JOURNAL OF CLINICAL MICROBIOLOGY, May 1983, p. 830–833
0095-1137/83/050830-04$02.00/0

Vol. 17, No. 5

Influence of Age, Sex, and Diet on Asymptomatic Colonization of Infants with *Clostridium difficile*

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Received 20 December 1982/Accepted 31 January 1983

A total of 40% of 107 stool samples from infants 1 to 52 weeks of age were found to contain *Clostridium difficile* antigens, detected by counterimmunoelectrophoresis. Within the group tested, there was no detectable variation by age or sex. Infants fed formula were nearly four times more likely to carry *C. difficile* than were those exclusively breast fed (62 versus 16%), whereas breast-fed infants also receiving formula or solids had an intermediate rate of colonization (35%). The distributions were similar when a subgroup with the highest levels of antigen was assessed separately. These data will be useful in considering potential pathogenic activities of *C. difficile* colonization in infancy.

Asymptomatic colonization of the intestines with *Clostridium difficile* occurs frequently during the first year of life. In neonatal intensive care units, carrier rates of 21 to 59% in the absence of gastrointestinal symptoms have been reported (6, 19; J. D. Siegel and B. Milvenan, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 205, 1980). Among normal neonates less than 4 weeks of age, rates of 0 to 29% have been observed (3, 6, 11, 13–15, 23, 24; 20th ICCAC, abstr. no. 205), and older infants up to a year or two of age have been found to have carrier rates of 4 to 46% (1, 5, 20, 22, 23, 24). In contrast, fewer than 4% of children older than 1 to 2 years of age carry this organism (22, 23), and these values are similar to those reported for healthy adults (8, 10, 22, 24). Unlike *C. difficile* carriers in older age groups, some infant carriers may be heavily colonized and have very high levels of *C. difficile* toxin A (S. Donta and T. Wilkins, personal communication) and toxin B (3, 5, 6, 17, 19; D. H. Batts, R. Holmes, R. Fekety, J. Silva, and D. Schaberg, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 204, 1980) without apparent symptoms.

Although *C. difficile* carriage in infancy is usually benign, epidemiological data may help in evaluating possible relationships with various pathological conditions known to occur within this age group. For example, it is possible that conditions such as protein loss during acute diarrhea (16) or sudden infant death syndrome (5; S. Arnon, D. Mills, P. Day, R. Henrickson, N. Sullivan, and T. Wilkins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B121, p. 38) might be influenced by colonization with this organism. We have performed quantitative determinations of *C. difficile* antigen in 107 stool samples from 91 infants to provide a better description of the effects of age, sex, and diet upon colonization with this organism.

MATERIALS AND METHODS

Infants were recruited from the well-baby clinics in the Departments of Child Health and Family Practice at the University of Missouri Health Sciences Center. Only infants in good health and with normal rates of weight gain were studied. A wide range of socioeconomic groups was represented. Dietary information was recorded prospectively. Data from studies of some of the infants were included in an earlier report from this center (5).

C. difficile antigens were detected by counterimmunoelectrophoresis (CIE) using a 1% agarose gel (type II; Sigma Chemical Co.) containing barbital buffer (immunoelectrophoresis buffer I; Bio-Rad Laboratories), pH 8.6, and hyperimmune *C. difficile* antiserum (7), which is known to react with a number of *C. difficile* antigens, including a major surface carbohydrate (18, 25). Paired wells were cut 3 mm in diameter and 9 mm (center to center) apart, with 12 to 14 pairs of wells in a single row, across a glass slide (8.6 by 10.2 cm) onto which had been poured 18 ml of molten agarose. Ten microliters each of antiserum diluted 1:2 and of samples prepared in serial $10^{0.5}$-fold dilutions were placed in the appropriate wells. Electrophoresis was carried out at a constant current of 35 mA per plate for 1 h on a tap-water-cooled base plate. Positive and negative antigen controls were included in each plate. Plates were examined immediately after electrophoresis, after incubation overnight at 4°C in a moist chamber, and again after flooding with 95% ethanol for 15 min. Preliminary trials indicated that the latter

TABLE 1. Frequency of *C. difficile* antigen in healthy infants, by age and diet

Age (wk)	No. with positive test/no. fed the following milk source:[a]						
	Breast exclusively (*A*)	Breast plus supplement[b] (*B*)	Formula exclusively (*C*)	Formula plus supplement (*D*)	Breast (*A* + *B*)	Formula (*C* + *D*)	Total
0–4	2/19	3/6	6/10	1/1	5/25	7/11	12/36 (33%)
5–8	3/12	1/3	4/5		4/15	4/5	8/20 (40%)
9–12	0/1	0/4	2/5	6/8	0/5	8/13	8/18 (44%)
13–16	0/2	2/5	1/2	1/2	2/7	2/4	4/11 (36%)
17–20	1/3	1/1	1/2	3/6	2/4	4/8	6/12 (50%)
21–52		1/4	2/2	2/4	1/4	4/6	5/10 (50%)
Total	6/37 (16%)	8/23 (35%)	16/26 (62%)	13/21 (62%)	14/60 (23%)	29/47 (62%)	43/107 (40%)

[a] The following *P* values were obtained by chi-square analysis of the total for each column: *A* versus *B*, not significant; *A* versus *C*, <0.001; *A* versus *D*, <0.01; *B* versus *C*, <0.05; *B* versus *D*, 0.05; *A* + *B* versus *C* + *D*, <0.001.

[b] Supplementation with formula or solids or both.

procedures enhanced the sensitivity of the assay severalfold and improved the clarity of questionable precipitin lines in many instances (it is necessary to use 1:2 diluted antiserum if ethanol is used).

The CIE assay was validated, in some instances, by concomitant cytotoxin assay, kindly performed by David Lyerly and Tracy Wilkins (Virginia Polytechnic Institute) or performed in our laboratories as described elsewhere (5). Because of the small sample size, the lowest fecal dilution tested in the cytotoxin assays was either 1:200 or 1:600 (final dilution). A positive cytotoxin assay was recorded if samples produced a tissue culture cytopathic effect that could be neutralized by pretreatment with *C. difficile* antiserum. In some cases such samples were also neutralized by nonimmune serum; these samples were considered inconclusive and were not used to evaluate the CIE assay.

Selective agar medium containing cefoxitin and cycloserine (9) was used for isolation of *C. difficile*.

RESULTS

To verify the utility of the CIE assay as an epidemiological tool, cultures were performed in 24 of the present cases, and cytotoxin assays were performed in 65 instances. CIE was positive in 8 of 12 culture-positive cases (67% sensitivity) and negative in 12 of 12 culture-negative cases (100% specificity). The cytotoxin assay was also 100% specific (16 of 16 positive assay results were also culture-positive) but, with the necessary dilutions, was less frequently positive than CIE. Whereas 14 of 16 cytotoxin-containing samples were positive by CIE, only 36 of 46 samples without detectable cytotoxin also lacked antigen detectable by CIE.

The distribution of *C. difficile* colonization, as determined by the presence of *C. difficile* antigen assayed by CIE, is shown in Table 1. *C. difficile* antigen was clearly more prevalent among infants receiving formula than among those fed mother's milk, and this difference appeared to be independent of age, within the age limit studied. Among breast-fed infants, a trend towards more frequent antigen carriage

TABLE 2. Frequency of high-level *C. difficile* antigen in healthy infants, by age and diet

Age (wk)	No. with high-level *C. difficile* antigen/no. fed the following milk source:[a]						
	Breast exclusively (*A*)	Breast plus supplement[b] (*B*)	Formula exclusively (*C*)	Formula plus supplement (*D*)	Breast (*A* + *B*)	Formula (*C* + *D*)	Total
0–4	0/9	3/6	3/10	1/1	3/25	4/11	7/36 (19%)
5–8	1/12	1/3	2/5		2/15	2/5	4/20 (20%)
9–12	0/1	0/4	2/5	3/8	0/5	5/13	5/18 (28%)
13–16	0/2	0/5	0/2	1/2	0/7	1/4	1/11 (9%)
17–20	1/3	1/1	0/2	2/6	2/4	2/8	4/12 (33%)
21–52		1/4	2/2	1/4	1/4	3/6	4/10 (40%)
Total	2/37 (5%)	6/23 (26%)	9/26 (35%)	8/21 (38%)	8/60 (13%)	17/47 (36%)	25/107 (23%)

[a] The following *P* values were obtained by Fisher's exact test or chi-square analysis of the total for each column: *A* versus *B*, <0.05; *A* versus *C*, <0.05; *A* versus *D*, <0.005; *B* versus *C*, not significant; *B* versus *D*, not significant; *A* + *B* versus *C* + *D*, <0.01.

[b] Supplementation with formula or solids or both.

TABLE 3. *Clostridium difficile* colonization rates by sex, stratified by diet

Predominant milk source	No. positive/no. observed (% positive)	
	Male	Female
Breast	9/33 (27)	5/25 (20)
Formula	11/17 (65)	15/25 (60)

was associated with the administration of supplementary foods. This trend appeared to be independent of age. Stools from supplemented breast-fed infants were somewhat less likely to demonstrate *C. difficile* antigen than were those from formula-fed infants, with or without supplementation.

Independent of diet (Table 1, right-hand column), there was no remarkable variation in antigen carriage rates by age group. This finding appeared to occur within each dietary subgroup, although the number of values in each of these subgroups is too small to draw definite conclusions.

A subgroup of 25 stools from *C. difficile* carriers with the highest levels of *C. difficile* antigen (titer, 1/100 to 1/300) was analyzed separately. We were particularly interested in this subgroup because its members might be more likely to be involved in any pathological events to be considered for *C. difficile*. The relationships between age and diet for this subgroup were similar to those of the whole group (Table 2).

The ratio of male to female carriers was 1.00. The distribution of *C. difficile* antigen by sex remained near unity when stratified by dietary subgroups (Table 3) or by age (Table 4). In the high-antigen subgroup, the male-female ratio was 13:12.

DISCUSSION

These data show that infant formula feeding and supplementation of breast feeding in the first year of life appreciably enhance the likelihood of intestinal colonization with *C. difficile*, as determined by CIE. The relationship appears to be uninfluenced by the age or sex of the infants. The data confirm and extend a previous report showing a relationship between diet and colonization rate as determined by stool culture (5).

A relatively homogeneous age distribution of *C. difficile* colonization rate was found, in both a qualitative and a quantitative sense, during early infancy. This finding would weigh against a primary role for *C. difficile* in diseases with a sharp age incidence peak within this range, including, for example, sudden infant death syndrome, unless age-specific cofactors (e.g., lack of toxin-neutralizing serum antibodies) were also involved. The age distribution of *C. difficile* colonization somewhat more closely parallels that of *Salmonella* gastroenteritis (2). An interaction between the two might therefore be postulated. Perhaps more likely, both *Salmonella* spp. and *C. difficile* might be limited by similar host mechanisms, such as intraluminal pH, oxidation-reduction potential, or the presence of hydrogen sulfide or certain organic acids (reviewed in reference 4).

These results differ somewhat from those of Holst et al., who found a *C. difficile* colonization rate of 86% between 1 and 8 months of age, but only 4% among infants younger than 1 month (12). The present study does not show a lower incidence qualitatively or quantitatively during the first 4 weeks of life. These differences during the first month of life are difficult to explain. It is quite possible that differences in feeding practices or environmental exposure to *C. difficile* during the immediate postnatal period could be important factors.

This study shows an equal distribution of *C. difficile* by sex. The absence of a sexual predominance has also been observed in neonatal intensive-care units (6; A. Zedd, T. Sell, D. Schaberg, R. Fekety, and M. Cooperstock, unpublished data).

It is now abundantly clear that healthy infants commonly may be colonized with *C. difficile*. The present study shows a facilitative effect of formula feeding. Both the age distribution of *C. difficile* colonization and the relationship of *C. difficile* colonization rates with diet appear to parallel findings for the genus *Clostridium* as a whole (21). The practical importance of these observations remains to be determined. It is possible that infants may represent an important reservoir for *C. difficile* strains capable of causing antibiotic-associated pseudomembranous colitis when transmitted to older children and adults. However, a preliminary study of six adults with community-acquired *C. difficile* coli-

TABLE 4. *C. difficile* colonization rates by sex, stratified by age

Age (wk)	No. positive/no. observed (% positive)	
	Male	Female
0–4	7/17 (39)	5/16 (31)
5–8	5/10 (50)	2/8 (25)
9–12	2/7 (29)	5/11 (45)
13–16	1/3 (33)	3/7 (43)
17–20	2/6 (33)	3/4 (75)
≥21	3/6 (50)	2/4 (50)
Total	20/30 (67)	20/30 (67)

tis in our institution revealed that none had intimate contact with infants under 2 years of age (A. Zedd et al., unpublished data). Concern has also been raised regarding nosocomial sources for *C. difficile* colitis. Many potential opportunities for the spread of *C. difficile* from units caring for neonates or young infants to units caring for older children or adults exist, as there are often shared diagnostic and therapeutic facilities and shared personnel in a variety of occupations. The availability of *C. difficile* typing methods will allow an enhanced understanding of these possibilities.

ACKNOWLEDGMENTS

We are deeply indebted to Tracy Wilkins for making his laboratory available to this study, for providing *C. difficile* antisera, and for critiquing the manuscript. We thank Karen Ehlert for assistance in preparing the manuscript and Deanna Fabacher for assistance in collecting specimens.

This study was supported in part by a grant from Ross Laboratories.

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