Longitudinal Investigation of Carriage Rates, Counts, and Genotypes of Toxigenic *Clostridium difficile* in Early Infancy

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ABSTRACT Asymptomatic infant carriers of toxigenic *Clostridium difficile* are suggested to play a role in the transmission of *C. difficile* infection (CDI) in adults. However, the mode of *C. difficile* carriage in infants remains to be fully elucidated. We investigated longitudinal changes in carriage rates, counts, and strain types of toxigenic *C. difficile* in infants. Stools collected from 111 healthy infants in Belgium periodically from birth until the age of 6 months were examined by quantitative PCR targeting 16S rRNA and toxin genes. Toxigenic *C. difficile* was detected in 18 of 111 infants (16%) in the period up to the age of 6 months. The carriage rate of toxigenic *C. difficile* remained below 5% until the age of 3 months. The carriage rate increased to 13% 1 week after weaning (average age, 143 days) and reached 16% at the age of 6 months. Counts of toxigenic *C. difficile* bacteria ranged from 10^4 to 10^8 cells/g of stool. Notably, two infants retained >10^8 cells/g of stool for at least several weeks. Average counts in the 18 infants hovered around 10^7 cells/g of stool from the age of 3 days until the age of 6 months, showing no age-related trend. Genotyping of toxigenic *C. difficile* isolates from the 18 infants revealed that 11 infants each retained a particular monophyletic strain for at least a month. The genotype most frequently identified was the same as that frequently identified in symptomatic adult CDI patients. Thus, toxigenic *C. difficile* strains—potential causes of CDI in adults— colonized the infants' intestines.

IMPORTANCE Our study provides longitudinal data on counts and strain types of toxigenic *C. difficile* in infants. We found that considerable numbers of toxigenic *C. difficile* bacteria colonized the infants' intestines. The results of strain typing suggest that toxigenic *C. difficile* carried by healthy infants could be potentially pathogenic to adults. These results and findings are informative not only for ecological studies but also for efforts to prevent or control the spread of CDI in adults.

Toxigenic *Clostridium difficile* is the main cause of health care-associated gastrointestinal infections. In general, *C. difficile* is not a normal inhabitant of the adult gastrointestinal tract, because its colonization is considered to be prevented by the presence of commensal microbiota (1–3). Colonization of the intestines by toxigenic *C. difficile* is the first crucial step in the development of *C. difficile* infection (CDI). Accumulating evidence suggests that disruption of the microbiota and frequent exposure to toxigenic *C. difficile* could increase the risk of CDI (4–6). Whereas asymptomatic carriage of toxigenic *C. difficile* is rare in adults, occurring in <5% of adults (7, 8), it is very common in infants (9, 10), probably due to the immaturity of the microbiota composition. Regardless of the high prevalence, a great majority of infants rarely show CDI-related abdominal symptoms. The presumed reason for this is that infants are not susceptible to *C. difficile* toxins owing to the poor development of the toxin receptors or cellular signaling pathways in the intestines (11). However, asymptomatic infantile carriers of toxigenic *C. difficile* are suggested to be potential reservoirs of pathogenic strains and to play a role in the transmission of CDI in adults (12–14). Investigations of the carriage rates of toxigenic *C. difficile* in infants can be informative for preventive control of CDI in adults.

According to a review by Jangi and Lamont (15), the carriage rates of *C. difficile* are 37% in healthy infants less than 1 month of age and 30% in those between 1 and 6 months of age. This rate drops considerably to 14% at between 6 and 12 months of age and decreases to 10% after 1 year of age. Because the carriage rate is likely to peak by 6 months of age, infants of these ages are thought to have a greater chance of spreading *C. difficile* strains. Therefore, it is of great importance to better understand the colonization patterns of *C. difficile* during the first 6 months of life.

Our aim was to investigate time-dependent changes in carriage rates, counts, and strain types of toxigenic *C. difficile* in infants. For this purpose, we examined stool specimens collected from 111 infants at 0 days (meconium), 3 days, 7 days, 30 days, 90 days, and 180 days of age and after weaning by using TaqMan-based quantitative PCR (qPCR) that was previously developed for the selective quantification of both total *C. difficile* strains (i.e., all types) and toxigenic strains (16). We isolated toxigenic *C. difficile* from stool specimens and genotyped the isolates by using capillary gel electrophoresis-based PCR ribotyping (CGE-PCR ribotyping).

TABLE 1 Stool specimens used in this study

Stool specimen	No. of specimens	Mean day of stool sampling ± SD[a]
Meconium	99	0.7 ± 1.0
Day 3	83	2.8 ± 1.0
Day 7	100	8.4 ± 2.1
Day 30	108	31 ± 3.4
Day 90	107	91 ± 5.9
Weaning	75	143 ± 20
Day 180	105	182 ± 7.9

[a] The day of delivery of each infant was defined as day 0.

MATERIALS AND METHODS

Stool specimens. Stool specimens collected from subjects in an observational study conducted in Belgium (ISRCTN66704989) (17) were used in this study. The study subjects were 111 infants (including 2 pairs of twins), 82 of whom were delivered vaginally and 29 of whom were delivered by cesarean section. Stool samples were taken at 0 days (meconium), 3 days, 7 days, 30 days, 90 days, and 180 days of age and after weaning (1 week after the introduction of solids, at 143 days of age on average) (Table 1).

This study was approved by the ethics committee of the hospital network of Antwerp (Ziekenhuisnetwerk Antwerpen) and conducted in compliance with ethical principles from the Declaration of Helsinki, guidelines of good clinical practice, and applicable regulations. Written informed consent was obtained from the infants' mothers.

Standard strain. *Clostridium difficile* DSM 1296[T] was selected as the standard strain for the generation of analytical curves for qPCR and was cultured anaerobically at 37°C in modified Gifu anaerobic medium broth (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 1% glucose. The number of bacterial cells in fresh cultures was determined by using the 4′,6-diamidino-2-phenylindole (DAPI) staining method. The suspension was stored at −80°C until use for DNA extraction. DNA was extracted from bacterial suspensions as described previously (18).

Quantification of *C. difficile* by qPCR. *Clostridium difficile* in stool specimens was quantified by using TaqMan-based qPCR with three primer-probe sets, namely, 16SrRNA-F/R/P, targeting toxin A-positive and toxin B-positive (A$^+$ B$^+$), toxin A-negative and toxin B-positive (A$^-$ B$^+$), and toxin A-negative and toxin B-negative (A$^-$ B$^-$) types; tcdB-F/R/P, targeting A$^+$ B$^+$ and A$^-$ B$^+$ types; and tcdA-F/R/P, targeting the A$^+$ B$^+$ type (Table 2), as previously described (16), with some modifications. Stool DNA extracted previously from stool samples of infants (17) was used for the analysis. In brief, for the preparation of a standard DNA solution, bacterial DNA was extracted from 2×10^8 cells of a pure culture of the DSM 1296[T] strain (A$^+$ B$^+$ type) and dissolved in 1 ml of Tris-EDTA (TE) buffer. Five microliters of 10-fold serial dilutions of

standard DNA was applied for PCR to obtain a standard analytical curve ranging from 10^0 to 10^5 cells/5 μl of template. Five microliters of the extracted stool DNA solution (10 mg stool DNA/ml) and 2- and 4-fold dilutions of this solution were applied for PCR as a template containing the corresponding amounts of DNA from 50, 25, and 12.5 μg of stool. The ABI Prism 7900HT sequence detection system (Thermo Fisher Scientific Inc., Waltham, MA) with 384-well optical plates was used as the qPCR platform. qPCR counts (cells per gram of stool) were calculated by applying the obtained threshold cycle (C_T) values for the stool specimens to the standard analytical curve. The lower detection limit was 2×10^4 cells (4.3 $\log_{10}$ cells)/g of stool.

Identification of toxin types of predominant *C. difficile* strains in individual stool specimens. The toxin type of the predominant *C. difficile* strains in each stool specimen was determined by comparing the three qPCR counts targeting the 16S rRNA gene for total *C. difficile* strains (A$^+$ B$^+$, A$^-$ B$^+$, and A$^-$ B$^-$ types), tcdB for TcdB-producing strains (A$^+$ B$^+$ and A$^-$ B$^+$ types), and tcdA for TcdA-producing strains (A$^+$ B$^+$ type), as previously described (16).

Isolation of toxigenic *C. difficile*. *Clostridium difficile* was isolated by stool culture with cefoxitin-cycloserine-egg yolk (CCEY) agar as previously described (16). In brief, plates of CCEY agar (BioConnections Ltd., Knypersley, United Kingdom) supplemented with 40 ml of egg yolk emulsion (BioConnections), two vials of cefoxitin-cycloserine (BioConnections), and 10 ml of lysed horse blood per liter of medium were prepared in-house and stored at 4°C for a maximum of 1 week before use. Frozen stool specimens were thawed and mixed with an equal volume of absolute ethanol. After incubation of the mixture at room temperature for 30 to 60 min, 100 μl of alcohol-treated stool or its 10-fold dilution was inoculated onto CCEY plates and cultured under anaerobic conditions at 37°C for 48 to 72 h in an anaerobic glove box (Coy Laboratory Products Inc., Grass Lake, MI). At least three suspected colonies were subjected to rapid identification of *C. difficile* by real-time PCR using the CD16SrRNA-F/R/P primer-probe set.

PCR identification of toxin types of isolates. The toxin type of each *C. difficile* isolate was identified by PCR for the tcdA and tcdB genes, as described previously by Lemee et al. (19). tcdA-specific primers (forward primer 5′-AGATTCCTATATTTACATGACAATAT-3′ and reverse primer 5′-GTATCAGGCATAAAGTAATATACTTT-3′) and tcdB-specific primers (forward primer 5′-GGAAAAGAGAATGGTTTTAT TAA-3′ and reverse primer 5′-ATCTTTAGTTATAACTTTGACATC TTT-3′) were used for amplification. If the sizes of the respective amplicons of tcdA and tcdB were 370 bp and 160 bp, the isolate was determined to be of the A$^+$ B$^+$ type. If the respective amplicons were 110 bp and 160 bp, the isolate was determined to be of the A$^-$ B$^+$ type.

Total bacterial counts in stool. The numbers of total stool bacteria were determined by the DAPI staining method as previously described (20).

TABLE 2 Oligonucleotides used in this study

Target	Gene	Oligonucleotide	Sequence (5′–3′)[a]
Total *Clostridium difficile* (A$^+$ B$^+$, A$^-$ B$^+$, and A$^-$ B$^+$ types)	16S rRNA gene	CD16SrRNA-F	GCAAGTTGAGCGATTTACTTCGGT
		CD16SrRNA-P	FAM-TGCCTCTCAAATATATTATCCCGTATTAG-TAMRA
		CD16SrRNA-R	GTACTGGCTCACCTTTGATATTYAAGAG
TcdB-producing *Clostridium difficile* (A$^+$ B$^+$ and A$^-$ B$^+$ types)	tcdB	tcdB-F	TACAAACAGGTGTATTTAGTACAGAAGATGGA
		tcdB-P	FAM-TTTKCCAGTAAAATCAATTGCTTC-TAMRA
		tcdB-R	CACCTATTTGATTTAGMCCTTTAAAAGC
TcdA-producing *Clostridium difficile* (A$^+$ B$^+$ type)	tcdA	tcdA-F	CAGTCGGATTGCAAGTAATTGACAAT
		tcdA-P	FAM-TTGAGATGATAGCAGTGTCAGGATTG-TAMRA
		tcdA-F	AGTAGTATCTACTACCATTAACAGTCTGC

[a] All oligonucleotides were described previously (16). FAM, 6-carboxyfluorescein; TAMRA, 6-carboxymethyltetramethylrhodamine.

TABLE 3 Bacterial counts of *Clostridium difficile* in 55 *C. difficile*-positive infants by qPCR

Subject information			qPCR counts of stool specimens using three primer-probe sets (log$_{10}$ cells/g stool) [c,d]																				
			Meconium			Day 3			Day 7			Day 30			Day 90			Weaning			Day 180		
ID	Sex[a]	Delivery mode[b]	CD16S rRNA-F/R/P	tcdA-F/R/P	tcdB-F/R/P	CD16S rRNA-F/R/P	tcdA-F/R/P	tcdB-F/R/P	CD16S rRNA-F/R/P	tcdA-F/R/P	tcdB-F/R/P	CD16S rRNA-F/R/P	tcdA-F/R/P	tcdB-F/R/P	CD16S rRNA-F/R/P	tcdA-F/R/P	tcdB-F/R/P	CD16S rRNA-F/R/P	tcdA-F/R/P	tcdB-F/R/P	CD16S rRNA-F/R/P	tcdA-F/R/P	tcdB-F/R/P
001	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			5.6	-	-
002	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			7.4	7.6	7.5
004	M	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.9	-	-	5.1	-	-
008	M	N	-	-	-	No sample			-	-	-	-	-	-	-	-	-	7.4	-	-	7.2	-	-
010	F	C	No sample			8.3	8.5	8.3	8.7	8.8	8.6	8.3	8.6	8.4	-	-	-	No sample			-	-	-
013	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.7	-	-	7.5	-	-
014	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			7.7	-	-
018	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8.7	-	-
019	F	C	-	-	-	-	-	-	No sample			8.3	-	-	-	-	-	No sample			-	-	-
024	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			8.0	8.1	8.1
025	M	N	-	-	-	No sample			No sample			-	-	-	No sample			4.5	4.7	4.6	6.9	7.0	6.9
028	M	N	No sample			-	-	-	No sample			-	-	-	-	-	-	-	-	-	5.4	-	-
029	M	N	-	-	-	-	-	-	-	-	-	-	-	-	5.6	-	-	-	-	-	7.2	-	-
030	M	C	-	-	-	No sample			-	-	-	-	-	-	-	-	-	-	-	-	7.2	-	-
033	F	C	-	-	-	No sample			-	-	-	7.2	-	-	-	-	-	-	-	-	7.4	-	-
037	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			8.0	-	-
039	F	N	-	-	-	No sample			-	-	-	-	-	-	7.2	-	-	7.0	-	-	7.1	-	-
040	M	C	-	-	-	8.3	-	-	No sample			6.2	-	-	7.4	-	-	No sample			-	-	-
041	F	N	-	-	-	7.7	-	-	4.3	-	-	5.2	-	-	-	-	-	No sample			No sample		
043	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			6.3	-	-
044	M	N	-	-	-	No sample			-	-	-	-	-	-	7.0	-	-	8.2	-	-	4.8	-	-
046	F	N	-	-	-	No sample			8.8	-	-	6.6	-	-	7.5	-	-	5.8	-	-	6.1	6.2	6.2
052	F	N	No sample			-	-	-	-	-	-	-	-	-	-	-	-	No sample			7.4	-	-
055	F	N	-	-	-	No sample			-	-	-	-	-	-	-	-	-	No sample			6.5	-	-
060	F	C	-	-	-	No sample			8.3	-	-	5.7	-	-	6.0	-	-	No sample			-	-	-
061	F	N	-	-	-	No sample			-	-	-	-	-	-	-	-	-	7.4	-	-	7.3	-	-
065	M	N	-	-	-	-	-	-	-	-	-	-	-	-	6.9	-	-	-	-	-	-	-	-
068	M	N	-	-	-	8.2	-	-	5.6	-	-	7.7	-	-	-	-	-	-	-	-	4.6	-	-
071	F	C	-	-	-	No sample			No sample			-	-	-	No sample			5.4	-	-	7.4	-	-
074	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.9	-	-
078	M	C	-	-	-	No sample			No sample			-	-	-	-	-	-	-	-	-	7.3	-	-
080	M	N	-	-	-	-	-	-	-	-	-	No sample			-	-	-	6.6	-	-	-	-	-
085	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.0	-	-	7.3	7.5	7.5
086	F	N	-	-	-	-	-	-	-	-	-	-	-	-	6.4	6.5	6.3	7.4	7.7	7.5	5.7	5.9	5.7
088	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.9	7.1	7.1	6.4	6.5	6.4
092	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.0	-	-	6.2	-	-
097	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7	6.9	6.9
101	M	C	No sample			-	-	-	No sample			-	-	-	-	-	-	7.5	7.8	7.7	6.6	6.7	6.6
102	M	N	-	-	-	5.8	6.0	5.8	-	-	-	5.6	5.7	5.7	5.4	5.6	5.5	7.2	7.4	7.3	6.0	6.3	6.2
105	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			5.1	-	-
109	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.1	-	-
110	M	N	No sample			No sample			-	-	-	-	-	-	-	-	-	-	-	-	5.5	-	-
113	F	C	-	-	-	No sample			5.8	6.1	5.9	6.4	6.6	6.4	6.8	7.0	6.9	7.6	7.8	7.6	6.7	6.9	6.7
117	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.8	-	-
118	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.9	-	8.1
119	M	N	-	-	-	-	-	-	-	-	-	No sample			No sample			No sample			7.3	7.5	7.5
121	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7	-	6.6	5.9	-	6.1
123	M	C	No sample			-	-	-	-	-	-	-	-	-	6.8	-	-	7.4	-	-	7.2	-	-
126	F	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8.1	-	-	7.0	-	-
130	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.9	-	-
133	F	C	-	-	-	No sample			-	-	-	-	-	-	-	-	-	-	-	-	7.9	-	-
136	F	N	-	-	-	No sample			-	-	-	-	-	-	-	-	-	8.1	8.3	8.1	6.9	7.0	7.0
137	M	N	-	-	-	No sample			-	-	-	-	-	-	8.8	8.8	8.9	7.9	8.2	8.1	7.0	7.1	7.0
138	M	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No sample			7.8	-	-
142	F	C	-	-	-	No sample			-	-	-	-	-	-	7.2	-	7.3	7.8	-	7.9	No sample		

[a] M, male; F, female.
[b] N, natural delivery; C, cesarean section.
[c] −, not detected.
[d] Toxin types of *C. difficile* predominating in each specimen were identified on the basis of qPCR counts for the three genes according to the criteria described in Materials and Methods. On the basis of toxin type, each specimen is highlighted as follows: red, A$^+$ B$^+$ type; blue, A$^-$ B$^+$ type; green, A$^-$ B$^-$ type.

TcdB production by *C. difficile* isolates. The TcdB production capacities of *C. difficile* isolates were determined by a cell cytotoxicity neutralization assay (CCNA) with a *C. difficile* Toxin/Antitoxin kit (Techlab, Blacksburg, VA), as previously described (16). In brief, identified *C. difficile* colonies were subcultured anaerobically at 37°C in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD) for 5 days. The suspension was then centrifuged at $16,000 \times g$ for 5 min, and the supernatant was filtered with a 0.45-μm membrane filter. The filtrate was added to a Vero-B4 cell culture with or without *C. difficile* antitoxin. After incubation of the culture at 37°C for 24 or 48 h, the presence or absence of TcdB was determined by judging the cytopathic effects.

Capillary gel electrophoresis-based PCR ribotyping. Strain types of *C. difficile* isolates were characterized by CGE-PCR ribotyping as described previously by Indra et al. (21), with some modifications. In brief, DNA was extracted from pure cultures of each isolate. The 16S rRNA-to-23S rRNA intergenic spacer region was amplified in a 25-μl PCR mixture composed of 0.5 U of Ex *Taq* DNA polymerase (TaKaRa Bio, Shiga, Japan), 1× Ex *Taq* buffer, 200 μM deoxyribonucleoside triphosphates, 0.5 μM primers, and 1 μl of template DNA. PCR amplification consisted of 1 cycle of 95°C for 30 s; 25 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 30 min. One microliter of the amplicon or its 10-fold dilution was mixed with 1 μl of GeneScan 600 LIZ (Thermo Fisher Scientific) and 9 μl of Hi-Di formamide (Thermo Fisher Scientific). After heating at 95°C for 3 min and subsequent incubation on ice for 5 min, the amplicon was analyzed by capillary gel electrophoresis using ABI Prism 3130xl genetic analyzers (Thermo Fisher Scientific). The fragment files (.fsa) obtained were imported into BioNumerics software version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium). Genetic relatedness based on fragment patterns was determined for fragments ranging in length from 200 to 600 nucleotides. A dendrogram was created by using the multiscale setting for comparisons and the Dice coefficient with the unweighted pair group method with arithmetic means (UPGMA) for clustering. The PCR ribotypes (PRs) were determined by querying the fragment files (.fsa) against the Web-based database WEBRIBO (http://webribo.ages.at/). If no PCR ribotype was matched, a new PCR ribotype (NPR plus one digit) specific to this study was allocated to the strain.

Multilocus sequence typing. Strain types of *C. difficile* isolates were characterized by multilocus sequence typing (MLST) with primers for seven housekeeping genes, *aroE*, *dutA*, *gmk*, *groEL*, *recA*, *sodA*, and *tpi*, as described previously by Rousseau et al. (13). In brief, DNA was extracted from pure cultures of each isolate in BHI broth. Each target gene was amplified in a 25-μl PCR mixture composed of 2 U Fast *Taq* polymerase (Roche, Basel, Switzerland), 1× PCR buffer, 200 μM deoxyribonucleoside triphosphates, 2 mM MgCl$_2$, 0.4 μM each primer, 10 μl of GC-RICH solution, and 1 μl of template DNA. PCR amplification consisted of 1 cycle of 95°C for 5 min; 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min; and 1 cycle of 72°C for 10 min. The amplicons were purified with ExoSAP-IT for PCR product cleanup (USB, Cleveland, OH) and sequenced with primers for each gene by using ABI Prism 3130xl genetic analyzers. The sequences obtained for the seven genes were analyzed by using BioNumerics. BioNumerics queried a database (*C. difficile* MLST database at http://bigsdb.pasteur.fr/) for the sequences of the seven genes from each strain and allocated a specific sequencing type (ST) to each strain automatically. A dendrogram was created by using the multiscale setting for comparisons and the UPGMA for clustering.

Statistical analysis. Characteristics of *C. difficile*-positive infants and *C. difficile*-negative infants were compared by using Fisher's exact test or a χ^2 test.

Accession number(s). Nucleotide sequences determined by MLST were deposited in the GenBank/EMBL/DDBJ database under accession numbers LC151619 to LC151870.

RESULTS

Carriage rates and bacterial counts of *C. difficile* in stool samples, as determined by qPCR. Infant stool samples collected 7 times from birth until 180 days of age were examined by qPCR with the CD16SrRNA-F/R/P, tcdB-F/R/P, and tcdA-F/R/P primer-probe sets to quantify total *C. difficile* strains ($A^+ B^+$, $A^- B^+$, and $A^- B^-$ types), TcdB-producing strains ($A^+ B^+$ and $A^- B^+$ types), and TcdA-producing strains ($A^+ B^+$ type), respectively (see Table S1 in the supplemental material). In the entire period up to the age of 6 months, *C. difficile* was detected in 55 of 111 infants (50%), and toxigenic *C. difficile* was found in 18 infants (16%). Details of the counts of *C. difficile* bacteria in the 55 *C. difficile*-positive infants are shown in Table 3. The number of toxigenic *C. difficile* bacteria ranged from 10^4 to 10^8 cells/g of stool. Certain infants (e.g., subjects 010 and 137) retained large quantities of toxigenic strains (10^8 cells/g of stool) for several weeks. The number of total stool bacteria in these infants ranged from $\sim 10^9$ to 10^{11} cells/g of stool (see Table S2 in the supplemental material). The proportion of *C. difficile* strains among the total bacteria reached as high as 10% in subject 010 at 3 days of age.

Clostridium difficile was not detected in meconium samples, but 6% of infants had acquired *C. difficile* by the age of 3 days. Thereafter, the carriage rate of *C. difficile* rose to 12% at 90 days of age, then rose to 31% at weaning, and finally rose to 45% at 180 days of age (Fig. 1). The rate of carriage of toxigenic *C. difficile* ($A^+ B^+$ type and $A^- B^+$ type) similarly increased from 2% at 3 days of age to 15% at 180 days of age (Fig. 1). Regardless of the age of infants, approximately two-thirds of all *C. difficile* carriers were carriers of a nontoxigenic strain ($A^- B^-$ type), and one-third were carriers of a toxigenic strain (mostly of the $A^+ B^+$ type).

Isolation of toxigenic *C. difficile* from stool samples and characterization of the isolates. For further characterization of toxigenic *C. difficile* in infantile intestines, we isolated *C. difficile* strains from stool specimens that were positive for toxigenic *C. difficile* upon qPCR analysis. *Clostridium difficile* was isolated from 36 of the 38 positive stool specimens (Table 4). PCR amplification of *tcdA* and *tcdB* revealed that the isolates from 31 of the 36 specimens were of the $A^+ B^+$ type, and those from the other 5 specimens were of the $A^- B^+$ type, in agreement with the toxin types of the predominant *C. difficile* strains identified by qPCR with stool DNA (Table 3). There were no specimens from which strains of two or more different toxin types were isolated. All the isolates from the 36 specimens were confirmed by CCNAs to be capable of producing the TcdB toxin.

Comparison of genotypes of toxigenic *C. difficile* isolates. A total of 36 toxigenic *C. difficile* isolates from 18 infants were genotyped by CGE-PCR ribotyping. A dendrogram was generated by comparing the fragment patterns of amplicons of the 16S rRNA-to-23S rRNA intergenic spacer region. The 36 strains were classified into 10 monophyletic strains (clusters a to j) (Fig. 2). The largest cluster was cluster c, which was composed of 14 individual strains from 5 infants, followed by cluster g, which was composed of 5 individual strains from 3 infants. A Web-based database, WEBRIBO, was queried for the fragment data on each isolate, and a matched PCR ribotype (PR) was assigned to each strain. Most clusters were assigned specific registered PRs, and clusters b, g, and h, which did not match any PRs in the database, were assigned in-house PRs (NPRs 1, 2, and 3). The discrimination based on the assignment of PRs was concordant with that of clustering analysis based on the fragment patterns, except in the cases of clusters c and d. The same PR, PR 014/0 (014 subtype 0), was allocated to clusters c and d. Each of the 11 infants from whom toxigenic *C. difficile* was isolated at least twice retained a single monophyletic
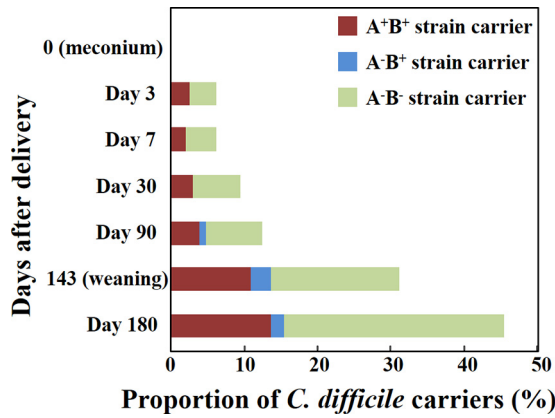
FIG 1 Carriage rates of each toxin type of *Clostridium difficile* in infants. On the basis of the qPCR counts for three genes (16S rRNA, *tcdA*, and *tcdB*), the toxin types (A$^+$ B$^+$, A$^-$ B$^+$, or A$^-$ B$^-$) of predominant *C. difficile* strains in individual specimens were identified. The rates of carriage of each toxin type of *C. difficile* were calculated with respect to each stool specimen group.

strain. In particular, infant 102 harbored the same monophyletic strain (PR 014/0) for a long period: from 3 days of age until 180 days of age (cluster c) (Fig. 2). MLST analysis showed a typing result very similar to that of CGE-PCR ribotyping (see Fig. S1 in the supplemental material) (although the resolution was slightly lower than that of CGE-PCR ribotyping), supporting our finding that each individual subject retained a particular single strain in their intestines.

Comparison of characteristics of *C. difficile*-positive and -negative infants at 6 months of age. To investigate factors that could be related to colonization with *C. difficile*, we compared a number of characteristics of *C. difficile*-positive ($n = 47$) and -negative ($n = 58$) infants at 6 months of age (Table 5). Although there was no significant difference in the male-to-female ratio and mode of delivery between the two groups, the proportion of infants who had continued to receive breast milk until 6 months of age was significantly higher in the *C. difficile*-negative group ($P < 0.001$). At 6 months of age, the percentage of infants who had begun ingesting solids was higher in the *C. difficile*-positive group than in the negative group ($P < 0.05$).

DISCUSSION

To our knowledge, this is the largest study to have investigated patterns of colonization by *C. difficile* in the infant gut. Previous studies were limited to reports of the bacterium's prevalence at a single time point or in relatively small numbers of infants (22, 23). We examined both the carriage rates and bacterial counts for total and toxigenic *C. difficile* strains in 111 infants over time, from immediately after birth until 180 days of age. We observed that carriage of *C. difficile* increased dramatically between 90 days of age and weaning (at an average of 143 days of age) (Fig. 1). In France, Rousseau et al. (12) examined stool samples of 10 healthy infants for *C. difficile* monthly during the first year of life; they reported that the *C. difficile* carriage rate rose sharply from 30% at 3 months of age to 80% at 4 months of age. This sharp rise was similar to what we detected, although their carriage rates were much higher than those reported here. In our study, regardless of the age of infants, carriers of toxigenic *C. difficile* accounted for approximately one-third of the total *C. difficile* carriers (Fig. 1).

This finding was also consistent with those of a single-point survey of 85 infants ranging in age from 1 month to 3 years by Rousseau et al. (12): they found that 38 infants (45%) carried *C. difficile* and that 11 infants (13%) carried toxigenic *C. difficile*. An advantage of our study was the quantitative analysis of both total and toxigenic *C. difficile* bacteria. Although there have been several reports of the quantification of *C. difficile* bacteria in infants (9, 24–26), we report here a longitudinal quantitative analysis of both total *C. difficile* and toxigenic strains in healthy infants. A total of 18 asymptomatic infants carried toxigenic *C. difficile*, with an average count of ~10^7 cells/g of stool (see Table S1 in the supplemental material). Eleven of the 18 infants retained toxigenic *C. difficile*, some at

TABLE 4 Isolation and characterization of toxigenic *Clostridium difficile*

Subject ID	Sampling day	Toxin type of *C. difficile* in stool[a]	Isolation of *C. difficile*	Isolate test		CCNA[c]
				PCR test[b]		
				tcdA/tcdB amplicon (bp)	Toxin type	
002	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
010	Day 3	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 7	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 30	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
024	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
025	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
046	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
085	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
086	Day 90	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
088	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
097	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
101	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
102	Day 3	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 30	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 90	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
113	Day 7	A$^+$ B$^+$	No	n/a	n/a	n/a
	Day 30	A$^+$ B$^+$	No	n/a	n/a	n/a
	Day 90	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
118	Day 180	A$^-$ B$^+$	Yes	110/160	A$^-$ B$^+$	Pos
119	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
121	weaning	A$^-$ B$^+$	Yes	110/160	A$^-$ B$^+$	Pos
	Day 180	A$^-$ B$^+$	Yes	110/160	A$^-$ B$^+$	Pos
136	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
137	Day 90	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	weaning	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
	Day 180	A$^+$ B$^+$	Yes	370/160	A$^+$ B$^+$	Pos
142	Day 90	A$^-$ B$^+$	Yes	110/160	A$^-$ B$^+$	Pos
	weaning	A$^-$ B$^+$	Yes	110/160	A$^-$ B$^+$	Pos

[a] Toxin types of *C. difficile* predominating in each specimen were identified on the basis of qPCR counts for the three genes.
[b] n/a, not applicable.
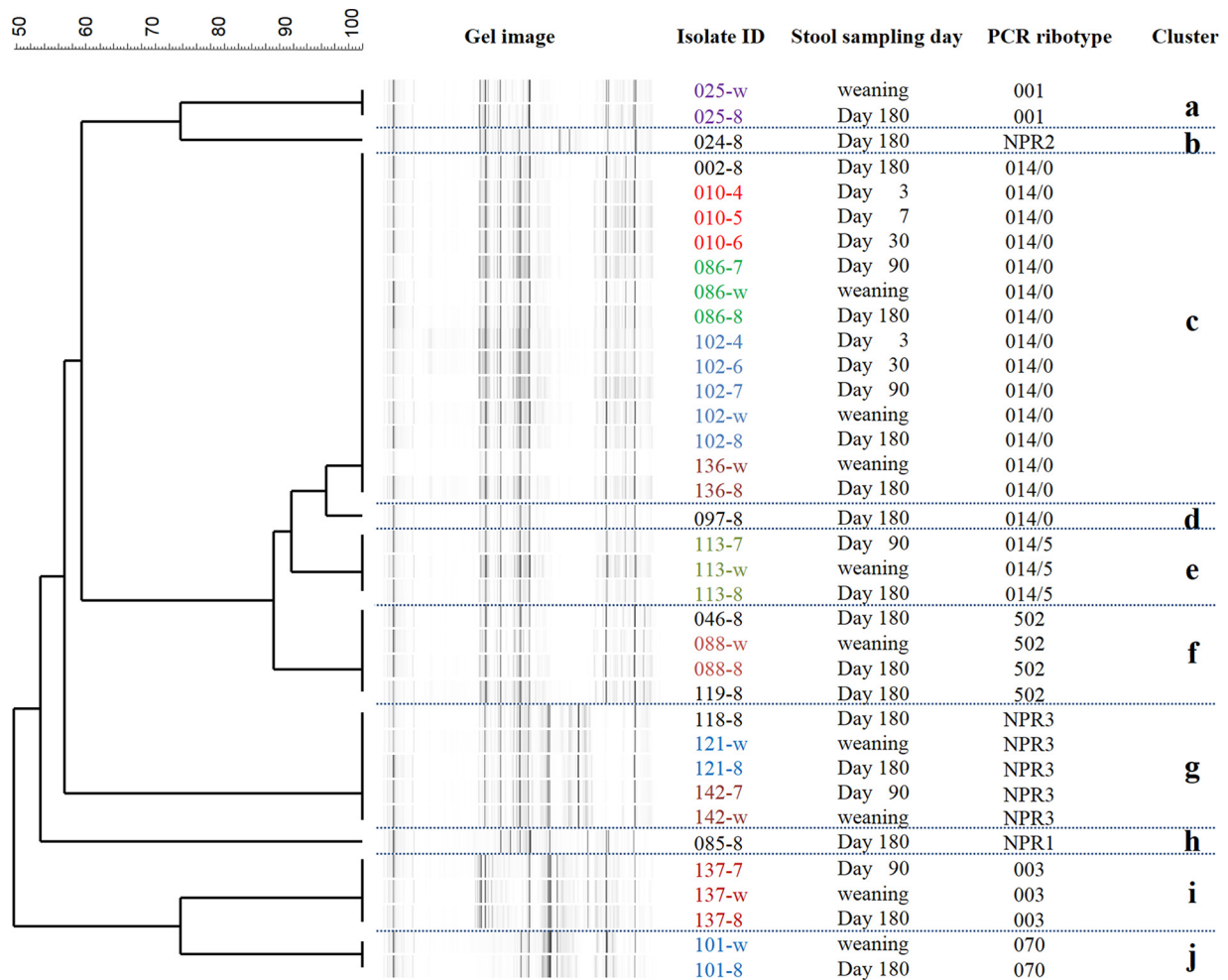[c] CCNA, cell cytotoxicity neutralization assay.

FIG 2 Dendrogram of toxigenic *Clostridium difficile* isolates from infants, as determined by CGE-PCR ribotyping analysis. The dendrogram was created with a multiscale setting for comparison and the unweighted pair group method with arithmetic means for clustering. Amplicons of the 16S-to-23S intergenic spacer region were analyzed by capillary gel electrophoresis with a 3130xl genetic analyzer. Genetic relatedness based on the fragment patterns was determined for fragments ranging in length from 200 to 600 nucleotides. PCR ribotypes were determined by querying fragment files (.fsa) against the Web-based database WEBRIBO (http://webribo.ages.at/). If no matched PCR ribotype was obtained, a new PCR ribotype (NPR plus one digit) specific to this study was allocated to the strain.

levels as high as 10^8 cells/g of stool (Table 3). In our previous study, the mean *C. difficile* count in asymptomatic elderly individuals was 10^4 cells/g of stool (16); thus, *C. difficile* levels as a proportion of the total microbiota population were relatively high in infants. Moreover, Naaber et al. (27) reported an average *C. difficile* count in patients ranging in age from 3 to 89 years (median, 72 years of age) with antibiotic-associated diarrhea of $10^{7.9}$ cells/g of stool. Thus, our asymptomatic infants carried quite high numbers

TABLE 5 Comparison of characteristics of *Clostridium difficile*-positive infants and *C. difficile*-negative infants at 6 months of age[a]

Characteristic	Value for group			
	C. difficile-positive infants infected with:			
	Toxigenic strains (*n* = 16)	Nontoxigenic strains (*n* = 31)	Total (*n* = 47)	*C. difficile*-negative infants (*n* = 58)
No. of males/no. of females	9/7	19/12	28/19	29/29
No. of infants with natural delivery/no. of infants with cesarean delivery	14/2	24/7	38/9	40/18
No. (%) of infants with continual intake of breast milk for 6 mo	2 (13)	8 (26)	10 (21)***	32 (55)
No. (%) of infants with initiation of solid food	15 (94)	30 (97)	45 (96)*	47 (81)

[a] Comparison between *C. difficile*-positive infants (total) and *C. difficile*-negative infants was done by using Fisher's exact test or a χ^2 test. *, $P < 0.05$; ***, $P < 0.001$.
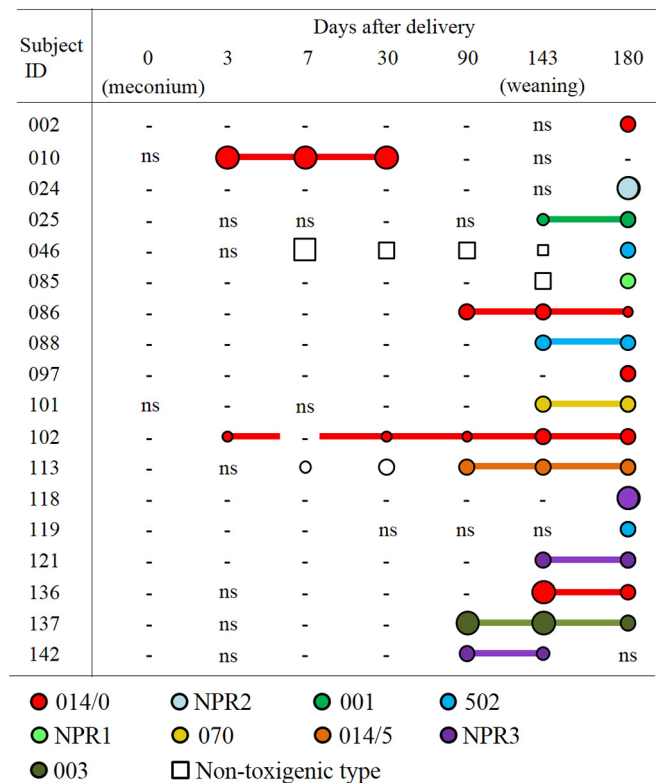
Subject ID	Days after delivery						
	0 (meconium)	3	7	30	90	143 (weaning)	180
002	-	-	-	-	-	ns	● (014/0)
010	ns	● (014/0)	● (014/0)	● (014/0)	-	ns	-
024	-	-	-	-	-	ns	◯ (NPR2)
025	-	ns	ns	-	ns	● (001)	● (001)
046	-	ns	□	□	□	□	● (502)
085	-	-	-	-	-	□	● (NPR1)
086	-	-	-	-	● (014/0)	● (014/0)	● (014/0)
088	-	-	-	-	● (502)	● (502)	● (502)
097	-	-	-	-	-	-	● (014/0)
101	ns	-	ns	-	-	● (070)	● (070)
102	-	● (014/0)	-	● (014/0)	● (014/0)	● (014/0)	● (014/0)
113	-	ns	◯	◯	● (014/5)	● (014/5)	● (014/5)
118	-	-	-	-	-	-	● (NPR3)
119	-	-	-	ns	ns	ns	● (502)
121	-	-	-	-	-	● (NPR3)	● (NPR3)
136	-	ns	-	-	-	● (014/0)	● (014/0)
137	-	ns	-	-	● (003)	● (003)	● (003)
142	-	ns	-	-	● (NPR3)	● (NPR3)	ns

Legend:
- ● 014/0
- ◯ NPR2
- ● 001
- ● 502
- ● NPR1
- ● 070
- ● 014/5
- ● NPR3
- ● 003
- □ Non-toxigenic type

FIG 3 Longitudinal changes in bacterial counts and genotypes of toxigenic *Clostridium difficile* isolates in 18 infants. Circles and squares indicate the detection of toxigenic *C. difficile* and nontoxigenic *C. difficile* by qPCR, respectively. The sizes of the symbols reflect bacterial numbers as determined by qPCR, where small symbols indicate $<6.0 \log_{10}$ cells/g stool, medium symbols indicate 6.0 to 8.0 $\log_{10}$ cells/g stool, and large symbols indicate $>8.0 \log_{10}$ cells/g stool. A minus symbol means that no *C. difficile* was detected by qPCR. "ns," no samples. Circles filled with color indicate the isolation of a *C. difficile* strain by stool culture, and each color corresponds to a genotype of toxigenic *C. difficile* identified by PCR ribotyping. For each infant, strains that were confirmed to be monophyletic are linked with a line.

of *C. difficile* bacteria, comparable to those in patients with antibiotic-associated diarrhea.

Even though *C. difficile* seems to be an early colonizer of the infant gut, its source remains unknown. We did not detect *C. difficile* in any meconium specimens (Fig. 1), and it was detected in only 1 of 109 maternal stool specimens collected before delivery (data not shown), in agreement with data from previous studies (28–32). Therefore, it is quite unlikely that *C. difficile* is transmitted from the mother to the infant gut. Acquisition of *C. difficile* from the hospital environment at birth is generally considered a possible transmission route (11). We considered that this may have been the case in infants in whom we detected *C. difficile* at 1 month of age. Interestingly, the rate of acquisition of *C. difficile* within the first month of life in infants delivered by cesarean section (20.7%; 6/29) was higher than that in naturally delivered infants (4.9%; 4/82) ($P < 0.05$); this suggests that infants delivered by cesarean section are less resistant to *C. difficile* colonization. On the other hand, the carriage rate of *C. difficile* rose dramatically between 90 days of age and weaning (at an average of 143 days of age) (Fig. 1). Taking into account the fact that infants of these ages spend most of their time at home, or in day nurseries, it is unlikely that they acquired *C. difficile* in the hospital. Therefore, we spec-

ulated on two potential sources of *C. difficile*: solid foods and the surrounding living environment. Unfortunately, we did not collect samples immediately before the initiation of solids, and this limited our ability to determine whether food diversification was the reason for *C. difficile* acquisition. In the previous study by Rousseau et al. (12), the proportion of *C. difficile*-positive infants jumped to 80% at 4 months of age, but only 25% of these positive infants had started food diversification. In contrast, Fallani et al. (33) reported that the proportion of infants carrying *C. difficile* significantly decreased after weaning. Accordingly, solid foods could not have been the main source of *C. difficile* in infants. Generally, infants become increasingly physically mobile at around 3 months of age or older and are inclined to put various objects into their mouths; therefore, we considered that oral intake of *C. difficile* from surfaces in living environments was reasonable. Although it is unclear whether individual houses are contaminated with *C. difficile*, one study demonstrated the contamination of day nurseries with *C. difficile* and also the transmission of certain *C. difficile* strains between these facilities and the children attending them (28). We therefore suggest that infants acquire *C. difficile* bacteria that are ubiquitous in their living environments, although further investigations are needed.

Clostridium difficile was detected at least twice in 29 of the 55 *C. difficile*-positive infants, and 27 of these 29 infants retained the same toxin types of *C. difficile* (Table 3). Moreover, both of our typing analyses (CGE-PCR ribotyping and MLST) revealed that the same monophyletic strain was retained in each infant (Fig. 2; see also Fig. S1 in the supplemental material). Figure 3 summarizes the longitudinal changes of *C. difficile* bacteria in the 18 toxigenic *C. difficile*-positive infants. In agreement with findings of previous studies (12, 23), specific single strains of the same genotype were retained over time in each of the 11 infants who yielded toxigenic *C. difficile* isolates on at least two occasions. These results suggested that *C. difficile* in a majority of infants was not transient, but rather was a colonizer, in their intestines. The reason why *C. difficile* is more commonly found in the infant gut than in adults has not been thoroughly elucidated. It was recently suggested that certain bacterial species belonging to *Clostridium* cluster XIVa can prevent colonization by *C. difficile* (2, 3). Intestinal microbiota that are of low complexity and immature and that lack such bacteria possessing possible *C. difficile*-inhibitory effects may be responsible for colonization (34). As an extrinsic factor, intake of breast milk decreased carriage rates of *C. difficile* (Table 5) and was an inhibitor of colonization by *C. difficile*, as reported previously in other studies (24–26, 35).

We report here an in-depth survey of genotypes of *C. difficile* isolates in Belgian infants. The most frequently detected toxigenic *C. difficile* strain in the CGE-PCR ribotyping analysis was of PR 014/0 (Fig. 2); in the MLST analysis, it was of ST33 (see Fig. S1 in the supplemental material). Fifteen of the 36 toxigenic *C. difficile* isolates (42%) were identified as being both ST33 and PR 014/0 isolates. A previous study showed that ST33 was the most frequently identified strain type of toxigenic *C. difficile* in French infants (13). Many PCR ribotyping-based studies have revealed that PR 014 is the most frequent strain type of toxigenic *C. difficile* in French infants (12, 13) and the second most frequent one after PR 001 in Swedish infants (23). PR 014 strains are also frequently isolated from adult CDI patients (36). Interestingly, a recent survey of CDI patients in Belgium revealed that PR 014 had become one of the most prevalent strain types identified in hospitals (37).

It is probable that PR 014 is the most common strain type, not only in healthy infants but also in adult CDI patients, in Belgium, and this may support the recently proposed hypothesis that asymptomatic infants are a reservoir of pathogenic strains and contribute to the mediation of CDI in adults (12, 14, 23). The results of a literature search support the associations of specific PRs between infants and adult patients in other countries, namely, PR 014 in France (12, 38) and PR 001 in Sweden (23, 39). The possibility of direct transmission of specific strains from asymptomatic babies to their mothers, who then suffer recurrent CDI, has been reported (14), although this form of transmission remains speculative. Furthermore, it was recently reported that close contact with children less than 2 years of age is a risk factor for community-associated CDI in adults (40). We consider it highly credible that CDI in adults can be mediated by asymptomatic infant carriers of toxigenic *C. difficile*.

In conclusion, we investigated longitudinal changes in the carriage rates, counts, and genotypes of toxigenic *C. difficile* bacteria in infants from after birth until the age of 6 months. We found that considerable numbers of toxigenic *C. difficile* bacteria colonized the intestines of healthy infants. The toxigenic strains were of the same genotype as that capable of causing CDI in adults, supporting the hypothesis that asymptomatic infants can mediate CDI in adults by transmitting pathogenic *C. difficile*. These findings are informative not only for ecological studies but also for efforts to prevent or control the spread of CDI in adults.

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