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Persistence of toxigenic *Clostridium difficile* in the gut microflora of healthy Swedish Infants

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Abstract

Clostridium difficile is the major cause of antibiotic-associated diarrhea in adults, whereas infants may be colonized without symptoms. We have determined the rate of *C. difficile* colonization in 184 healthy Swedish infants followed from birth for 0 to 1-3 years of age, investigated which environmental factors that promote *C. difficile* colonization, and identified individual *C. difficile* ribotypes and their toxin genes in a subgroup of 42 colonized infants. In all, roughly one third of the infants harboured *C. difficile* at 6 months of age, 60 % at 12 months, one third at 18 months and 4% at 36 months of age. Delivery by caesarean section was a risk factor for *C. difficile* colonization, as was absence of elder siblings, while breastfeeding was protective. Long-term (≥6 months) colonization by a single ribotype occurred in 36% (15/42) of the infants. The most common ribotypes were 001 (32%) and 014 (19%), and among strains that persisted for ≥6 months in the microbiota of an infant, 87% were either ribotype 001 or 014. A majority of the strains (73%) carried toxin genes (*tcdA* and *tcdB*) and produced the toxin B *in vitro*. None of the strains harboured genes for the binary toxin. Our results indicate that certain toxigenic ribotypes of *C. difficile* have a pronounced capacity to persist in the infantile gut microbiota, which may function as a reservoir for strains causing *C. difficile* infection (CDI) in adults.

Introduction

Clostridium difficile is a common cause of antibiotic-associated diarrhea. *C. difficile* is a sporeforming obligate anaerobe colonizing the gut of 2-15% of healthy adults (1,2) but usually being present in low counts, since a complex bowel microbiota prevents its expansion (3). *C. difficile* is frequent in infants´ gut microbiota, with isolation rates ranging from 20 to 100% (4,5). The highest carrier rate has mostly been observed during the first six months of life. After that, the colonization rate declines in parallel with an increased complexity of the gut microbiota, leading to suppression of *C. difficile*. *C. difficile* spores are ubiquitous in the environment and infants can acquire *C. difficile* in the hospital milieu, while transfer of *C. difficile* from mother to child during delivery occurs rarely (5). Risk factors for *C. difficile* colonization include delivery by caesarean section, and bottle-feeding. For unknown reasons, young infants normally remain healthy despite high quantities of toxin-producing *C. difficile* in the colon (6). An increase in *C. difficile*-related hospitalizations among infants has been noted in the United States (7), and may indicate an increased or prolonged *C. difficile* colonization and/or colonization with more pathogenic strains. Here, we report the *C. difficile* colonization pattern in healthy Swedish infants in the infant birth-cohort, ALLERGYFLORA, from one week to three years of age, and the relation between *C. difficile* colonization and environmental and lifestyle factors. In addition, individual *C. difficile* ribotypes, toxin genes and toxin B production *in vitro* was analysed.

Materials and Methods

The ALLERGYFLORA birth cohort

The Swedish ALLERGYFLORA birth cohort includes 184 Swedish infants born at term (≥38 gestational weeks) in 1998-2003 at the Sahlgrenska University Hospital (Göteborg, Sweden).

The main aim of the ALLERGYFLORA study was to investigate whether the infantile gut microbiota affects later allergy development and quantitative culture of faecal samples was performed from 1 week up to 3 years of age. Informed consent was obtained from the parents and the study was approved by the Human Research Ethics Committee of the Medical Faculty, Gothenburg University, Sweden.

Selection of infants for *C. difficile* ribotype studies

Isolates from 42 infants colonized by *C. difficile* and born in 1998-2003 were characterized (see below). Twenty-nine were delivered vaginally and 13 by caesarean section. The characteristics of this subgroup of infants were similar to those of the entire cohort except for a higher frequency of sectio delivery (Table 1). All 42 infants provided faecal samples at 1, 2 and 4 weeks and 2 and 6 months, 40 infants were sampled at 12 months, 22 at 18 months and 16 at 36 months of age.

PCR ribotyping of *C. difficile* isolates

C. difficile isolates were subjected to PCR ribotyping (8). Ribotype designations according to the Anaerobic Reference Unit (Cardiff, UK), were used when possible.

Toxin gene carriage and toxin B production

C. difficile toxin genes were identified by PCR. For *tcdA* as described by Kato *et al*. (9). Cepheid Xpert™ *C. difficile* assay was used to detect *tcdB* and the binary toxin (*cdtA/B*). All isolates were also analyzed for toxin B by the cell cytotoxicity neutralization assay (CCNA) using the *C. difficile* toxin/antitoxin kit (TechLab, Blacksburg, VA).

Table 1. Characteristics of the infants

	Entire cohort	Strain-typed cohort
	N (%)	N (%)
Total	184 (100)	42 (100)
Vaginal delivery	157 (85)	29 (69)
Girls	91 (49)	23 (55)
Siblings	90 (49)	17 (41)
Breastfed at 6 mo	128 (70)	27 (66)
GI problems in first year:		
Diarrhoea	22 (12)	6 (14)
Colic	34 (18)	6 (14)
Constipation	35 (19)	7 (17)
Antibiotics in first year	47 (26)	12 (29)
0-6 months	15 (8.2)	4 (9.5)
6-12 months	37 (20)	8 (19)

Results

Colonisation of infants with *C. difficile* over time (Figure 1a and 1b)

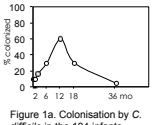

Figure 1a. Colonisation by *C. difficile* in the 184 infants followed from 1 week of age

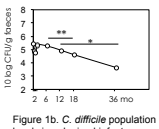

Figure 1b. *C. difficile* population levels in colonised infants

Colonisation with *C. difficile* related to delivery and feeding (Figure 2a and 2b)

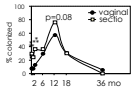

Figure 2a. *C. difficile* colonisation related to delivery mode

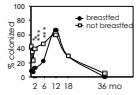

Figure 2b. *C. difficile* colonisation related to type of feeding

Ribotypes

In total, 22 *C. difficile* ribotypes were identified. Nine of these resembled ribotypes recognized by the Anaerobic Reference Unit (Cardiff, UK), the most common being 001 and 014, isolated from 19 and 11 of the infants, respectively. In addition, we identified 6 ribotypes that did not show identity with any of the international recognised ribotypes. In addition, ribotypes of non-toxigenic strains are referred in roman figures (Figure 3). All isolates were negative for the binary toxin and none of the *C.difficile* strains belonged to the 027 ribotype

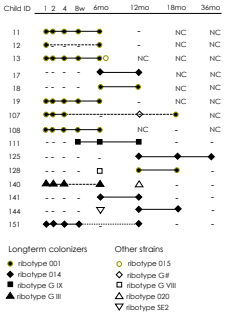

Figure 3. Persistence of *C. difficile* strains in the infantile gut microbiota. Fifteen infants were persistently colonized by a single *C. difficile* strain over at least 6 months. A solid line indicates the presence of the strain on consecutive sampling occasions; a dashed line denotes that the strain was not found on an intervening occasion. NC = no culture performed.

Conclusion

Colonisation of infants with toxigenic *Clostridium difficile* is frequent. The factors that influence the colonisation are: (i) the mode of delivery and (ii) feeding. The most common ribotypes identified among the colonising strains were 001 and 014. These ribotypes were also the long-term colonisers

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