen, 10% hydrogen, 5% carbon dioxide gas mixture) (Coy Laboratory Products; Grass Lake, MI, USA) at 37 °C in biological triplicates in 200 μL Brain Heart Infusion liquid media (Fisher Scientific; Waltham, MA, USA) with 1% yeast extract (Fisher Scientific; Waltham, MA, USA) and 0.03% L-Cysteine HCl (Alfa Aesar; Haverhill, VA, USA) (BHIS) and incubated overnight (18–24 h) at 37 °C under anaerobic conditions. Cultures were normalized by

optical density (OD) and sub-cultured (1:100) into 200 μL of fresh BHIS in a 96-well plate sealed with a gas-permeable membrane (Breathe-Easy®) (Millipore-Sigma; Burlington, MA, USA) and incubated at 37 °C with continuous double orbital shaking for an additional 24 h. OD600 was measured every 30 min in a BioTek Epoch 2 (BioTek; Winooski, VT, USA). Toxin production in each strain was assessed using a cytotoxicity assay as previously described [19]. Briefly, *C. difficile* cultures were removed from the anaerobic chamber and passed through a 0.2 μm PVDF membrane (Corning® FiltrEX™) (Corning Incorporated; Corning, NY, USA). The cell-free supernatant was then serially diluted 1:10 in phosphate buffered saline. Next, 10 μL of each dilution was added to a well in a 96-well plate containing 10,000 Vero cells (ATCC CCL-81) in 90 μL of DMEM with L-Glutamine, 4.5 g/L Glucose and Sodium Pyruvate (Corning Incorporated; Corning, NY, USA) + 1% penicillin and streptomycin (Gibco™) (Fisher Scientific; Waltham, MA, USA). Vero cells were incubated with bacterial supernatant for 18–24 h. Cytotoxicity was determined by morphological changes (cell-rounding) observed under a light microscope. The normalized cytotoxicity titer was recorded as the dilution where 100% of cells showed cell-rounding divided by the OD600 of the bacterial culture. These methods have been used by authors of this study [19] as well as other investigators [20,21].

Doubling time during exponential phase of each bacterial isolate grown in BHIS liquid media under anaerobic conditions was calculated using the GrowthCurver (version 0.3.1) [22] package in R Studio (R version 4.0.2). Differences in growth rate by host-species were tested by two-sided Student's t-test implemented in R.

2.4. Whole genome sequencing and analysis

DNA was extracted from *C. difficile* cultures using the Nextera DNA Flex Microbial Colony Extraction Demonstrated Protocol (Illumina) and quantified with the Quant-iT PicoGreen® dsDNA assay kit (ThermoFisher). The whole-genome sequencing library was generated from 1.5 ng DNA using the Illumina DNA Prep kit and IDT for Illumina unique dual indexes at 1:4 scale reaction volumes. DNA concentration in the libraries was assessed by Quant-iT™ PicoGreen™ dsDNA assay. Samples with library yields of less than 1 ng/ul were re-prepared and re-pooled. After pooling, the final library was run on the Agilent BioAnalyzer to check the size distribution and absence of additional adaptor fragments. Sequencing was performed on an Illumina HiSeq 2500 instrument using a high output v4 flow cell, producing 2 × 125 bp paired end reads. Extraction blanks and nucleic acid-free water were processed along with the isolates to empirically assess environmental and reagent contamination. A laboratory-generated mock community, consisting of DNA from *Vibrio campbellii* and lambda phage, was also included as a positive sequencing control.

Sequencing reads were filtered to remove low quality reads using Cutadapt [23] and Trimmomatic [24] and sequences with low complexity using Komplexity [25]. *C. difficile* genomes were assembled using MEGAHIT (v1.2.9) and deposited in GenBank (BioSample Accession numbers provided in Supplementary Table 1). Genome quality was assessed with CheckM (v1.1.3) [26]. *C. difficile* reference genomes were downloaded from NCBI RefSeq (N = 43) and were selected to provide a set of high-quality complete genomes covering all phylogenetic clades. Additional reference genomes were obtained from a

previous study of pediatric patients with inflammatory bowel disease and pediatric oncology patients at the Children's Hospital of Philadelphia (N = 29) [17]. Prokka (v1.14.6) [27] was used for gene annotation of each assembly. Sequence typing was performed for each isolate using MLST v.2.19.0 by analyzing housekeeping genes from assembled contigs and comparing the sequence variation against characterized *C. difficile* sequence types in the PubMLST database [28]Single-nucleotide variants (SNVs) were calculated by performing a pairwise alignment of single copy core genes using the Biostrings library (v2.54.0) in the R language for statistical computing. The presence of toxin genes, antibiotic resistance genes, and phage DNA was identified using Blast+ (v2.8.1) to isolate sequences to large and binary toxin genes of the CD196 Clostridium difficile strain, the Comprehensive Antibiotic Resistance Database [29] and the NCBI Virus database, respectively. For identification of phage alignments, blastn alignments that had a percent identity of >95% and an alignment length >20% were accepted. For the analysis of transposons, sequences described by Knight et al. [30] were downloaded from GenBank, except for Tn6190 which had no provided sequence reference from the paper. We replaced Tn6190 with the transposon it was found homologous to, Tn916. For the comparison of canine and bovine samples to geographically linked pediatric episomes [17], alignments were required to have 90% identity over at least 1000 bp.

3. Results

3.1. C. difficile isolates from bovine, canine, and pediatric populations

We obtained a set of bovine and canine isolates from the same geographic area collected between 2017 and 2019. We collected 15 *C. difficile* isolates from dairy calves from six dairy farms (Fig. 1A), one isolate from an adult dog, 35 isolates from healthy pre-weaned puppies from 10 litters, and one isolate from a healthy weaned puppy (Fig. 1B).

For comparison in humans, we used previously sequenced genomes from *C. difficile* isolates from a recent study of pediatric inflammatory bowel disease and oncology patients from the Children's Hospital of Philadelphia [17] collected between 2015 and 2018. The data from this study provided 29 genomes from humans in the same geographic region. The genomes from pediatric subjects were assembled from a combination of long-read and short-read sequences, some of which yielded circular chromosomes and allowed the identification of plasmids as circular DNAs independent of the host chromosome. Identifiers and characteristics (e.g., clade membership, sequence type, presence of toxin genes, phages) for all study and reference genomes are compiled in Supplemental Table 1.

3.2. Phenotypic characteristics of C. difficile isolates

Growth rates of *C. difficile* isolates were highest for bovine-derived isolates (mean (SD) doubling time 0.63 h ±0.11) and lowest in the pediatric-derived isolates (0.49 h ±0.15). There was a significant difference in doubling times between the human- and bovine-derived isolates ($p = 0.018$, Tukey test) (Fig. 2A). Mean doubling times in canine isolates (0.55 h ±0.16) were not statistically different from human-derived ($p = 0.23$) or bovine-derived isolates ($p = 0.26$). Overall, 36% (4/15) of bovine isolates, 43% (16/37) of canine isolates, and 10% (3/28) of pediatric isolates did not produce any detectable toxin during *in* vitro

conditions. Among toxin-producing strains of *C. difficile*, the normalized cytotoxicity titers were significantly lower ($p < 0.001$, Student's t-test) among the bovine isolates (median value 201) than among the canine isolates (median value 15267) and the pediatric isolates (median value 1800) (Fig. 2B). Growth rates were significantly different ($P = 0.003$) between toxigenic (mean doubling time 0.62 h) and non-toxigenic strains (mean doubling time 0.52 h).

3.3. Characteristics of the C. difficile core genome

Genome quality metrics and general features for the genomes are presented in Table 1. Assembly of the bovine and canine isolate genomes resulted in a range of contig numbers (382–1333), and genomes ranged in size from 3.84 to 4.24 Mbp, with a median of 3993 genes, 1760 (44.1%) of which were considered core genes (i.e., genes present at least once in all genomes analyzed). The high numbers of contigs for canine and bovine isolates is likely due to the fact we used short reads for de novo assembly of the genomes, which could lead to the assembly of DNA regions with low complexity and increased fragmentation. Thus, these numbers are not directly comparable for the human- and animal-derived isolates.

We assessed the phylogenetic relationships among *C. difficile* isolates based on the core genes and organized the genomes into clades and sequence types based on the placement of reference genomes from RefSeq (Fig. 3). Clade membership was highly dependent on host species ($P = 2 \times 10^{-14}$, Fisher's exact test), with all genomes in this study appearing in clades 1, 2, or 5. All but two pediatric isolates were placed in Clade 1, which has previously been associated with *C. difficile* infection in hospital settings [31]. All canine isolates were placed in Clade 1. In contrast, all bovine isolates but one were placed in Clade 5, which is reported to be associated with livestock [32]. Variation in sequence type was much lower for the animal isolates than for the pediatric isolates (Fig. 3): bovine isolates consisted only of ST 3 (n = 1) and ST 11 (n = 14), while canine strains consisted of ST 2 (n = 18), ST 3 (n = 13), ST 13 (n = 3) and ST 15 (n = 3). In contrast, 16 different sequence types were represented among the 28 pediatric isolates. Only three of the sequence types found in animal isolates were also found in the pediatric isolates: ST 2 (n = 2), ST 3 (n = 3) and ST 11 (n = 1).

Differences in number of single nucleotide variants (SNVs) in the core genes between isolates are shown in Fig. 4. Fewer differences in SNVs were observed within host species than between host species (Table 2, $P = 97.8*10^{-169}$). Bovine isolates differed by the lowest number of SNVs overall, relative to canine and pediatric isolates ($P = 4.9*10^{-14}$). The number of SNVs between canine isolates varied, with 32 of 37 isolates grouped into two distinct clusters. Clustering sometimes occurred within farm and within litters, but the median difference in SNVs was not always lower within farms and within litters than between farms and litters (Supplemental Figs. 1–2).

3.4. Characteristics of the C. difficile accessory genome

The total number of genes in each genome varied between host species ($P = 2.2*10^{-7}$, Fig. 5A). Bovine *C. difficile* genomes contained 205 fewer genes on average relative to genomes from other sources ($P = 0.001$ for canine, $P = 1.9*10^{-7}$ for pediatric). Genomes

from canine and pediatric sources also differed in the number of genes present (P = 0.029), but this number did not differ from the RefSeq reference genomes (P = 0.29). The total number of genes between human isolates from Clade 5 and the bovine isolates were not significantly different (P = 0.69, Supplemental Fig. 3), while both groups had a significantly lower number of genes compared to all other isolates (P = 0.001 for human clade 5, P = $1*10^{-7}$ for bovine).

To gain further insight on shared vs. unique genes between isolates, we carried out hierarchical clustering based on accessory gene presence or absence (Fig. 5B). Canine and bovine samples clustered strongly by host and separately from pediatric and reference genomes. Pediatric isolates were overall less similar in the composition of their accessory genomes relative to bovine and canine isolates (P = $2.6*10^{-8}$) and did not form a distinct cluster in the dendrogram.

We next examined the distribution of putative gene functions across host species (Fig. 5C). After alignment to the KEGG database, a total of 1606 KO (functional orthologs) were detected, 107 (6.7%) of which were differentially present/absent between host species. Genes unique to the bovine strains were assigned most often to ABC transporter or two-component system pathways. Canine, pediatric, and reference genomes each had more than 40 genes that were absent from bovine genomes. Flagellar assembly, phosphonate and phosphinate metabolism, and vancomycin resistance were other prominent pathways for genes that were missing from bovine isolates but present in other hosts. Comparison of the human Clade 5 isolates to the bovine isolates revealed no significant difference in Kos (Supplemental Fig. 4), while both groups showed 62 Kos that were significantly present/absent compared to all other isolates.

Lastly, we focused on factors affecting virulence and pathogenicity in the *C. difficile* genomes from our study. Genes in the pathogenicity locus (PaLoc), production site for *C. difficile* toxins A and B, were present in 27 of 29 genomes from pediatric sources (93%) but only in 21 of 37 genomes from canine sources (57%), while the bovine sources presented genes from PaLoc in 11 of 15 isolates (73%, Fig. 6A). Genes involved with production of binary toxin *C. difficile* transferase (CDT), which is associated with more virulent strains of *C. difficile* [33], followed a similar pattern (Fig. 6B). Full-length CDT genes were present in almost all of the bovine isolates (93%), while 21 of 37 canine isolates (57%) only had truncated CDT genes. Out of 29 pediatric isolates, 2 had full-length CDT genes and 24 had truncated CDT genes. In all, presence of toxin-associated genes was lower in isolates from canine sources relative to pediatric (P = 0.004) and bovine (P = 0.005) isolates.

To identify molecular determinants of antimicrobial resistance in our *C. difficile* isolates, we checked for genes associated with antibiotic resistance using the CARD database (Fig. 6C). Genes that conferred resistance to multiple antibiotics were present in all isolates and consisted of either efflux pumps or genes altering the antibiotic target site, including *mel*, *ermB*, and *cdeA* genes. Isolates from bovine sources were more likely to contain genes associated with resistance to aminoglycosides (P = $5.8*10^{-7}$) and macrolides (P = $1.9*10^{-10}$) and tetracyclines, relative to canine, pediatric, and reference genomes, including the *ANT(6)-Ia*, *ANT(6)-Ib*, *aad*, *mphN*, *tetM*, *tetS,* and *tet44* genes. A greater fraction of

bovine and canine isolates contained genes conferring resistance to tetracyclines relative to those from pediatric sourcesI, including *tetM* and *tetS* genes, though this effect was not statistically significant (P = 0.15). We found only one example of a pediatric isolate containing genes conferring resistance to specific antibiotics (aminoglycosides).

Because *C. difficile* pathogenesis and antibiotic evasion can be modulated by mobile DNA elements [34–36], we sought to understand gene flow among our strains. The animal-derived genomes were first surveyed for phage sequences by alignment to *C. difficile* phage genomes from the NCBI virus database. Segments of a total of eighteen phage genomes were detected (at least 20% of the target length) across the isolates (Fig. 6D). Canine isolates had the greatest number of aligned phages genome segments ($P = 3.92*10^{-5}$), with segments resembling Clostridium phage ΦCD27, Clostridium phage ΦCD505, Clostridium phage ΦMMP02, and Clostridium phage ΦMMP03 found in more than half of the canine isolates.

To further investigate gene flow between the human pediatric isolates and the bovine and canine strains, sequence reads from animal isolates were aligned to a collection of episomes identified in the pediatric subjects (Fig. 7). Sequencing of the pediatric isolates was carried out using both long and short sequence reads, so that all episome families assembled as complete DNA circles and were judged to be episomes on that basis. Many of the canine and bovine samples contained reads aligning to several of the human isolates. Most prominently, 17 of the canine isolates showed high coverage of a small circular plasmid of about 7 Kb related to the known plasmid pCD6 and carrying the mecR1 methicillin resistance gene. This plasmid was shared within the Clade 1 pediatric and canine isolates only, suggesting possible inheritance by descent. Other plasmids and phages showed segmental alignments, suggestive of long-term sharing of mobile DNAs but not recent transfer. A few elements that assembled as circles did not show strong resemblance to known mobile DNAs, but some encoded putative transposase or integrase genes, suggesting the potential to move in and out of the *C. difficile* chromosome, as has been suggested previously [36].

Genome sequences were also investigated for the presence of known conjugative transposons harboring anti-microbial resistance genes (Supplementary Fig. 5). Previously Knight et al. [30] identified four widely circulating conjugative transposons commonly associated with *C. difficile*, Tn6194, Tn6190/Tn916, Tn6164, and Tn1549, which transduce *ermB*, *tetM*, *tet-44*, and *vanB2*, respectively. Tn6194 was only detected in bovine samples. Tn6164 was present in the majority of canine and human samples, and in a minority of bovine samples. Tn6190/Tn916 was identified in bovine and canine samples but not human samples. No high-quality alignments were seen for Tn1549. None of these transposons were associated with the well-vetted episomes described above, suggesting that they are likely integrated in the main chromosome.

4. Discussion

Concerns that animals may represent a reservoir for zoonotic *C. difficile* has highlighted the need to compare characteristics of *C. difficile* isolates from humans and domestic animals. We found that isolates mostly clustered by host species based on core genome

alignment, but not necessarily by site (i.e., farm, litter). This could indicate host-specific circulation of strains, but it could also be related to clade- and sequence type-specificity of the isolates. Within clades and sequence types, strains of *C. difficile* tend to have unique clinical, microbiological and ecological features [4]. All canine isolates were from Clade 1, with most belonging to only two sequence types (ST 2 and 3), and all but one bovine isolate were from Clade 5 and ST 11. Isolated examples of co-clustering of pediatric isolates within the two canine and single bovine clusters are consistent with the possibility of transmission between hosts. However, because interactions of the pediatric subjects with dogs and cows are unknown, it is unclear whether such transmission would occur via a direct (i.e., animal-to-human) or indirect (e.g., via the environment or other fomite) pathway.

Many studies have documented the existence of highly similar or even identical strains of *C. difficile* both within geographically proximal human/animal populations [9–11] as well as across geographically distinct health care facilities and farms [30,37–39], suggesting the possibility of interspecies transmission. The emergence of *C. difficile* isolates previously associated with animals in human populations, as occurred with ribotype 078, the primary strain lineage of ST 11 [40], suggests that an animal reservoir could be the source of novel infections in people. However, the frequency with which spillover from domestic animals to people occurs is unknown. On dairy and beef farms, studies showed no colonization of farmers in close contact with colonized dairy animals [43, 44], and a large-scale study of pet owners showed no concurrent colonization of pets and their owners with *C. difficile* [45]. These findings, along with the apparent clustering of sequence types by host species observed in our study and others [30,41,42], suggest infrequent spillover. The diversity of sequence type in pediatric isolates in contrast to what appears to a relatively stable population of sequence types in bovines and canines also suggests that human-to-human transmission is the dominant mode of spread of this organism within the human population.

With the possibility of interspecies transmission of *C. difficile* comes the possibility of transmission of antibiotic resistance genes. While all isolates had genes that conferred resistance to multiple antibiotics, the bovine isolates had the most resistance genes to individual classes of antibiotics, including aminoglycosides, macrolides, and tetracyclines. This could be due to selective pressure from on-farm antibiotic use. Dairy calves are often fed milk replacer containing aminoglycosides such as neomycin or streptomycin, and non-lactating dairy animals are sometimes treated with macrolides such as tulathromycin, tilmicosin, or gamithromycin, which could create a pool of macrolide resistance genes on the farm that calves could acquire via the environment. We could not confirm use of these drugs on the farms from which these isolates came, but the practices described above occur routinely on dairy farms. Other studies have also found high levels of tetracycline and macrolide resistance in *C. difficile* isolates from veal calves [46,47] and dairy farms [48]. One study even suggested that tetracycline selection pressure drove the rapid clonal expansion and spread of a livestock-associated tetracycline-resistant strain of *C. difficile* (RT078) that has increasingly been found in community-acquired infections [49]. Antimicrobial resistance in *C. difficile* appears to be predominantly mediated by transposons and other mobile elements [36, 50, 51] and *C. difficile* can readily acquire genes from the host's intestinal metagenome [50]. Stepping back, these data highlight the existence of an

extensive reservoir of antibiotic resistance genes in domestic animals poised for transfer into humans given sufficient pressure from antibiotic use.

Phylogenetic analyses of our *C. difficile* isolates showed clustering by host species, and phenotypic and genetic characteristics followed similar patterns. Toxin genes and production, which are major parameters of virulence, differed by host species. Most of the pediatric isolates carried genes for both of the endotoxins (*TcdA* and *TcdB*) and the truncated binary toxin (*CDT*), while all of the bovine isolates carried genes for the full-length or truncated binary toxin and at least one of the exotoxins. This is consistent with previous findings where up to 100% of animal isolates were CDT-positive [52], and where Clade 5 organisms tended to be dominated by CDT-producing strains [30]. The bovine-derived strains also displayed lower cytotoxicity titers, indicative of less extensive toxin production, and higher doubling times, reflective of slower growth. Toxin production in *C. difficile* occurs in the stationary phase of growth [53], and growth rates have been found to be inversely correlated with disease severity [54,55]. The distribution of toxin genes, cytotoxicity titers, and growth rates in the bovine-derived strains might partly explain why all bovids in this study were clinically healthy carriers of *C. difficile*. Slightly more than half (21/37) of our canine isolates were positive for any toxins, which is consistent with findings of another study [56].

As was found in other studies, certain categories of genes appeared preferentially associated with certain clades, and, by extension, host species. In both our study and another [57], bovine isolates from similar geographic regions had unique genes putatively associated with the two-component system and ABC transporters. The two-component signaling-transduction systems are involved in sensing and responding to numerous environmental stimuli [58], and *C. difficile* has a large number of two-component regulatory systems for processes ranging from quorum sensing [59] to toxin production [60, 61]. The unique systems in cattle and dogs may reflect unique environmental conditions faced by the bacteria within the gut of each host species. ATP-binding cassette (ABC) transporters translocate a wide variety of substrates across biological membranes using energy from ATP hydrolysis [62]. The specificity of these genes in the bovine strains may reflect exposure to substrates found in cattle but not other species, which could be derived from animals' environment, diet, or exposure to drugs.

Mobile DNAs – phage, plasmids and transposons—are known to transport determinants of virulence and antibiotic resistance in *C. difficile* [34–36]. The pediatric isolates studied here were sampled from sites proximal to the location of the animal isolates, allowing interrogation of possible episome transfer. The most commonly shared episome was a 7 Kb plasmid related to pCD6, found in pediatric and canine isolates, which encodes the mecR1 methicillin resistance determinant [63]. The canine and human isolates containing this plasmid were all from Clade 1, consistent with inheritance by descent within this clade. Additional shared matches to multiple further episomes were found, though typically only as partial genome segments. These findings suggest long-term sharing of a pool of mobile DNAs. However, no examples of recent transfer of full episomes, as marked by high identity of episomes against a background of different host cell genomes, were seen.

A targeted analysis of gene segments matching database *C. difficile* phages was also carried out. Certain phage segments were found in only one type of host species: φcd506, φcdhm13, and φcdhm11 matches were found only in isolates of animal origin, while φcd38–2, φcd111, φcd146, and φcd6356 were found only in isolates of pediatric origin. Other authors similarly found φcd506 in a *C. difficile* isolate of canine origin and φcd111 and φcd146 in isolates of human origin [64]. While these phages were demonstrated to be able to infect both human and animal *C. difficile* isolates [64], the repeated finding of these phages in isolates of specific host species suggests the possibility that phage specificity exists for *C. difficile*. However, the distribution could also be due to the fact that the pediatric isolates were associated with clinical infection while the animal isolates were associated with colonization only. For example, φCD38–2, for which extensive alignments were found only in isolates of pediatric origin, upregulates toxin production in *C. difficile* [65] and may therefore lead to or exacerbate *C. difficile* infection. Given the interest in better defining host range of phages in *C. difficile* both for epidemiological and potentially therapeutic purposes [34,64,66] and the role of phages in transducing and spreading antimicrobial resistance genes, these findings are of interest and should be further explored.

A limitation to our study is that the majority of published human-associated *C. difficile* genomes are from hospital settings. Thus, there is a possibility that we may miss community transmission of *C. difficile* strains in patient subsets not associated with healthcare settings. However, we have included *C. difficile* strains isolated from the high-risk pediatric population that have been shown to likely be community-acquired [67]. This important comparison allows us to postulate that both hospital and community-acquired *C. difficile* are host adapted to humans. Another limitation is that isolates were collected at different time periods, with human pediatric isolates collected before the animal-derived strains. Analyses of contemporarily collected strains may have revealed different findings.

In summary, *C. difficile* strains were mostly phylogenetically clustered by host species and displayed clade-specific, and by extension, host specific phenotypic and genetic characteristics. However, co-clustering of some pediatric isolates among animal-derived isolates suggests the possibility of interspecies circulation of *C. difficile*. While the directionality of transmission cannot be definitively established, the possibility of animal-to-human transmission warrants concern, especially in light of the reservoir of antimicrobial resistance genes in animal-derived strains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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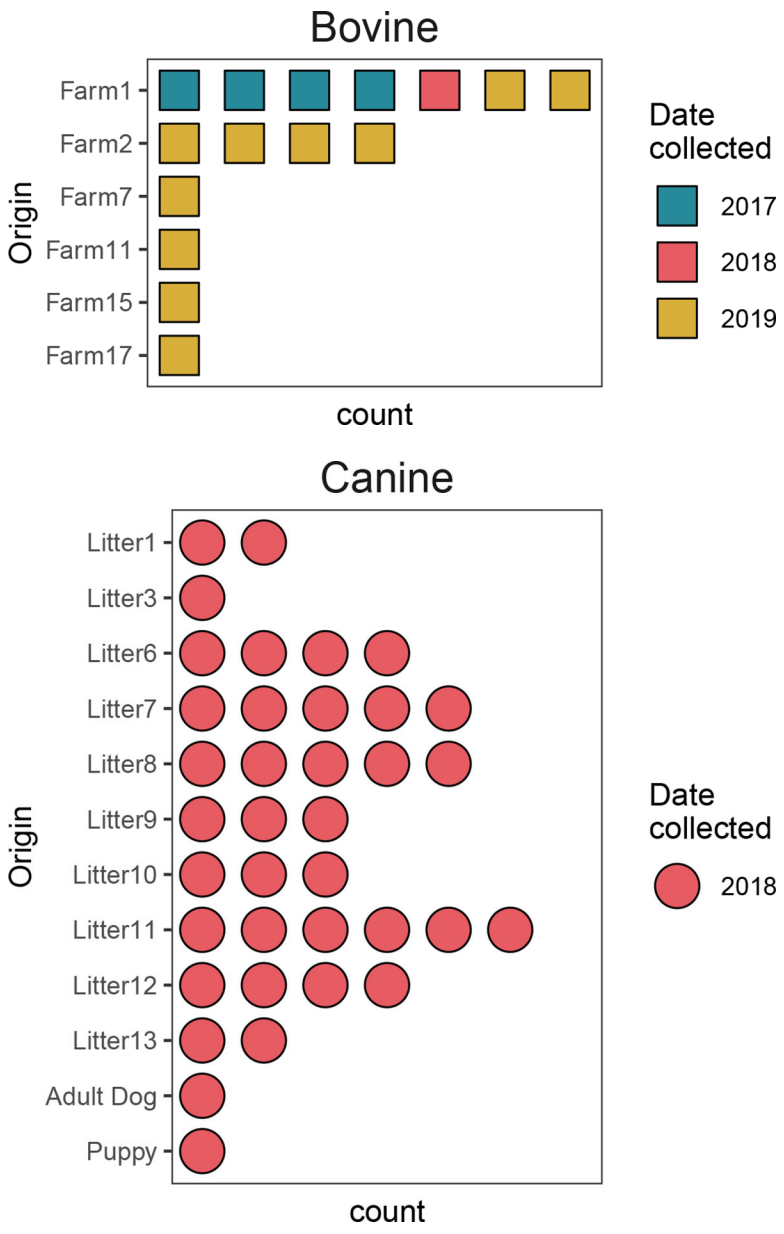


Fig. 1.
Host species, locations, and dates of collection of animal-derived isolates of *C. difficile*.

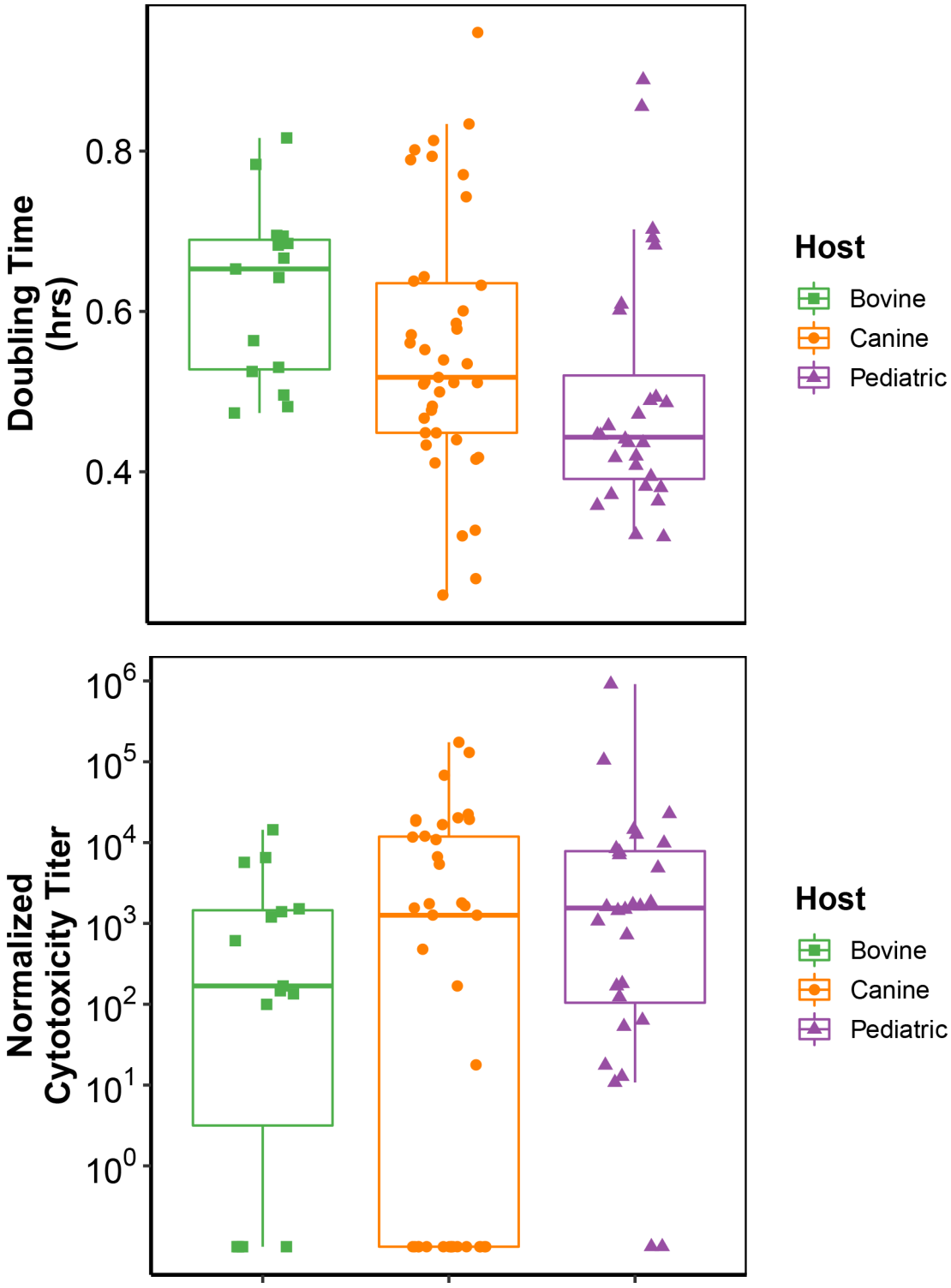


Fig. 2.

Growth and cytotoxicity characteristics of *C. difficile* isolates from bovine, canine, and human pediatric patients. (A) Doubling times during exponential growth in rich liquid

media. (B) Normalized toxin titers using Vero cell cytotoxicity assay after 24 h of liquid media growth of *C. difficile* isolates.

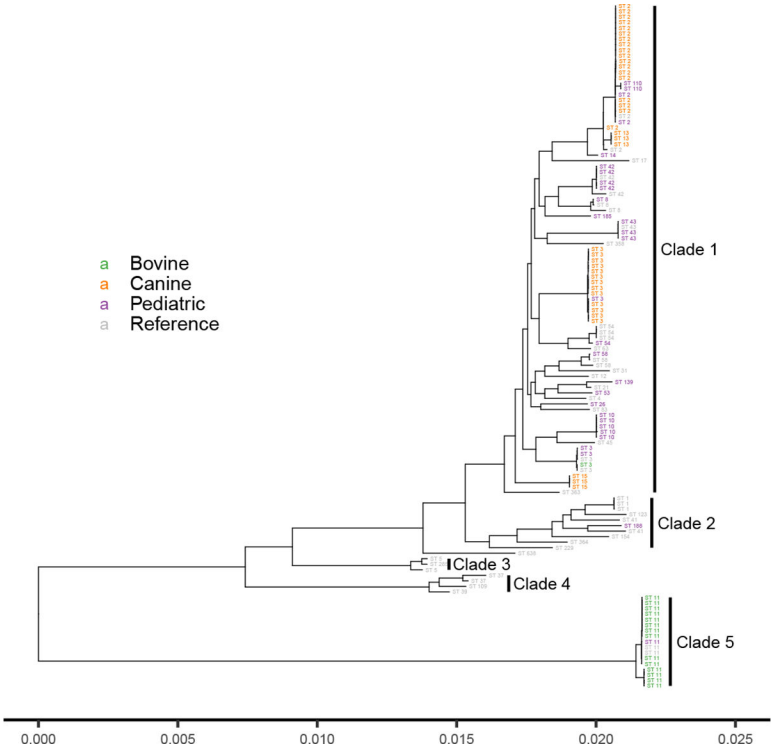


Fig. 3.

Phylogenetic tree of *C. difficile* isolates from bovine, canine, and pediatric human hosts. The scale represents the number of substitutions within the core genes used to infer the tree.

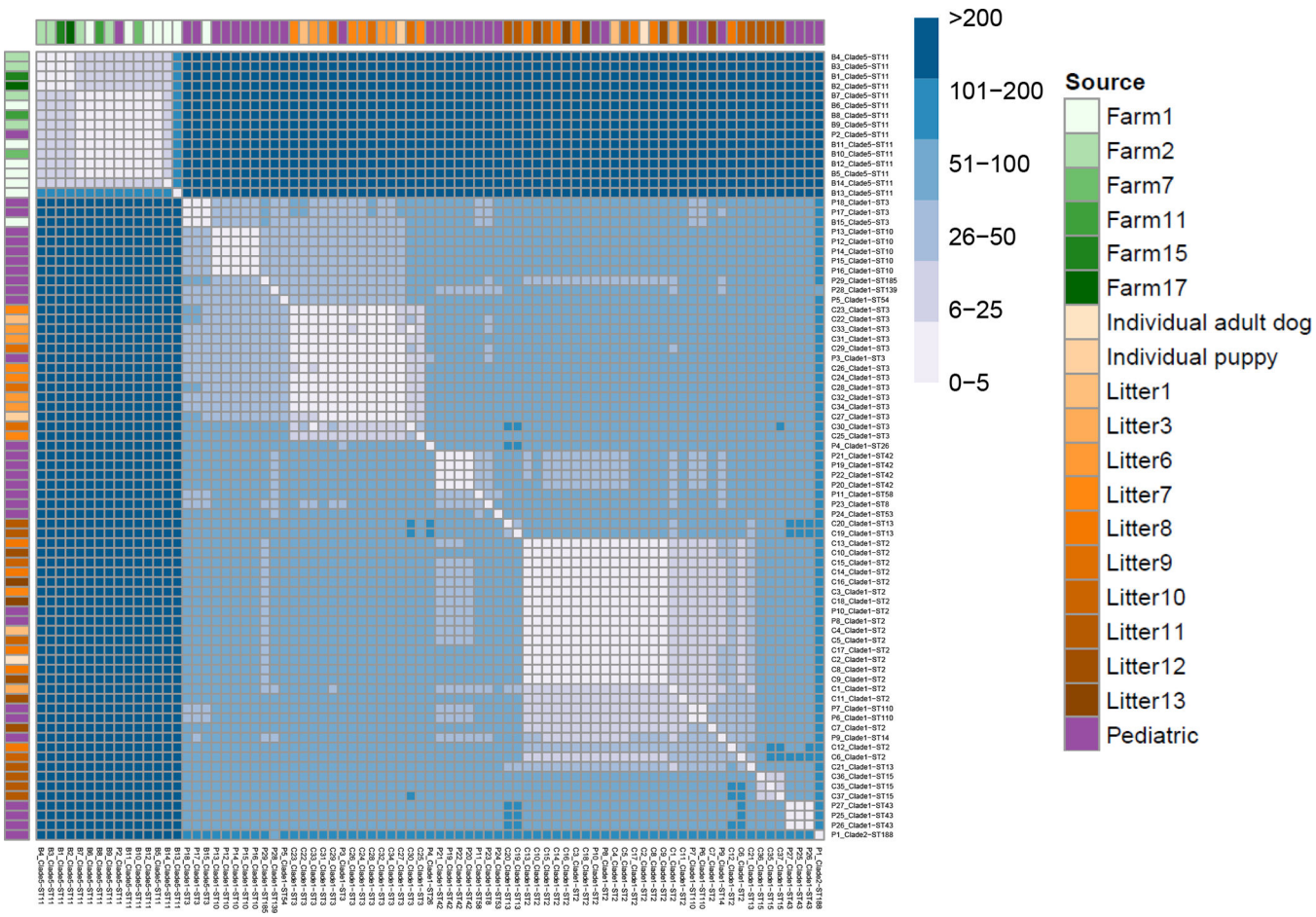
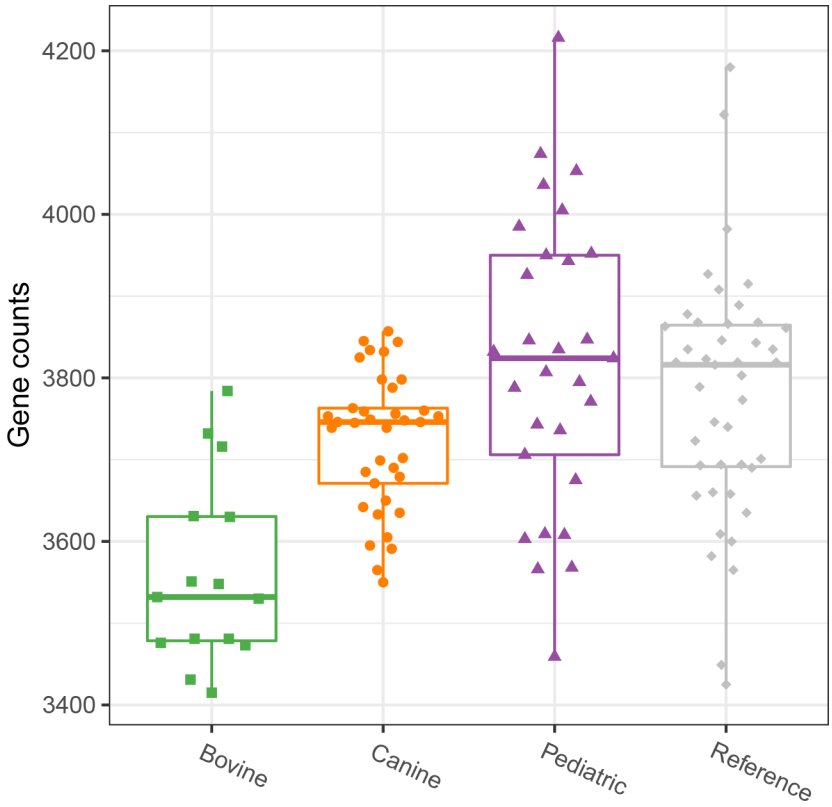
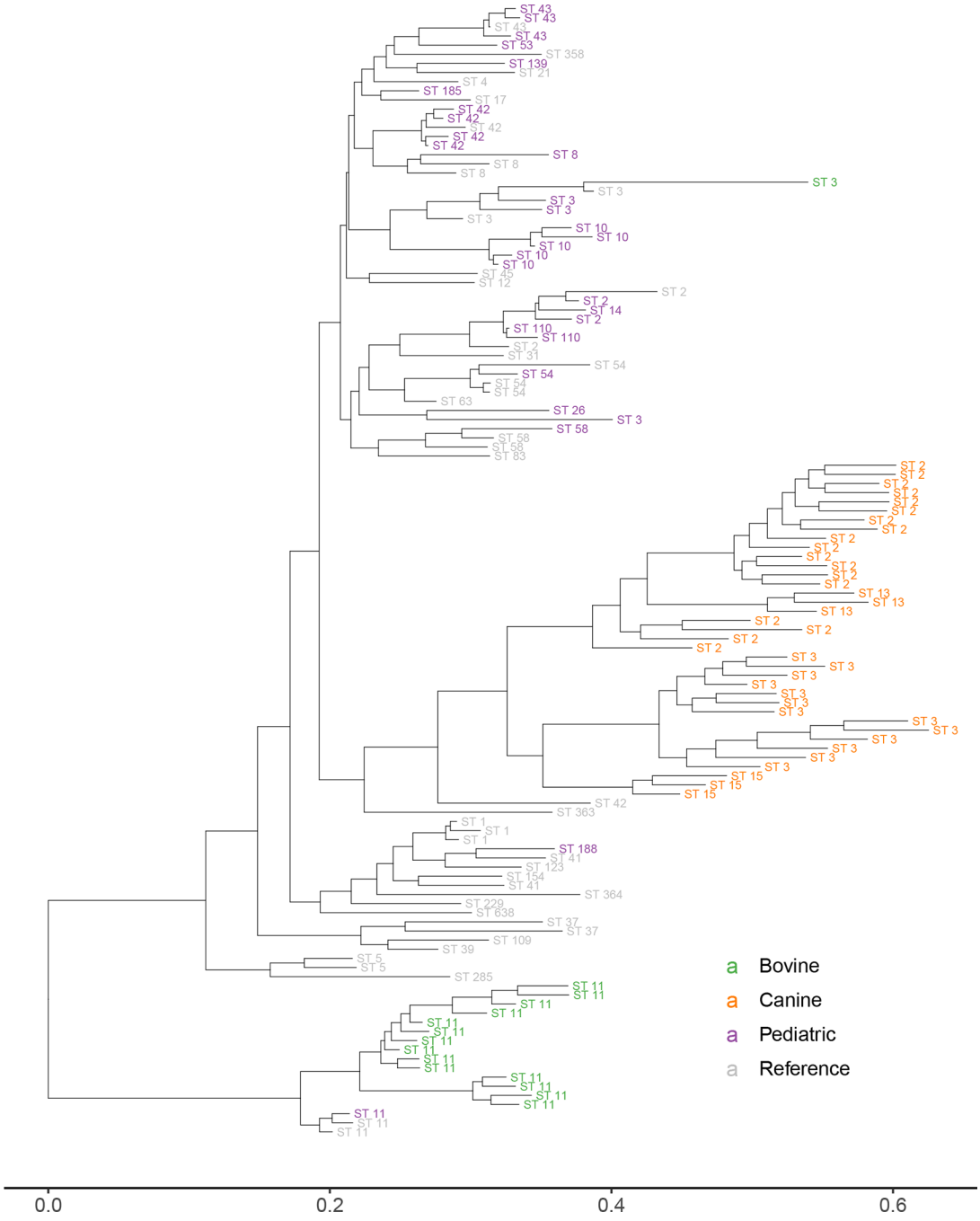


Fig. 4.

Number of single nucleotide variants between *C. difficile* isolates from bovine, canine, and pediatric human hosts. Colors next to the rows and columns indicate the farm for bovine isolates, or the litter for canine isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)





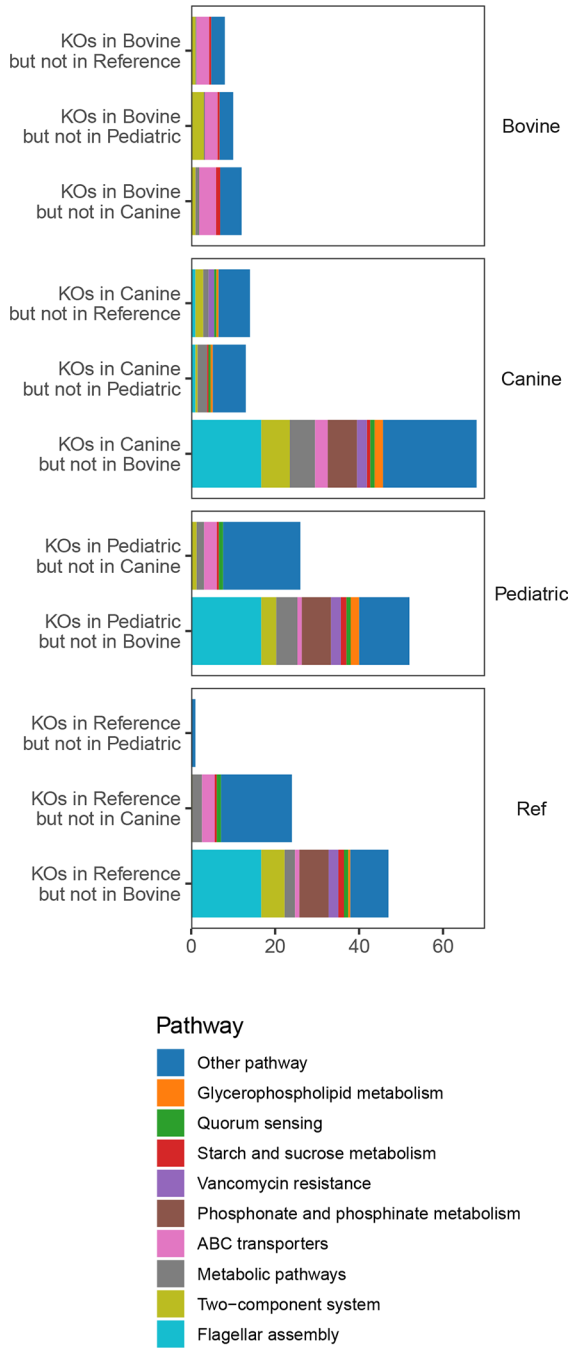
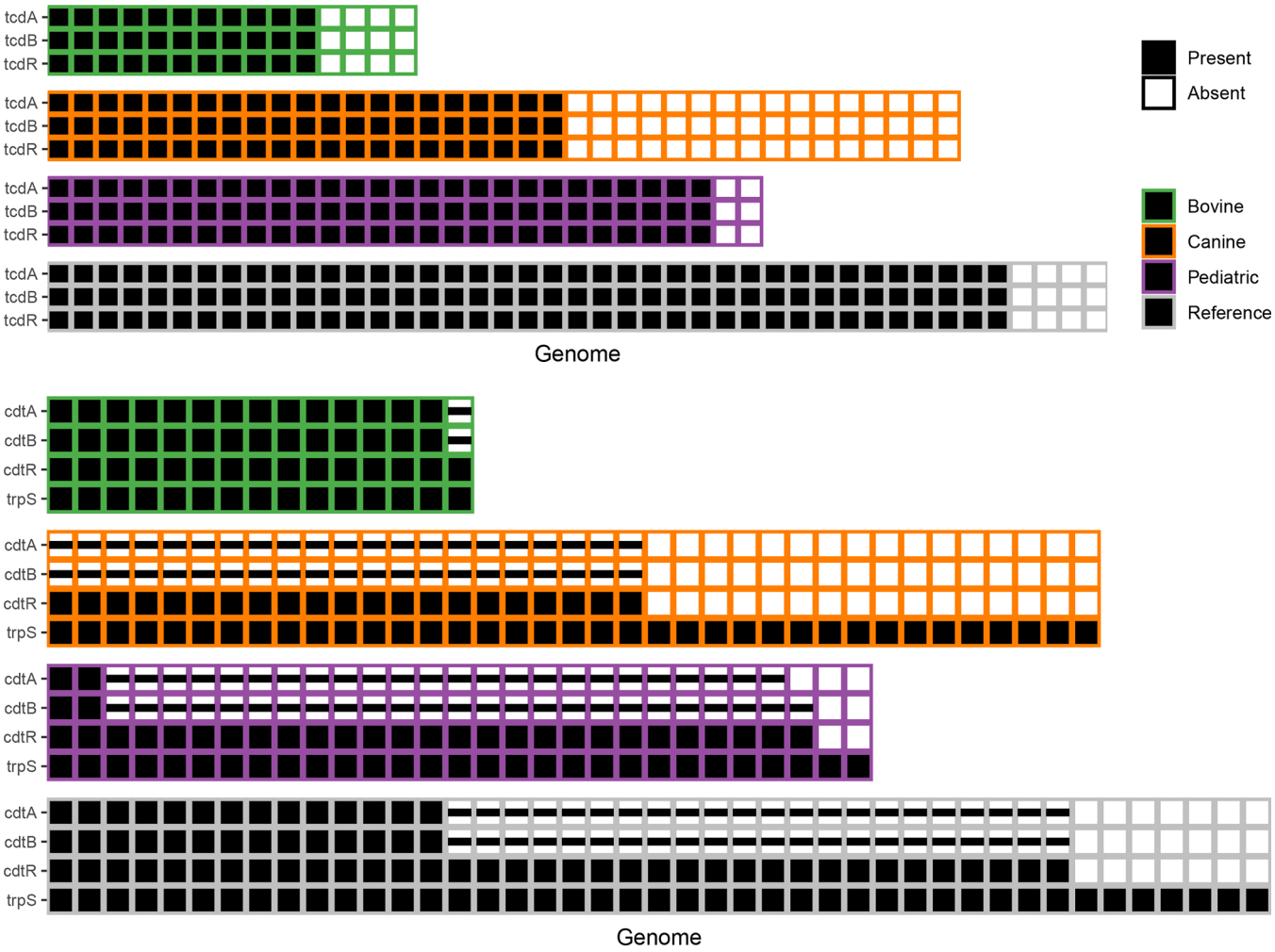
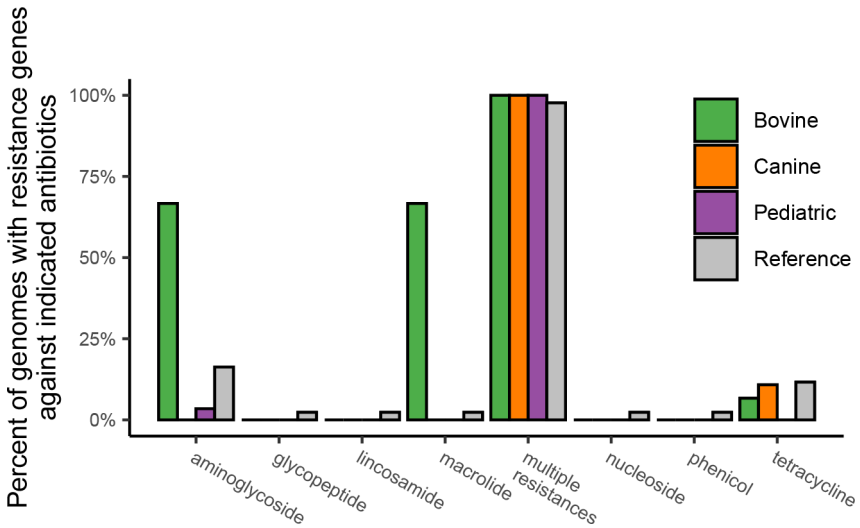


Fig. 5.

Accessory genome of *C. difficile* isolates from bovine, canine, and pediatric human hosts. (A) Total number of genes observed. (B) Hierarchical clustering of genomes based on gene presence/absence. (C) Pathway assignments for genes observed in isolates from one source but absent in isolates from another source.





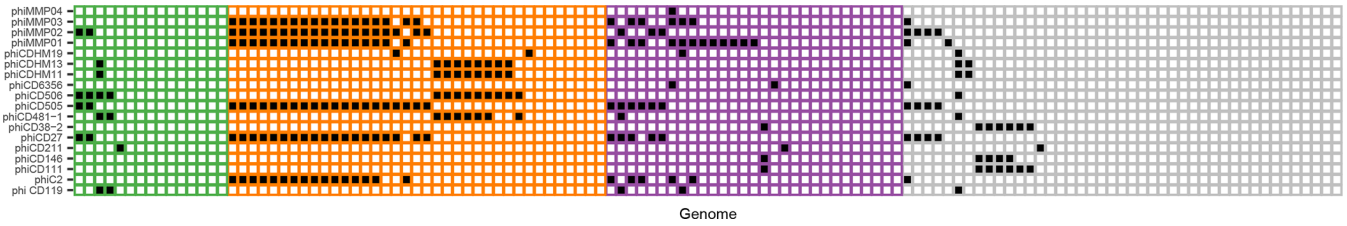


Fig. 6.

Toxin genes, antibiotic resistance genes, and phage genome segments in *C. difficile* isolates. (A) Presence and absence of genes involved with production of *C. difficile* toxin A and toxin B. (B) Genes involved with production of binary toxin *C. difficile* transferase (CDT). Horizontal lines in boxes indicate truncated CDT genes (C) Frequency of antimicrobial resistance genes. (D) Phage genomes found in *C. difficile* isolates from canine, bovine, and pediatric patient populations, and in reference genomes.

Fig. 7.

Analysis of sharing of human pediatric episomes with canine and bovine isolates. Pediatric episomes were identified by deep sequencing using both long and short reads, allowing identification as extrachromosomal circles. Samples are indicated in the columns, episomes in the rows. The extent of alignments are shown by the scale on the right. Selected gene types are summarized to the left. Only isolates that showed a match to an episome are shown. Details are in Supplementary Report 1.

Table 1

Genome content of 124 *C. difficile* isolates from pediatric patients, dogs and dairy calves.

	Host population		
	Pediatric patients	**Canine population**	**Bovine population**
Mean (SD) genome size (Mbp)	4.25 (0.14)	4.13 (0.07)	3.95 (0.08)
Median (range) GC%	28.6 (28.4–29.3)	28.7 (28.4–29.3)	28.5 (28.4–28.6)
Mean (SD) number of predicted genes	3832 (180)	4060 (133)	3716 (115)
Mean (SD) coding % of genome (number of nucleotides within a coding sequence relative to all nucleotides in the genome)	82.6 (0.21)	84.9 (0.30)	83.9 (0.44)

Table 2

Variations in single nucleotide variants in *C. difficile* isolates within and between host species

Comparisons	Median (interquartile range) difference in numbers of single nucleotide variants between isolates
CANINE	
Within canine isolates	58 (16.5–71)
Between canine and pediatric isolates	61 (51–77)
Between canine and bovine isolates	538 (535–543)
Between canine and reference isolates	90 (63–142)
BOVINE	
Within bovine isolates	12 (3–117)
Between bovine and pediatric isolates	534 (529–539)
Between bovine and reference isolates	533 (516–540)