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Prospective study of *Clostridium difficile* colonization and para-cresol detection in the stools of babies on a special care unit

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SUMMARY

Infants' stools were examined for the presence of *Clostridium difficile* and its cytotoxin in a study performed over a one-year period on a special care baby unit. Overall, 21 % of infants were colonized, but the organism was only recovered in a seven-month period during which its weekly prevalence in the group varied from zero to 44 %, with a distinct clustering of colonized infants being observed. Tests for the presence of cytotoxin in the stools and in supernatants of broth that had been inoculated with each isolate were negative. The factors predisposing to colonization were a prolonged stay in the unit, low birth weight, younger gestational age and being nursed in an incubator. The organism was recovered only once from an environmental screen.

An antibiogram, used in conjunction with toxin production, was helpful in distinguishing these isolates from a collection obtained from other units in the hospital. We conclude that *Cl. difficile* was acquired by nosocomial spread although we did not establish the precise mechanism involved.

The detection of para-cresol by gas–liquid chromatography was found to be specific but insufficiently sensitive as a screening test for the organism's presence in the stools. It could only be demonstrated in infants whose birth-weights were less than 2500 g, and no association was observed between the type of feed and para-cresol presence in stools.

INTRODUCTION

Clostridium difficile was first reported to be a normal and harmless commensal of the infant bowel by Hall & O'Toole (1935). More recently both the organism and its cytotoxin have been acknowledged as playing a pathogenic role in pseudomembranous colitis (Larson *et al.* 1978). Subsequent studies have implicated *Cl. difficile* in other intestinal disorders (Greenfield *et al.* 1983) including neonatal necrotizing enterocolitis (Cashore *et al.* 1981).

The observation of clustering of cases of colitis has led to epidemiological investigations to establish the prevalence of the organism among different groups of hospitalized patients. Any attempts to interpret the results of these studies have been frustrated by the discrepancy in observed carriage rates and methods used, and the infrequent recovery of the organism from the hospital environment.

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We have carried out a prospective study in a group of premature infants, all of whom required admission to our special care unit. We intended to clarify the modes of acquisition and transmission of *Cl. difficile* on the unit and to assess the relevance of various host factors. We attempted to demonstrate identity between isolates by using an antibiogram together with the preliminary phage typing system recently described by Sell, Schaberg & Fekety (1983).

In addition to the established techniques used for the demonstration of *Cl. difficile*, we tested stools for the presence of para-cresol by gas–liquid chromatography as an indirect method for the detection of the organism in the bowel.

MATERIALS AND METHODS

The subjects

The 146 pre-term infants studied were consecutive admissions to the special care baby unit at Westminster Hospital, London, between 1 August 1982 and 31 July 1983. Babies admitted for overnight observation were not included in the study. The unit comprises two adjacent rooms, the larger (10 × 8 m) normally accommodating a total of eight incubators or cots, and the smaller (8 × 4 m) accommodating four cots.

Faecal samples

These totalled 490 and were collected from infants within 48 h of birth or admission to the unit, subsequently on the third day, and thereafter weekly whilst they remained on the unit.

Stools (approximately 0·5 g) were transferred from soiled nappies into bijou bottles containing 4·5 ml pre-reduced salt solution (Holdeman, Cato & Moore, 1977) and prepared as described by Stark & Lee (1982), giving an approximate 1:10 dilution. On the day of collection they were stored at −70 °C.

After thawing, aliquots were dispensed as follows: 2 drops from a 30-dropper pipette (Microbiological Supply Co.) were inoculated on to selective cycloserine–cefoxitin–fructose agar plates (Oxoid), which were incubated at 37 °C for 48 h in an anaerobic jar; 1 ml was inoculated into Robertson's cooked meat medium, heat-shocked at 80 °C for 12 min (Stark & Lee, 1982) and subcultured before and after 3 days incubation at 37 °C onto kanamycin horse blood agar plates. Of the remaining suspension 1 ml was kept at −70 °C for use in the estimation of para-cresol; 2 ml were centrifuged at 3000 rev./min for 20 min, and the supernatants were filtered (0·45 μm filters) for use in the detection of cytotoxin (Larson *et al.* 1978).

Environmental samples

Environmental cultures were taken in a minor study from pre-selected sites between the period of 27 January and 31 March 1983. They included air samples on settle plates (kanamycin blood agar) for two hours, and cultures of floors and incubators, each over an area of approximately 50 cm^2 using a sterile cotton-tipped applicator which had been moistened in sterile saline. A selective agar plate was then inoculated by rolling the cotton tip on to the agar surface; the tip was then

inoculated into a Robertson's cooked meat medium which was heat-shocked, incubated for 3 days, and finally subcultured.

Contact plates, 55 mm diameter (Sterilin) containing selective agar, were used for a major environmental screen for sampling from a wide range of inanimate sites. These included chairs, incubators, scales, discard-bins, benches, empty feeding bottles, cupboards, floors and the breast-milking machine. Also impression cultures from the finger pads of the nurses on the unit were taken.

Bacteriology

After incubation all plates were examined under long-wave (360 nm) high-intensity ultraviolet light (Mineralight, model UVGL 58) for the chartreuse fluorescence characteristic of *Cl. difficile* colonies. A semi-quantitative scoring of the density of growth on the primary plates was made, and these values were compared with the viable counts (colony-forming-unit/g wet faeces) obtained from simulated faecal samples which had been processed in an identical way. The semi-quantitative counts were scored as follows: 1+ for growth on inoculum well and the first set of streaks equivalent to 10^3–10^4 c.f.u./g; 2+ for growth up to the second set of streaks equivalent to 10^4–10^5 c.f.u./g, and 3+ for growth up to the third and fourth set of streaks equivalent to 10^5–10^6 c.f.u./g.

Colonies of distinctive morphology were Gram-stained and subcultured into Robertson's cooked meat medium. After three days incubation the cultures were checked for purity and identified by biochemical reactions to glucose (+ve), fructose (+ve), mannose (+ve), lactose (−ve) and sucrose (−ve) (Holdeman, Cato & Moore, 1977), and by the detection of para-cresol production in Robertson's cooked meat medium. Broth from the Robertson's cooked meat medium was decanted, clarified by centrifugation, filtered and tested for the presence of cytotoxin using a tissue culture assay (Larson *et al.* 1978).

Typing

Cl. difficile isolates from the infants in this study and from patients in two other units of the hospital were individually tested for susceptibility to ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (10 μg), cefuroxime (30 μg), chloramphenicol (10 μg), clindamycin (2 μg), methicillin (10 μg), metronidazole (5 μg), penicillin (1·5 units), tetracycline (10 μg), vancomycin (30 μg) and gentamicin (10 μg) using a conventional disc sensitivity method.

In addition, representative isolates from the cluster period were seeded on a soft agar lawn, on to which 30 μl of stock phage preparations (numbers, 2, 3, 4, 6, 7, 8, 9 and 10) were spotted with a micropipette; confluent lysis on the lawn was taken to indicate sensitivity to the respective phage.

Gas–liquid chromatography

A pye Unicam 104 series gas chromatograph was used for the detection of para-cresol in faecal suspensions using a glass analytical column as described by Phillips & Rogers (1981).

Serial para-cresol standards were prepared in sterile distilled water at concentrations of 100, 50, 25, 12·5 and 6·25 μmol/ml. Qualitative analysis was performed

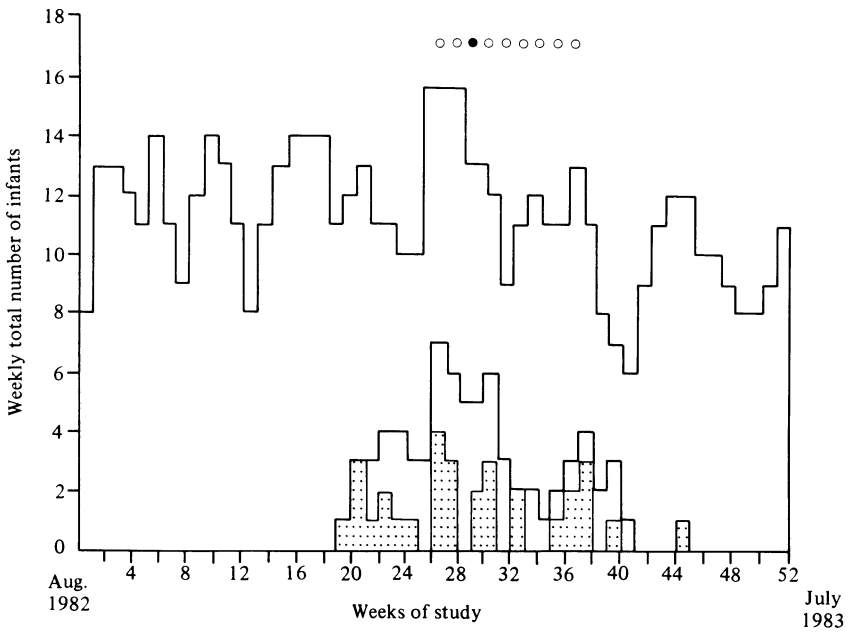

Fig. 1. The weekly prevalence (lower bold line) and the weekly incidence (shaded bars) of infants colonized by *Clostridium difficile* in relation to the weekly total number of infants (upper line). The circles indicate the times when minor (○) and major (●) environmental samples were taken.

by extraction of para-cresol from 1 ml of each standard solution by 0·5 ml of diethyl ether. This was vortex-mixed and cleared by low-speed centrifugation, and 2 μl of the ether extract was injected into the chromatograph. Each faecal suspension was similarly treated. Para-cresol concentration was estimated from serial standards in each set of gas chromatographs.

RESULTS

Colonization pattern

Cl. difficile was isolated from 31 infants (21 %) in the study, of whom 22/69 (32 %) were incubator infants and 9/77 (12 %) were cot infants. Throughout the 7 months cluster period the weekly prevalence varied from 0 % to 44 % (Fig. 1), and the weekly incidence paralleled the fluctuation in number of babies present on the unit.

The majority of the infants were colonized by *Cl. difficile* after the second week of life, and the mode of delivery did not influence the frequency of colonization at any time after birth (Table 1). A prolonged stay on the unit, a lower birth weight, a younger gestational age and nursing in an incubator each predisposed to colonization (Table 2).

Of the 19 incubator infants who at the time of transfer to cots were culture negative, seven were subsequently found to be colonized. The median duration of carriage was 8·2 days overall, and the organism persisted in 84 % (26/31) of infants until discharge from the unit. On one occasion *Cl. difficile* was recovered from a

Table 1. *The number of infants newly colonized by* Clostridium difficile *in relation to their age in weeks following either Caesarean section or vaginal delivery*

Age post-delivery (weeks)	Delivery		
	Caesarean	Vaginal	Total
0–1	1	1	2
1–2	6	4	10
2–3	4	5	9
3–4	3	4	7
4–5	1	1	2
5–6	0	0	0
6–7	1	0	1
Total	16	15	31

Table 2. *Comparison between infants during the cluster period* (1 *December* 1982 *to* 30 *June* 1983)

Factors	Culture-positive ($n = 31$)	Culture-negative ($n = 49$)	P value (chi square)
Duration on unit (days)	30*	13·4*	< 0·025
Birth weight of infant			
< 2500 g	27	14	< 0·005
> 2500 g	4	35	
Gestational age of infant			
< 37 weeks	27	18	< 0·005
> 37 weeks	4	31	
Infant nursed in			
incubator then cot	22	14	< 0·005
cot	9	35	
Sex of infant			
male	19	26	NS
female	12	23	
Type of delivery			
vaginal	15	22	NS
Caesarean section	16	27	
Antibiotic therapy at first stool culture			
yes	20	26	NS
no	11	23	
Diet of infant			
expressed breast milk	3	2	
formula-milk	17	29	NS
mixed formula and EBM	11	18	

Note: values represent actual number of infants in the group except where noted.
* Represents median value, NS = no significant difference.

heat-shocked culture taken from a floor surface in the larger room during a minor environmental screen, but the staff screens were negative. Cytotoxin was not demonstrated in any infants' stool filtrates.

Typing

All isolates, including the environmental isolate, were indistinguishable in being non-toxigenic and having identical antibiotic and phage sensitivity patterns, but

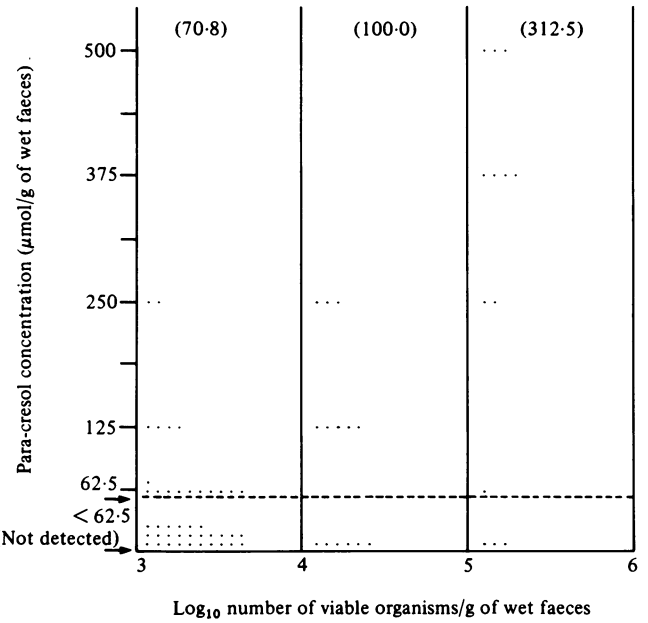

Fig. 2. The relationship between the estimated absolute para-cresol concentrations and semi-quantitative viable counts of *Clostridium difficile* in infants' faecal samples. Figures in parentheses indicate median values of para-cresol concentrations.

Table 3. *Association between the culture of* Clostridium difficile *and the detection of para-cresol in infants' faeces*

	Clostridium difficile		
Para-cresol	Isolated	Not isolated	Total faecal samples
Detected	35	0	35
Not detected	35	70	105
Total	70	70	140

sensitivity to clindamycin, methicillin and tetracycline differed when compared with the isolates from two other units in the hospital which, furthermore, were toxigenic. This difference was not confirmed by phage susceptibility, where all the isolates were lysed by phages 6, 7 and 10 but unaffected by phages 2, 3, 4, 8 and 9.

Para-cresol estimation

A total of 140 faecal samples were examined and 70 were negative for both *Cl. difficile* and para-cresol. Para-cresol was detected in 35 out of the 70 positive cultures as shown in Table 3. The distribution of approximate para-cresol concentrations in relation to the semi-quantitative viable counts of *Cl. difficile* in infants' stools is shown in Fig. 2. Para-cresol was present in the stools of 19 infants all of whom were less than 2500 g in birth weight, however, the type of infant feed did not influence para-cresol detection.

DISCUSSION

Despite the current interest in *Cl. difficile*, its epidemiology is still poorly understood. With the exception of the preliminary use of bacteriophages and bacteriocins (Sell, Schaberg & Fekety, 1983), no suitable method for typing *Cl. difficile* isolates has yet been found.

Viscidi, Willey & Bartlett (1981) found their normal neonatal colonization rate with *Cl. difficile* to be as low as 4%, but more recent short-term studies indicate that the frequency of neonatal colonization may be variable (Cooperstock *et al.* 1983; Larson *et al.* 1983). Malamou-Ladas *et al.* (1983) reported that the isolation rate of *Cl. difficile* was particularly high in premature babies (54%) when compared with full-term neonates (20%). By contrast Viscidi, Willey & Bartlett (1981) found that the incidence was comparable between these two groups. We have carried out a one-year study in which *Cl. difficile* was isolated from 21% of infants overall, but the pattern of colonization, with fluctuations in the weekly prevalence, may explain the wide disparity in reported colonization rates in young infants. Most of the peaks observed in the total number of infants on the unit reflected a prolonged stay for some, rather than an increase in the admission rate.

With our typing methods, all the isolates, including the environmental isolate, were indistinguishable and we conclude that these represented the same strain. An outbreak with only one strain suggests acquisition from a common source, although its origin was not evident. We found no association between mode of delivery and infant colonization rate, and although vertical transmission might occur, vaginal carriage in healthy females has been found to be low (Gorbach *et al.* 1973). We only recovered the organism on one occasion from a heat-shocked culture taken from a floor surface in the middle of the larger room. Survival of *Cl. difficile* in an aerobic environment is possible because of its ability to form resistant spores, which may permit low-level contamination to continue as a source of infection. This and other exogenous sources may be important in nosocomial transmission of *Cl. difficile* (Rogers *et al.* 1981; Malamou-Ladas *et al.* 1983). Once the infants had acquired *Cl. difficile*, the pattern of colonization indicated infant-to-infant cross-infection.

An assessment of the characteristics of infants who were culture-positive supports the notion of nosocomial spread. The distribution of the organism was shown to be uninfluenced by infants' sex, diet, type of delivery, or by preceding antibiotic administration. The majority of the infants were not colonized until between the second and fourth week of life. The group consisted of infants of lower birth weight who often required longer hospitalization with nursing and medical care in an incubator. Thus infants, nursery staff and the inanimate environment may all be relevant to persistence of *Cl. difficile* in this clinical situation.

Isolation of *Cl. difficile* in two neonates, both of whom subsequently developed necrotizing enterocolitis, marked the start of the cluster period. These findings do not establish a cause-and-effect relationship between the recovery of *Cl. difficile* and the occurrence of enterocolitis. If there were such an association then further cases of colitis among susceptible neonates might have been expected to have occurred during the cluster period. However, none of the remaining colonized

babies was symptomatic. This agrees with Sherertz & Sarubbi's (1982) conclusion that necrotizing enterocolitis and *Cl. difficile* appear coincidentally in the nursery.

In this study, para-cresol was only detected in the faeces of those pre-term neonates who were colonized with *Cl. difficile*. In a comprehensive survey of the amino acid-fermenting clostridia, Elsden, Hilton & Waller (1976) found that *Cl. difficile* possessed the ability to produce para-cresol from tyrosine. This characteristic appears to be shared only with *C. scatologenes*. Although other gut anaerobic bacteria may produce para-cresol *in vitro* (Bone, Tamm & Hill, 1976), we did not detect it in the *Cl. difficile*-negative samples. The detection in stools of para-cresol by gas–liquid chromatography was specific but insufficiently sensitive as a marker for the presence of the organism; comparable results have been obtained when screening for isocaproic acid produced by the organism (Potvliege, Labbé & Yourassowsky, 1981). A dilution factor of 1:10, which was introduced during the collection of the faecal samples, may have accounted for this reduced sensitivity.

An analysis of the relationship between the approximate quantity of para-cresol and the numbers of *Cl. difficile* present in the stools indicates that when the organism is present at concentrations above 10^3 c.f.u./g, corresponding high concentrations of para-cresol could be detected. This production of para-cresol by *Cl. difficile* may be an advantage in enabling the organism to compete for an ecological niche in the intestinal tract. This view is supported by the *in vitro* observations of Hafiz & Oakley (1976) that *Cl. difficile* had a remarkable tolerance for para-cresol, which could therefore be incorporated into a selective culture medium.

Bone, Tamm & Hill (1976) found an average urinary excretion of 51·8 mg para-cresol per day in normal adults. Urinary excretion has been observed to increase as weanling pigs grow (Yokoyama *et al.* 1982). We have found that infants with birth weights of over 2500 g had none detectable in their stools, and we assume that in older infants the majority of the para-cresol produced in the intestine is absorbed and excreted in the urine, although we did not test for this.

Para-cresol, a phenolic compound, is classified as a type-B toxic agent and can cause a rapid circulatory collapse and death in humans (Merck Manual). Yokoyama *et al.* (1982) have recently proposed that intestinal production may be responsible for a growth-depressing effect on the weanling pig. Wysowski *et al.* (1978), implicated the improper use of a phenolic disinfectant in the nursery as a cause of neonatal hyperbilirubinaemia. The possibility that an increased exposure to para-cresol following *Cl. difficile* colonization could similarly lead to adverse systemic effects requires further investigation.

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