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Original Article

Degree of concordance of *Clostridioides difficile* strains in adults with community-associated *C. difficile* infection and infants with *C. difficile* colonization

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Abstract

Background: Infants asymptomatically excrete *Clostridioides difficile* during their first year of life, suggesting that they may represent a source of infection for adults who acquire community-associated *C. difficile* infection (CA-CDI). The genetic relationship of *C. difficile* strains from asymptomatic infants and adults with CA-CDI is not well defined.

Methods: In this study, 50 infants were recruited at birth, and stool samples were collected at routine well-child visits. Adult stool samples collected during the same period and geographical area from patients who were diagnosed with CA-CDI were selected for comparison. *C. difficile* was cultivated and probed by PCR for toxin genes and were typed by PCR fluorescent ribotyping. Isolates from adults and infants with shared ribotypes were subjected to whole-genome sequencing (WGS).

Results: Of these 50 infants, 36 were positive for *C. difficile* at least once in their first year of life, with a peak incidence at 6 months. Among 180 infant stool samples, 48 were positive. Of 48 isolates from positive stools, 29 were toxigenic by polymerase chain reaction (PCR) and 8 of 48 stool samples were positive for toxin by enzyme immunoassays (EIAs). Ribotypes F106 and F014-020 were present in both colonized infants and adults with CA-CDI. WGS identified 1 adult–infant pair that differed by 5 single-nucleotide polymorphisms (SNPs). Also, 4 additional adult–infant clusters differed by ≤16 SNPs.

Conclusions: Infants that are colonized with *C. difficile* share ribotypes with adults from the same geographical region with CA-CDI. Selected isolates in the 2 populations show a genetic relationship by WGS.

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In the mid-2000s,[1-3] cases of *Clostridioides difficile* infection occurring outside acute-care hospitals and long-term care facilities were increasingly recognized by public health officials, heralding the era of community-associated *C. difficile* infection (CA-CDI). Subsequently, nearly 50% of adults infected with *C. difficile* were categorized as CA-CDI.[4,5] The varied, sometimes prolonged incubation period of *C. difficile* infection has rendered it difficult to identify the reservoirs of the organism in the community. Although the sources of adult community-associated infection likely are multiple, studies have indicated that infants may serve as one nidus of transmission[6]; a large proportion of infants excrete toxigenic *C. difficile* asymptomatically during their first year of life.[7,8] To probe the possible role of colonized infants as a source of CA-CDI in adults, the current study enrolled a cohort of healthy, full-term infants at birth and cultured their stool during regular well-child visits over their first year of life. Isolates of *C. difficile* then were compared by ribotyping and by whole-genome sequencing (WGS) to those derived from a concomitant cohort of adults with CA-CDI from the same metropolitan area.

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Methods

This study was reviewed and approved by the Institutional Review Board of University Hospitals Cleveland Medical Center.

Patient recruitment

Healthy infants attending a suburban, demographically diverse pediatric practice were enrolled at birth and prospectively followed at the 2-, 6-, and 12-month well-child visits. At birth and at each visit, a stool sample was collected and refrigerated for up to 3 days before being stored at −80°C for batch testing. These handling procedures resulted in <10% decrease in cultivatable *C. difficile* colony numbers compared with samples cultured on the day the stool was produced.

A contemporaneous set of sequential adult stool samples submitted to the clinical microbiology laboratory at University Hospitals Cleveland Medical Center, located ~9.65 km (~6 miles) from the pediatric practice, was used for comparison. Samples were identified as *C. difficile*–positive by commercial polymerase chain reaction assay (PCR) and stored at −80°C. A subset of these samples was categorized by the hospital infection prevention service as "community onset" according to National Healthcare Surveillance Network case definitions[9]; these were deidentified and selected for further analysis.

Microbiology and ribotyping

Infant stool samples were tested for presence of free toxin using a commercially available enzyme immunoassays (EIAs) for toxins A and B (Abbot Laboratories, Abbot Park, IL). Infant and adult samples were cultivated for *C. difficile* using previously defined methods.[10] In short, frozen stool was transferred to an anaerobic chamber and thawed for 10 minutes at 37°C. A 10-mg aliquot of thawed stool suspension was plated onto prereduced *C. difficile* brucella agar containing 0.1% taurocholic acid and 5 mg/L lysozyme.[11] Putative *C. difficile* colonies were selected based on typical morphology and identity was confirmed by a latex agglutination assay (Microgen Bioproduct, Surrey, UK). We performed PCR amplification of the *tcdC* and *cdtB* genes to identify toxigenic *C. difficile* isolates and binary toxin-positive isolates, respectively.[10]

The ribotype of each isolate was determined by fluorescent PCR ribotyping according to previously published methods.[12] In short, genomic DNA was extracted by heating an overnight culture to 95°C for 15 minutes and amplified by PCR using the primers, conditions, and program reported by Martinson et al.[12] Fluorescent amplicons were then sequenced for fragment analysis by Capillary Electrophoresis at the University of Michigan Biomedical Research Core facility. Fluorescent ribotypes were designated using the Montana State University Fluorescent PCR Ribotyping Pipeline reference library.[12]

Whole-genome sequencing

Samples from the infant and adult CA-CDI sets that expressed the same ribotypes were further subjected to WGS. DNA was extracted using the QIAamp DNA Micro Kit (Hilden, Germany). The preparation of the libraries was performed using the Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA), and paired-end reads (2 × 150 bp) were generated using the Illumina MiniSeq reagent kit and MiniSeq instrument (Illumina, San Diego, CA). Whole-genome single-nucleotide polymorphism (WgSNP) analysis was performed using the cloud based BioNumerics version 7.6 software platform (Applied Maths NV, Sint-Martens-Latem, Belgium, available at http://www.applied-maths.com) after dynamic trimming and mapping of the raw reads to a reference genome isolate (National Center for Biotechnology Information [NCBI] Cdiff Refseq 630). The reference genome de novo assembly was completed using SPAdes assembler on the Bionumerics calculation engine using the Bowtie2 mapping algorithm.

We used the following settings for the Bowtie mapping algorithm: minimum total coverage of 3, minimum forward coverage of 1, minimum reverse coverage of 1, single-base threshold of 0.75, double-base threshold of 0.85, triple-base threshold of 0.95, and gap threshold of 0.5. An SNP analysis was performed using the patient isolates (adult and infant). Using SNP filtering, positions with at least 1 unreliable (N) or ambiguous (non-ACGT) base was removed. Each retained SNP position has minimum 5× coverage, at least covered once in both forward and reverse directions. Further, advanced cluster analysis (similarity matrix) was constructed using similarity coefficient for categorical (differences). A dendrogram was created using global closest pair method (UPGMA) with no resampling. All of the analyses were performed within the BioNumerics version 7.6 software platform.

Whole-genome multilocus sequence typing (wgMLST) with assembly-free and assembly-based calls was performed using the calculation engine on the Bionumerics version 7.6 platform, employing the sequences derived from the WGS analysis. Quality assurance was performed to ensure accuracy of the calls. Isolates so analyzed were assigned both to their MLST Pasteur sequence type (ST) and their public ST.

Results

In total, 50 infants were recruited as newborns between June 2016 and March 2017; 11 infants (22.0%) were lost to follow-up by the 12-month well-child visit. All infants were full term, and 36 (72.0%) were born via vaginal delivery. Most of the infants were exposed to perinatal antibiotics (Table 1). Over the course of the study, 180 infant stool samples were collected. No infant had a *C. difficile*–positive stool at the newborn baseline, but 36 (72.0%) were positive for *C. difficile* at least once during subsequent study visits. Among these 36, 9 infants (25.0%) tested positive on multiple well visits, and 3 (8.3%) infants tested positive at all 3 postnatal visits. The incidence peaked at 6 months, with 25 of 43 (58.1%) infants testing positive for *C. difficile* (Fig. 1). No infant displayed gastrointestinal symptoms during any of their study visits.

Of the 36 infants with positive cultures for *C. difficile*, 25 (69.4%, or 50% total infants) carried the enterotoxin gene *tcdC* (Table 2). Of the 36, 8 (22.2%, or 16% of all infants) carried binary toxin-positive strains, all from isolates that also were *tcdC* positive. Also, 5 (13.9%, or 10.0% of all infants) had 1 or more stools that tested positive for enterotoxin by EIA (Table 2). The proportions of the 180 total stool samples that exhibited these characteristics is presented in Table 2. The 48 infant stool samples with positive cultures for *C. difficile* had a density of organisms that ranged from $\log_{10}$ 2.0 to $\log_{10}$ 4.6 colony-forming units (CFU) per gram of stool. Of 48 infant samples, 20 (41.6%) harbored >$\log_{10}$ 4.0 CFU per gram of stool, all with toxigenic organisms.

Of 37 stool specimens from adults with CA-CDI, 35 (94.6%) had toxigenic *C. difficile* recovered by culture. Of these 35 isolates, 4 (18.2%) were binary toxin positive. The density of *C. difficile* in these samples ranged from $\log_{10}$ 2.0 to $\log_{10}$ 6.0 CFU per gram of stool. Also, 11 adult samples (31.4%) contained > $\log_{10}$ 4.0 CFU per gram of stool.

In total, 38 infant *C. difficile* isolates and 35 isolates from the contemporaneous set of adults with CA-CDI were submitted for fluorescent PCR ribotyping. We were unable to recover the 10 infant samples that were not submitted for ribotyping from the stored frozen isolates. Among the 38 infant isolates, 18 (47.3%) matched existing ribotypes in the reference database.[12] For the infant isolates that matched existing ribotypes, 8 ribotypes were identified, with F014-020, F106, and F010 having the greatest representation (Fig. 2). Among the 35 adult isolates, 29 (82.9%) matched existing ribotypes in the reference database.[12] For the adult CA-CDI isolates that matched existing ribotypes, 12

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Table 1. Infant Characteristics at Birth[a]

Characteristic	Infants (N = 50)[a]
Gestational age, weeks	39.3 (38.3–40.2)
Sex, male	23 (46.0)
Birth method	
Vaginal	36 (72.0)
Cesarean	14 (28.0)
Feeding plan	
Breast milk	43 (86.0)
Formula	1 (2.0)
Both	6 (12.0)
Antibiotics	
Prenatal	5 (10.0)
Intrapartum	26 (52.0)
Neonatal	0 (0.0)

[a]Continuous data are presented as median (interquartile range). Categorical data are presented as number (% of N).

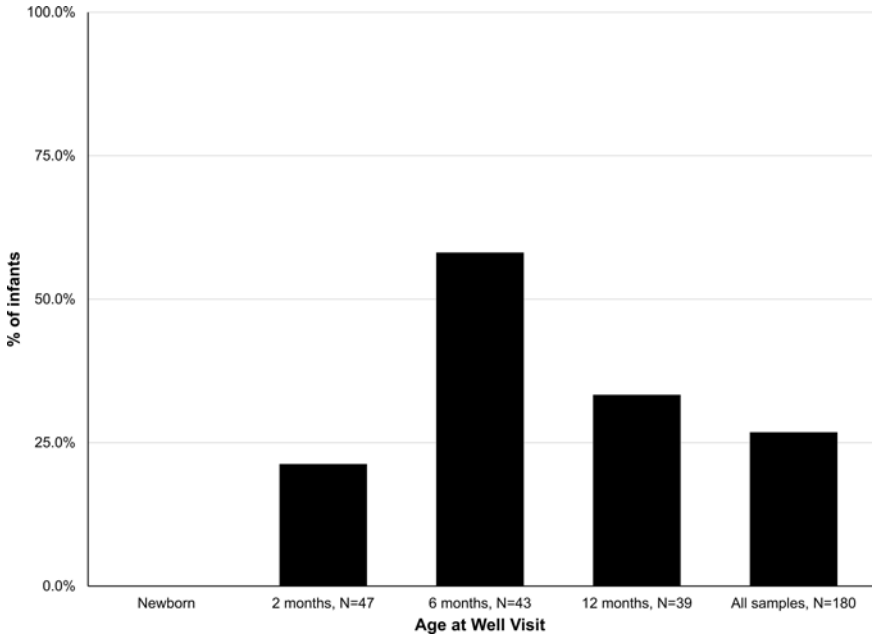

Fig. 1. Longitudinal prevalence of *Clostridioides difficile* positive stools in healthy infants.

ribotypes were identified, and F014-020 was the most frequently represented strain (Fig. 2).

Furthermore, 2 ribotypes were present in both adults with CA-CDI and in infants: F106 was recovered from 4 infants and 1 adult and F014-020 was recovered from 3 infants and 12 adults (Fig. 2). All of these isolates were toxigenic. These organisms were submitted for wgMLST analysis to determine the degree of genetic similarity of *C. difficile* between the adult and infant samples. One adult–infant pair within cluster 1, both derived from ribotype F106 and from ST33 (Pasteur) or ST42 (Public), differed by 5 SNPs (adult-11-F106 and infant-1-F106) (Fig. 3 and Supplementary Figs. 1 and 2 online). Within this cluster 1, an additional infant (infant-2-F106), differed by 15 SNPs. A third adult–infant pair in cluster 2, adult-12-F014 and infant-5-F014, both derived from the same ribotype and belonging to the same ST group (ST1 [Pasteur] or ST110 [Public]), differed by 14 SNPs. A fourth adult–infant pair in cluster 3, adult-3-F014 and infant-6-F014, derived from the same ribotype and the same ST group (ST1 [Pasteur] or ST2 [Public]), differed by 15 SNPs (Fig. 3 and Supplementary Figs. 1 and 2 online). An apparent cluster (cluster 4) including several isolates (infant-7-F014, adult-6-F014, adult-5-F014, and adult-10-F014) all were derived from the same PCR

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Table 2. Results of Infant Stool Analyses

Variable	Infants (N = 50), No. (%)	Samples (N = 180), No. (%)
Culture positive for *C. difficile*	36 (72.0)	48 (26.7)
Culture positive for toxigenic *C. difficile*	25 (50.0)	29 (16.1)
Culture positive for binary-toxin *C. difficile*	8 (16.0)	8 (4.4)
Stool with detectable toxin by EIA	5 (10.0)	8 (4.4)

Note. EIA, enzyme immunoassay.

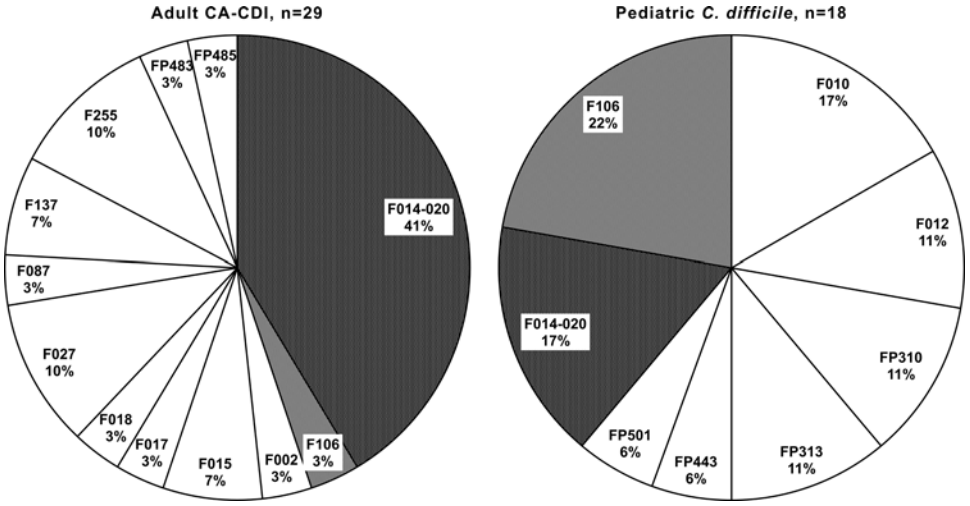

Fig. 2. Ribotype concordance in a contemporaneous population of adults with CA-CDI and infants with *Clostridioides difficile*. Ribotypes present in both populations are denoted in the shaded pie slices.

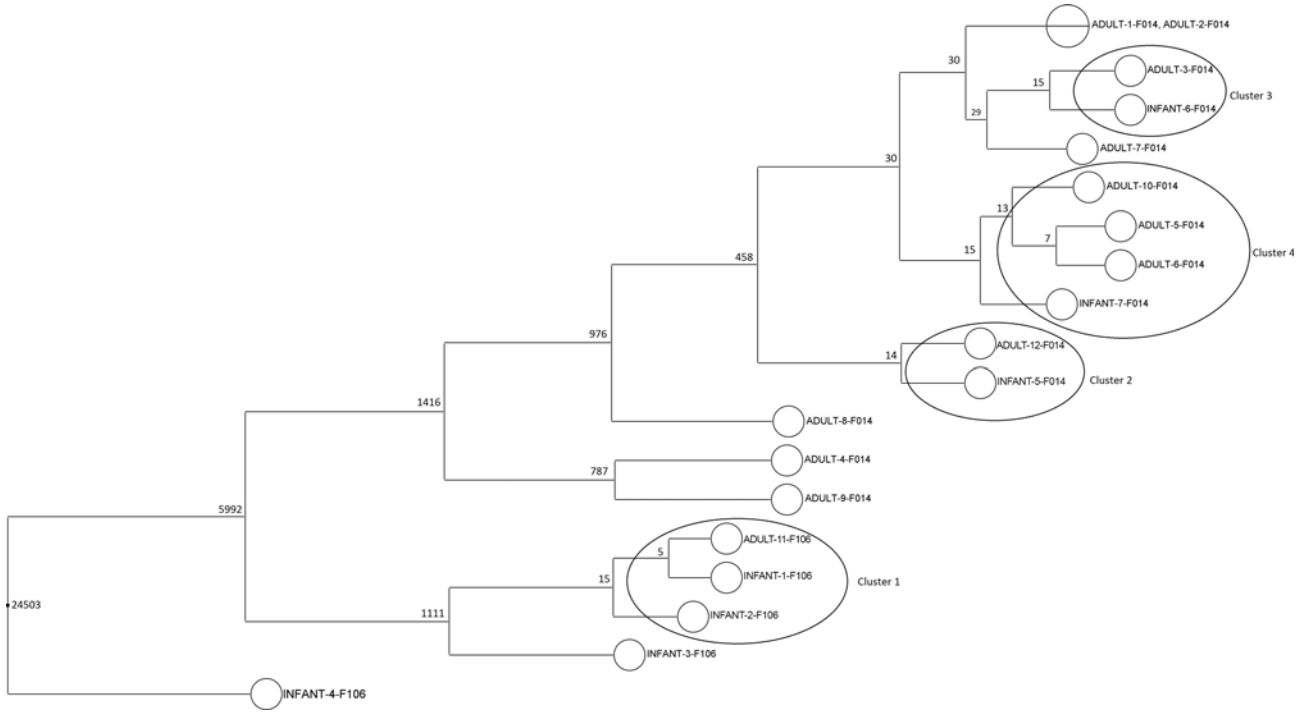

Fig. 3. Dendrogram representing the genetic relatedness of isolates subjected to whole-genome sequencing. Isolates are designated as "adult" or "infant," followed by their study number and polymerase chain reaction (PCR) ribotype assignment. Potentially related adult-infant pairs are grouped together in circles. The number designated at each branch point represents the single-nucleotide polymorphism (SNP) distance between the isolates.

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ribotype and the same ST group (ST1 [Pasteur] or ST2 [Public]) and differed by ≤15 SNPs (Fig. 3 and Supplementary Figs. 1 and 2 online).

Discussion

In this study, we explored the potential role of *C. difficile*–colonized infants in the epidemiology of CA-CDI in adults. We confined the study to adults with community-associated infection because we hypothesized that epidemiologic associations between babies and adults would be most apparent among those who had acquired *C. difficile* outside acute-care and long-term care wards. Our data indicated that at least some organisms involved in asymptomatic infant carriage and those simultaneously isolated in adult CA-CDI within the same geographical region exhibited genetic similarities. Using this protocol, we were unable to establish the directionality of putative transmission events; the results are as consistent with adult-to-infant transmission as transmission from the baby to the adult, or acquisition by both populations from a common reservoir. These results do indicate, however, that asymptomatic infant carriage and adult CA-CDI are not caused by mutually exclusive strains.

Two ribotypes in particular, comprising 39% of isolates from infants, were identifed in a subset (45%) of organisms cultured from the contemporaneous population of adults with CA-CDI. WGS comparisons revealed 1 infant–adult pair that differed by 5 SNPs and several others that differed by ≤16 SNPs. To add perspective to the degree of relatedness implied by these SNP differences, Eyre et al[13] determined that the evolutionary mutation rate of *C. difficile* isolates cultured repeatedly from the same adult was slightly less than 1 SNP per year.[13] Although it is likely that the mutation rates vary from strain to strain and are different in organisms derived from different ecological contexts, the results from the current study are consistent with a common bacterial ancestor encountered ~5 years prior to our study for the pair differing by 5 SNPs. Those differing by 16 or fewer SNPs may be unrelated (according to the definitions suggested by Eyre et al[13]) or from an event proportionately further in the past. The babies and adults included in the current report had no apparent epidemiological link, and notwithstanding these estimates of genetic relatedness, it is not possible to confidently time a past transmission event in the absence of data pertaining to social interactions. Nonetheless, the current data support the possibility that infant colonization and adult CA-CDI are not distinct epidemiological phenomena.

Prior European studies have implicated babies as reservoirs for CA-CDI in adults. Rousseau et al[14] prospectively followed a small cohort of infants in Paris and found that ribotypes of asymptomatically excreted *C. difficile* that were similar to disease-producing ribotypes in French adults with CDI. Further strain characterization was not performed, however, and the adult isolates were not collected concomitantly to the infants'. Additional studies performed by investigators in Oxford, United Kingdom, employed high-throughput WGS to compare colonizing *C. difficile* strains in infants to those from adults with CDI in central England. Small proportions of isolates from both groups were genetically identical (ie, 0 SNPs), and 38% of infant isolates exhibited 10 or fewer SNPs compared with adult samples.[15] Our study, while smaller in scale, reflects similar characteristics in an American population.

Even though, to the best of our knowledge, no study to date has directly demonstrated infant-to-adult household transmission of *C. difficile*, such transmission is credible. Family-member contact with colonized infant stool is frequent and usually unprotected, providing abundant opportunity for transmission. Indeed, the density of organisms in at least some of the infants in our study, >$\log_{10}$ 4 per gram of stool, exceeds that of most adult carriers[10] and was similar to our adult samples from patients with CA-CDI. Moreover, apparent intrafamilial transmission of other enteric bacteria has been documented,[16,17] and spread of *C. difficile* from a recently infected adult to other noninfant household contacts has been reported in prospectively studied families in Quebec.[18] Ultimately, however, the establishment of asymptomatic infant *C. difficile* excretion as a contributor to CA-CDI in adults will require longitudinal studies documenting the transmission of genetically related isolates from colonized infant to initially *C. difficile*–negative adult contacts. The current study provides the motivation to study the epidemiological role of infant *C. difficile* colonization in this more granular fashion.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/ice.2020.1290

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Conflicts of interest. All authors report no conflicts of interest relevant to this article.

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