Colonization of the Large Bowel by *Clostridium difficile* in Healthy Infants: Quantitative Study

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Colonization of the large bowel of healthy infants by *Clostridium difficile* was studied. Feces were collected from five breast-fed and five formula-fed infants throughout the first year of life, and levels of *C. difficile* were quantitated. Three breast-fed and five formula-fed infants were colonized for periods of between 8 and 42 weeks, and another infant harbored the organism only during week 1. Colonization of breast-fed infants commenced before or during weaning, with levels reaching 10^3 to 10^5 organisms per g of wet feces. Colonization of formula-fed infants commenced before solid foods were given, with levels of 10^3 to 10^7 organisms per g of wet feces. Isolates from eight of the babies were shown to produce cytotoxin in vitro. Single fecal specimens from 60 more children aged up to 4 years were also examined, and it was found that the carriage rate of *C. difficile* fell sharply after 1 year of age, although in the second year it was still higher than in adults. These findings are discussed in relation to the microbial ecology of the large bowel and the paradox that levels of *C. difficile* in the large bowel of healthy infants are similar to those causing pseudomembranous colitis in patients.

Clostridium difficile is now widely accepted as the major causative organism of pseudomembranous colitis (PMC). The organism can be isolated at high frequency from the stools of PMC patients and also from patients receiving antimicrobial therapy but without PMC, but is found only rarely in normal adults. Furthermore, *C. difficile* cytotoxin has been demonstrated in the feces of 97% of PMC patients (4). The etiology of PMC does not depend on the presence of the organism alone, but disease seems to be precipitated by factors which disturb the ecology of the gastrointestinal tract, notably antibiotic therapy but also surgery (2, 11) or cancer chemotherapy (10).

In contrast to adults, infants commonly harbor *C. difficile* in the intestinal tract. Hall and O'Toole (15) isolated the organism from infant feces as early as 1935, and in 1940 Snyder (22) reported isolations from 10 of 22 formula-fed infants ranging in age from 8 to 48 weeks. In later studies Larson et al. (18) and Kelsey and Vince (17) isolated *C. difficile* from 5 of 8 and 3 of 17 healthy babies, respectively. In a previous survey in this laboratory *C. difficile* was demonstrated in the feces of 4 of 10 normal infants aged between 1 and 45 weeks (P. L. Stark and A. Lee, J. Pediatr., in press).

C. difficile is not unique in its selective colonization of the human intestine during infancy. Other species (e.g., *C. butyricum*) have been shown to occur frequently in infants but only rarely in adults, reflecting fundamental differences in the composition and physicochemical conditions of the intestinal milieu in the different age groups (Stark and Lee, J. Pediatr., in press). However, *C. difficile* is exceptional in that it exists as a harmless commensal in a considerable proportion of healthy infants but causes the symptoms of PMC in certain patients. The reasons for this difference are unknown, and to date most work has concentrated on the pathogenic role of the organism.

The present study was undertaken to define the role of *C. difficile* in the microbial ecology of the normal infant gastrointestinal tract. Five breast-fed and five formula-fed infants were studied prospectively throughout the first year of life, using an improved selective medium to quantitate the levels of organisms present in the feces. Specimens from 80 other individuals of different ages were also examined to determine the incidence of the organism in other age groups.

MATERIALS AND METHODS

Subjects. Serial fecal specimens were collected from five breast-fed and five formula-fed babies who were born at the Women's Hospital, Sydney, Australia, and thereafter lived in typical Sydney suburban homes. Between three and five samples were collected from each baby during the first week of life, one at week 4 and then at approximately 8-week intervals for the next 11 months. Single fecal specimens were also collected from 13 infants of <1 year of age who were temporary residents of the Karitane Mothercraft Hospital (Sydney), 47 children aged between 1 and 4 years

TABLE 1. Incidence of *C. difficile* in the large bowel of infants, children, and adults

Age (yr)	No. of individuals sampled	No. positive for *C. difficile*
<1	13	7
1	10	3
2	17	1
3	12	0
4	8	0
20–40	28	1

who were living at home with their Sydney families, and 28 adults who were staff or postgraduate students at the University of New South Wales.

None of the subjects studied suffered from gastrointestinal or any major illness or received antibiotic therapy during the course of the survey.

Specimen collection and transport. Fecal samples (approximately 0.5 g) were collected into bijou bottles containing 4.5 ml of prereduced salts solution (16), 10% glycerol, cysteine HCl (0.5 g/liter), and resazurin (1 mg/liter) overlaid by a thin layer of paraffin oil. Samples were frozen at −60°C on the day of collection (9).

Culture and identification of *C. difficile*. Serial 10-fold dilutions of fecal suspensions were prepared in prereduced brain heart infusion (BHI), and 0.1-ml aliquots were spread across the surface of prereduced *C. difficile* selective agar plates. The selective agar was modified from George et al. (12) and contained cefoxitin (16 μg/ml) and cycloserine (500 μg/ml) in supplemented BHI agar (16). Quantitative studies showed that recovery of *C. difficile* on this selective agar was comparable to that on supplemented BHI agar without selective agents.

Isolates were subcultured onto supplemented BHI agar, and the pure cultures so obtained were used to inoculate BHI broths for gas-liquid chromatography. Fecal dilutions were prepared, and plates were inoculated and incubated at 37°C for 48 h in an anaerobic

chamber containing an atmosphere of 10% H_2 in CO_2 circulated over a palladium catalyst.

Identification was based on Gram stain morphology, gas-liquid chromatographic analysis of volatile fatty acids produced as end products of carbohydrate metabolism, and fermentation reactions as determined in API 20A strips (API Diagnostic Reagents).

Toxin assay and neutralization. *C. difficile* isolates were grown in BHI broths containing 10% glucose and cysteine HCl (0.5 g/liter) for 5 days at 37°C in the anaerobic chamber. The broths were centrifuged, and supernatants were concentrated by $(NH_4)_2SO_4$ precipitation and then filter sterilized as previously described (8). The culture filtrates were divided into small aliquots and frozen at −60°C until use.

Each culture filtrate (0.1 ml) was added to two tubes containing monolayers of human lung fibroblast cells (MRC5 strain). Medium 119 containing Hank salts and 2% fetal calf serum (0.9 ml) was then added to each tube. For neutralization tests, culture filtrates were preincubated for 30 min at 37°C with an equal volume of *C. sordellii* or *C. histolyticum* antitoxin (Wellcome Research Laboratories) before being added to the cell cultures. Tests were read after 6, 18, and 24 h of incubation at 37°C. A positive test was one that showed cytopathic changes in at least 50% of the cell monolayers and in which the effect could be completely neutralized by *C. sordellii* antitoxin but not by *C. histolyticum* antitoxin.

RESULTS

Incidence of *C. difficile* in the large bowel of infants, children, and adults. The frequency with which *C. difficile* was isolated from the feces of individuals of different ages is shown in Table 1. Isolation rates were highest in babies of <1 year of age, whereas the organism could still be isolated from 30% of babies in their second year of life, compared with only approximately 3% adults.

Prospective study of the colonization by *C.*

TABLE 2. Colonization of the large bowel of breast-fed infants by *C. difficile*

Age (wk)	Viable count of *C. difficile*[a]					Total no. colonized (total no. examined)
	1[b]	2	3	4	5	
1	<3	3.3–4.2[c]	<3	3.3–4.6	<3	2 (5)
2–8	<3	<3	<3	4.9	<3	1 (5)
9–16	<3	<3	NE[d]	3.2	<3	1 (4)
17–24	<3	<3	<3	4.9	<3	1 (5)
25–32	<3	NE	5.2	4.2	<3	2 (4)
33–40	5.2	<3	5.2	NE	<3	2 (4)
41–48	3.1	<3	<3	<3	NE	1 (4)
49–56	NE	<3	<3	<3	NE	0 (3)
57–64	<3	NE	<3	NE	NE	0 (2)

[a] Expressed as log_{10} number of viable organisms per gram of wet feces. Double rule indicates time when first solid food was given.

[b] Each column represents a single baby identified by number.

[c] Where more than one isolation was made during the first week, the range of counts is given.

[d] NE, Not examined.

TABLE 3. Colonization of the large bowel of formula-fed infants by *C. difficile*

Age (wk)	Viable count of *C. difficile*[a]					Total no. colonized (total no. examined)
	6[b]	7	8	9	10	
1	<3	<3	3.1	<3	6.1	2 (5)
2–8	<3	<3	6.3	6.1	5.1	3 (5)
9–16	3.4	5.8	5.3	4.1	4.1	5 (5)
17–24	4.2	4.5	6.2	<3	3.1	4 (5)
25–32	NE[c]	7.1	5.1	3.3	4.1	4 (4)
33–40	<3	4.7	5.2	NE	NE	2 (3)
41–48	NE	NE	NE	4.4	4.5	2 (4)
49–56	<3	<3	<3	<3	<3	0 (3)
57–64	NE	<3	<3	NE	NE	0 (2)

[a] Expressed as $\log_{10}$ number of viable organisms per gram of wet feces. Double rule indicates time when first solid food was given.

[b] Each column represents a single baby identified by number.

[c] NE, Not examined.

difficile of the large bowel of infants during the first year of life. Fecal samples were collected from 10 babies at intervals during the first year of life and cultured to quantitate the numbers of *C. difficile* present. Of the five breast-fed babies, one yielded the organism only during the first week of life, one was colonized during the period of exclusive breast feeding, and two were colonized during weaning (Table 2). By comparison, four of the formula-fed infants were colonized before solid foods were given and one was colonized shortly thereafter (Table 3).

The duration of colonization was longer in formula-fed infants. *C. difficile* could be demonstrated in the feces of formula-fed infants for periods lasting from 14 to 42 weeks compared with 8 to 29 weeks in the breast-fed group. However, *C. difficile* could not be demonstrated in any of the babies in the prospective study after 45 weeks of age.

The counts of *C. difficile* varied between 10^3 and 10^7 organisms per g of wet feces for formula-fed infants and 10^3 and 10^5 organisms per g of wet feces for the breast-fed group. By comparison, levels of *C. difficile* in the older children and adults did not exceed 10^4 organisms per g of wet feces (Table 4).

Toxin assay and neutralization. Isolates from the nine babies in the prospective study who yielded *C. difficile* on culture were tested for cytotoxin production, using human lung fibroblast tissue cultures. Isolates from eight of these babies were found to produce cytotoxin in broth culture whereas those from the remaining baby were negative, indicating that either cytotoxin had not been produced or titers were too low to cause typical actinomorphic changes in fibroblast cells. In each case in which a cytopathic effect was observed, it could be neutralized by

C. sordellii antitoxin but not by *C. histolyticum* antitoxin.

DISCUSSION

This study has shown that *C. difficile* may be isolated from 90% of healthy infants during the first year of life and must be considered as part of the normal microbial flora for individuals in this age group. This isolation rate is higher than the 30 to 50% reported by other authors (1, 18, 24). In 8 of 10 babies studied prospectively, the organism was isolated repeatedly over a period of months, demonstrating that a stable colonization of the large bowel had occurred, whereas in one infant *C. difficile* had only a transient presence in the gastrointestinal tract during the neonatal period. This is in contrast to the suggestion of Larson et al. (18) that the colonization of neonates by *C. difficile* is transient. Colonization occurred in both breast- and formula-fed infants, although it generally commenced earlier in the formula-fed group. The latter finding supports earlier observations, made using less sensitive

TABLE 4. Levels of *C. difficile* in the large bowel of individuals of different ages

Age (yr)	No. of individuals	Viable count of *C. difficile*[a]
<1 (breast fed)	4	4.3 ± 0.81
<1 (formula fed)	5	4.8 ± 1.2
1	3	3.3 ± 0.15
2	1	4.2
24	1	3.7

[a] Expressed as $\log_{10}$ number of viable organisms per gram of wet feces. Where more than one specimen was examined, the mean viable count ± 1 standard deviation is given.

isolation techniques, that colonization of the infant intestine by clostridia is inhibited during exclusive breast feeding (P. L. Stark and A. Lee, J. Med. Microbiol., in press).

The carriage rate for *C. difficile* in infants fell after 1 year of age but in the second year was still higher than in adults. The 3% carriage rate found for adults in this survey is in agreement with that reported for adults in the United States (13).

The high incidence of colonization of infants by *C. difficile* suggests that this organism is more widespread in our environment than previously suspected. Hafiz et al. (14) have isolated the organism from the genital tract of females. However, it is more likely that infants colonized during weaning obtained the organism from other carriers, e.g., food or their surroundings, rather than from the birth canal. The babies in turn may be an exogenous source of *C. difficile* for adults who are receiving antibiotics.

It is paradoxical that *C. difficile* can be both a harmless commensal in the gastrointestinal tract of infants and the causative agent of PMC and, furthermore, that the incidence of PMC in infants is very low (19) despite the fact that this age group is frequently colonized by the causative organism. Levels of *C. difficile* between 10^5 and 10^9 bacteria per g of feces have been reported for PMC patients (3–5, 24). By comparison, levels of over 10^6 bacteria per g of feces were recorded for six babies in the present survey at some time during the first year of life. Caution must be observed when comparing these values because of the different media used for quantitation, the possible effect of freezing the specimens, and the different fluid contents of diarrheal specimens from patients compared with normal specimens from infants; however, it appears that levels of *C. difficile* in babies can be in the same range as those from PMC patients without producing disease.

There have been conflicting reports about the in vivo production of cytotoxin by *C. difficile* in infants. Larson et al. (18) have demonstrated cytotoxin in the stools of two healthy neonates who were colonized by *C. difficile*. Cashore et al. (6) found specific cytotoxicity in the stools of five neonates suffering from neonatal necrotizing enterocolitis. Rietra et al. (20) demonstrated cytotoxicity resembling the effect of *C. difficile* toxin in the feces of 14% of healthy infants between 0 and 5 months of age. However, other authors have been unable to detect cytotoxicity in the stools of a total of 49 healthy neonates or 77 neonates with necrotizing enterocolitis (3, 7, 23). These discrepancies may be related to the different assay techniques used and possibly to the different ages of the infants examined. In the present study, infants' stools were not tested for cytotoxicity since it was considered that testing samples which were already diluted 1:10 in a medium designed for the protection of anaerobic bacteria, and not necessarily for the preservation of cytotoxin activity, would not help resolve this important issue. Ideally, two fecal samples, one for culture and a further undiluted sample for cytotoxin assay, should be collected from each baby at each examination but this was not practical in the present study, which relied on the cooperation of mothers to collect specimens over a relatively long follow-up period. This study has shown, however, that infant strains of *C. difficile* are cytotoxigenic in vitro and therefore negates the possibility that infants colonized by the organism do not develop PMC because they select only nontoxigenic strains. Bartlett et al. (4) have recently demonstrated that *C. difficile* elaborates a second toxin which differs from the cytotoxin in that it produces a positive response in the ileal loop assay and a more florid inflammatory reaction after intracecal injection into hamsters. It is not known whether this second toxin is produced in the infant gut or which of the two toxins is responsible for the pathological changes seen in PMC.

In attempting to explain the paradox, it is also necessary to consider the differences in bacterial populations and physicochemical conditions existing in the large bowel of infant and PMC patients. The majority of patients have previously been exposed to antibiotics, and this would profoundly influence the composition of the microbial flora. Previous reports have shown that numbers of bifidobacteria, *Eubacterium* spp., *Bacteroides* spp., and anaerobic cocci are reduced in PMC patients (10). Presumably, it is this perturbation of the microbial ecology which allows multiplication of *C. difficile* to occur; however, it appears from infant studies that multiplication of the organism does not in itself produce disease. Other factors such as bacterial activation of toxin or changes in pH, Eh, intestinal motility, or the mucin lining the mucosal epithelium must contribute to the pathogenic process.

The gastrointestinal microbial flora of the infant during the time of colonization by *C. difficile* also lacks some of the anaerobic genera found in normal adults. Six of the babies in the prospective study were included in a detailed investigation of changing bacterial populations in the large bowel of infants (Stark and Lee, J. Med. Microbiol., in press), and it was interesting to find that the time when *C. difficile* disappeared from their feces coincided with increases in levels of *Bacteroides* spp., *Eubacterium* spp., and anaerobic cocci. This again suggests that these genera suppress the multiplication of *C. difficile* in the large bowel. Other authors have

also shown that certain gram-positive anaerobic rods inhibit the multiplication of *C. difficile* in vitro (21).

In conclusion, there are still many questions to be answered about the role of *C. difficile* in health and disease. To understand the mechanisms of pathogenicity involved in PMC it is clearly not sufficient to test only for the presence of the organism and cytotoxin production, but also to consider the total environment inside the large bowel of patients, including all bacterial populations present and their interactions with *C. difficile* and its toxins and a variety of physicochemical parameters. The infant large bowel, colonized by *C. difficile* but without disease, could serve as a useful comparative model for the identification of those factors in the large bowel of patients which contribute to the etiology of PMC.

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