J Clin Pathol 1986;**39**:1013–1018

Asymptomatic carriage of *Clostridium difficile* in patients with cystic fibrosis

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SUMMARY Faecal samples from 37 patients with cystic fibrosis and 40 control patients at the Brompton Hospital and the London Chest Hospital were examined for the presence of *Clostridium difficile*. The organism was isolated from 2 (17%) of control patients who were receiving antibiotics and from one (3·6%) of control patients who had no antimicrobial treatment. Thirty two per cent of the patients with cystic fibrosis excreted *C difficile*, though none of them had diarrhoea. Two of the three isolates from control patients and nine of the 12 isolates from patients with cystic fibrosis produced toxin B (cytotoxin) in vitro. Toxin B was present in the stools of one of the control patients and three of the patients with cystic fibrosis; toxin A (enterotoxin) was not detected in the faeces of the patients with cystic fibrosis. Two cytotoxigenic strains of *C difficile* isolated from patients with cystic fibrosis were examined in hamsters; both were virulent, and the animals died.

Clostridium difficile is the primary cause of most cases of pseudomembranous colitis as well as many cases of diarrhoea associated with antibiotics.[1] A combination of antibiotics, which may disrupt the normal gut flora and make the patient susceptible to infection with *C difficile*, and a hospital stay, which would increase the risk of exposure to this organism, would result in an increased risk of *C difficile* mediated gastrointestinal disease. Patients often exposed to both of these risk factors are those with cystic fibrosis. These patients receive almost continual antimicrobial prophylaxis and are often inpatients, although symptoms of diarrhoea associated with antibiotics are rare—despite recent work that shows a relatively high carriage rate of *C difficile*.[2–4]

The purpose of this study was to confirm this apparent high carriage rate both in inpatients and outpatients with cystic fibrosis at two different hospitals in London, England; to compare the carriage rate with that of other subjects with respiratory problems who were taking antibiotics; and further, to try to determine why the carriage of *C difficile* in these patients is asymptomatic.

Accepted for publication 22 April 1986

Material and methods

PATIENTS AND SPECIMENS

Faecal samples were obtained from 77 patients at the London Chest Hospital (LCH) and the Brompton Hospital, London (BH). Thirty seven of these patients had cystic fibrosis (mean age 19·1 years, range 6–32 years). Eight of the patients with cystic fibrosis were inpatients at LCH, 10 were inpatients at BH, and 19 were outpatients at BH; all but two were receiving antibiotics, seven receiving only nebulised antibiotics; and most were receiving treatment for pancreatic insufficiency, which was well controlled in all cases.

The control group consisted of 35 inpatients at LCH and five inpatients at BH (mean age 56 years, range 27–78 years), who were suffering from respiratory tract diseases unrelated to cystic fibrosis. These included emphysema, asthma, chronic obstructive airways disease, and carcinoma. Twelve of these patients were receiving antibiotics. None of the patients had diarrhoea or other gastrointestinal illness at the time of study.

Faecal samples (about 10 g) were sent directly to the diagnostic laboratories of LCH from the wards, or by post from the patients' homes, in sterile plastic universal bottles. They were stored undiluted at −20°C for no more than one month before cultivation.

ISOLATION AND IDENTIFICATION OF *C difficile*

About 0·1 g of undiluted faeces was seeded directly on to selective agar. The medium was a modification of that described by George *et al*[5] and consisted of Columbia agar base with the addition of 0·05% w/v cysteine hydrochloride, 0·03% w/v sodium formaldehyde sulphoxylate, 7% v/v defibrinated horse blood, and 500 mg/l of D-cycloserine (Sigma) and 20 mg/l of cefoxitin (Merck, Sharp, and Dohme) as selective agents. The medium was stored at room temperature in an anaerobic environment for 24 hours before use. Seeded plates were incubated at 37°C for 48 hours in anaerobe jars containing an anaerobic environment generated by a gas kit system (Oxoid, Basingstoke). Anaerobiosis was monitored by resazurin indicator strips (Oxide). Fresh catalyst (reactivated by heating at 160°C for two hours) was used in each anaerobe jar.

A similar sample of faeces (0·1 g) was inoculated into Robertson's cooked meat medium (Southern Group Laboratories), which was incubated aerobically at 37°C for 48 hours and then subcultured on to the selective agar, incubated as described above.

After incubation the primary isolation agar plates were examined for colonies typical of *C difficile*, and a semiquantitative estimate of the amount of growth was recorded: + for growth at original inoculation site only; ++ for growth also in primary streaks; +++ for growth also in secondary streaks. When *C difficile* was recovered only after enrichment in Robertson's cooked meat medium growth was recorded as E positive.

Colonies typical of *C difficile* were presumptively identified by their characteristic pale green fluorescence under long wave (360 nm) ultraviolet light,[5 6] their typical cellular appearance in a Gram stained preparation after growth on Columbia blood agar,[6] and the smell of paracresol. Full identification was confirmed by conventional techniques including gas-liquid chromatography, as described in detail elsewhere.[6]

STORAGE OF ISOLATES

Cultures of *C difficile* were stored at room temperature in Robertson's cooked meat medium.

TESTS FOR TOXIGENIC STATUS OF ISOLATES

Isolates of *C difficile* were tested for their ability to produce toxin B (cytotoxin) in vitro by screening for cytopathic effects in African green monkey kidney (Vero) cells by the "conventional tube method," as described elsewhere.[7] The toxin B titre was also determined in all positive stools. Stools from patients with cystic fibrosis, which were positive for toxin B, were also screened for the presence of toxin A (enterotoxin) by an ELISA technique.[8]

PATHOGENICITY OF *C difficile* ISOLATES IN HAMSTERS

Two of the cytotoxigenic isolates of *C difficile* that produced toxin B in vivo were investigated for their ability to cause disease in hamsters. Each strain was tested in a pair of animals. The methods used have been described in detail elsewhere.[9 10] Briefly, hamsters pretreated with clindamycin were housed individually in sterile isolator cages and given 0·5 ml of a washed suspension of *C difficile* containing 10^4 organisms/ml. One strain was given five days and the other nine days after clindamycin. At death faecal material was analysed for the presence of *C difficile* and toxin B.

GROWTH IN FAECAL EMULSIONS

The stools from six patients with cystic fibrosis who did not harbour *C difficile* were screened for their ability to support the in vitro growth of a toxigenic strain of *C difficile* (strain B-1), using a recently developed in vitro model of colonisation resistance, described in detail elsewhere.[11] Briefly, stools diluted 20-fold in sterile distilled water were seeded with an actively growing culture of *C difficile*, and growth and toxin B production over 48 hours were monitored. Two of the faecal emulsions that were inhibitory were filter sterilised and the growth of *C difficile* was monitored in the sterile filtrates to determine whether the inhibition was due to antagonism exerted by the normal flora, as is the case in healthy adults, or, due to other cell free factors.

DETERMINATION OF FAECAL PH

Determinations of pH on sterile aqueous filtrates of 1/10 dilutions of faeces were done with a pH meter (model 38B, Electronic Instruments Ltd, Richmond, Surrey, England).

Results

ISOLATION OF *C difficile*

C difficile was isolated from two of the twelve control patients (17%) who had received antibiotics. Both patients had been treated with cephalosporins (table 1). In contrast, *C difficile* was isolated from only one of the 28 control patients (3·6%) who had had no antibiotic treatment. In total, three of 40 (7·5%) control patients carried *C difficile* in their stools. All three were inpatients on a single ward at LCH. Two of these isolates produced toxin B in vitro, and toxin B was detected in the stools of one of the patients carrying *C difficile* (table 1).

Twelve (32%) of the patients with cystic fibrosis carried *C difficile* in their stools. There was an appreciable difference in the isolation rate of *C difficile* between patients at LCH (62·5%) and those at BH

Table 1 *Details of control patients carrying C difficile*

Age	*Sex*	*Cytotoxigenic status of C difficile*	*Toxin B in stool*	*Faecal pH*	*Antibiotic*	*Diagnosis*
68	F	Positive	Positive		None (prednisolone)	Lung cancer
64	M	Negative	Negative		Cefuroxime	Lung cancer
31	F	Positive	Negative	6·8	Ceftazidime	Bronchiectasis

(24%). All of those with cystic fibrosis from whom *C difficile* was isolated had received antibiotics by mouth or injection (table 2), giving a carriage rate of 43% among those taking antibiotics by these routes. The organism was not isolated from any of those patients with cystic fibrosis who had not received antibiotics or received only nebulised antibiotics. All the patients with cystic fibrosis carrying *C difficile* at LCH were inpatients on the same ward, which was also the ward on which the control patients with *C difficile* were nursed. Five of the patients from BH with cystic fibrosis who carried *C difficile* were outpatients and two were inpatients on separate wards. In one of the patients with cystic fibrosis *C difficile* was isolated from an enrichment culture but not from the direct faecal culture. All but one of the patients carrying *C difficile* were receiving either a cephalosporin or a penicillin (tables 1 and 2). In addition, many of the patients, were receiving an aminoglycoside. Similar treatment was received by some of the patients from whom *C difficile* was not isolated (table 3).

C difficile AND FAECAL TOXICITIES

Two of the three isolates of *C difficile* from control patients produced toxin B in vitro, but toxin B was only detected in one of these two stools (table 1). Nine of the 12 isolates of *C difficile* from the patients with cystic fibrosis were cytotoxigenic, and toxin B was detected in three of these stools at titres of 1/1024, 1/4096, and 1/16 384 (table 2). These three stools, however, did not contain detectable amounts of toxin A.

FAECAL PH

Faecal pH values were determined for the one control patient and the five with cystic fibrosis who had no toxin B in the faeces but who were excreting cytotoxigenic *C difficile* and for two of the patients with cystic fibrosis who had toxin B in their stools. The purpose of these measurements was to determine if the absence of faecal toxin B in patients colonised with toxigenic *C difficile* was due to pH mediated toxin degradation. Tables 1 and 2 show the results. None of the faeces was sufficiently acidic to degrade toxin B.

Table 2 *Details of patients with cystic fibrosis carrying C difficile*

Age	*Sex*	*Cytotoxigenic status of C difficile*	*Toxin B titre in stool*	*Relative concentration of C difficile in stool*	*Faecal pH*	*Hospital*	*In/out patient*	*Antibiotics*	
								By mouth or injection	*Nebulised*
18	M	Positive	1/40 96	2+	5·9	London Chest Hospital	In	Tobramycin Azlocillin	
16	M	Negative	—	+			In	Tobramycin Azlocillin	
19	M	Positive	1/16 384	3+	6·7		In	Tobramycin Azlocillin	
23	M	Negative	—	2+			In	Tobramycin Azlocillin	
21	F	Negative	—	3+		Brompton Hospital	Out	Flucloxacillin	Tobramycin Colistin
15	F	Positive	—	3+	5·8		Out	Ceftazidime	Ticarcillin
21	F	Positive	—	+	6·3		Out	Tobramycin Ticarcillin	Piperacillin
19	M	Positive	—	E positive	6·0		Out	Tobramycin Flucloxacillin	Gentamicin Carbenicillin
17	M	Positive	—	+	5·9		Out	Flucloxacillin	
16	M	Positive	1/1024	+			In	Ticarcillin Gentamicin	
26	M	Positive	—	+	6·0		In	Ceftazidime Tobramycin	
17	M	Positive	—	2+			Out	Flucloxacillin	

Table 3 *Systemic antibiotic treatment of patients who did not carry C difficile*

	No of patients	*Hospital*	*Antibiotics*
Controls	10	London Chest Hospital	Erythromycin (n = 4), cefuroxime, ceftazidime, tetracycline, co-trimoxazole, gentamicin + ampicillin, not known
Patients with cystic fibrosis	4	London Chest Hospital	Ceftazidime (n = 2), ceftazidime + flucloxacillin, tobramycin + azlocillin
	12	Brompton Hospital	Carbenicillin, flucloxacillin, flucloxacillin + amoxycillin (n = 2), flucloxacillin + tetracycline, gentamicin + ticarcillin, gentamicin + ticarcillin + carbenicillin, gentamicin + carbenicillin, gentamicin + ceftazidime, netilimicin + azlocillin (n = 2), erythromycin + ceftazidime

Table 4 *Survival of C difficile over 48 hours in faecal emulsions prepared from stools of patients with cystic fibrosis not carrying organism*

Patient Age	*Change in numbers of C difficile*	*Production of toxin B (titre)*	*Faecal pH*	*Antibiotics*
18	10 fold increase	Yes (16384)		Ceftazidime
40	10 fold decrease	No		Ticarcillin, gentamicin
21	10 fold decrease	Yes (256)		Tobramycin, azlocillin
6	10^2 fold decrease*	No	6·1	Azlocillin, netilimicin
20	10^6 fold decrease	No	7·1	Ethambutol, rifampicin, erythromycin
23	10^6 fold decrease*	No		Gentamicin, carbenicillin

*Sterile faecal emulsion filtrate inhibitory to the same degree.

GROWTH OF *C difficile* IN FAECAL EMULSIONS

Growth of *C difficile* in faecal emulsions prepared from patients with cystic fibrosis who were not excreting the organism was monitored to determine the degree and nature of colonisation resistance (table 4). *C difficile* grew in the faecal emulsion from one of these patients and produced toxin B to a titre of 1/16384. Three of the faecal emulsions were mildly inhibitory, resulting in a decrease in the numbers of *C difficile* of between 10 and 100-fold. In all cases the total number of vegetative cells of *C difficile* was greater than the number of spores present, showing that survival was not simply a function of presence of spores. In one of these three cases a small amount of toxin B was produced (titre 1/256), implying that at some stage the cells were also metabolically active. In two cases the faecal emulsions were extremely inhibitory resulting in a 10^6-fold decrease in total numbers of *C difficile*. In the one case analysed the sterile filtrate was equally as inhibitory. This inhibition was not due to an acidic pH as the stool had a pH value of 7·1.

PATHOGENICITY OF *C difficile* IN HAMSTERS

Both strains of *C difficile* tested were pathogenic in hamsters. The strain given five days after clindamycin had killed both animals within 24 hours, and the strain given nine days after clindamycin had killed both animals after 72 hours. Toxigenic *C difficile* and free toxin B were found in the caecal contents at death in both cases.

Discussion

This study clearly shows a high asymptomatic carriage rate of toxigenic strains of *C difficile* in patients with cystic fibrosis. In all, 32% of these patients carried *C difficile* in their faeces and all of them were receiving antibiotics. Interestingly, none of the patients on nebulised antibiotics alone carried the organism. This carriage rate is similar to that of 31% observed by Wu *et al*[2] but higher than that of 22% observed by Welkon *et al.*[4] Analysis of the data provided by this and other workers shows that the increased rate of colonisation of patients with cystic fibrosis is, most likely, a consequence of the antibiotics that are often given with this disease as opposed to a direct result of the underlying disease itself. We found that none of the patients with cystic fibrosis who were not taking antibiotics or only nebulised antibiotics carried *C difficile* compared with 43% of those receiving antibiotics by mouth or injection; and Wu *et al*[2] failed to isolate *C difficile* from any of their patients with cystic fibrosis who were not receiving antibiotics. Unfortunately, it is impossible to determine how many of those patients who were not taking antibiotics in the study by Welkon *et al*[4] carried *C difficile*. As 92% of them had had anti-

biotics during the previous six months and only one subject who carried *C difficile* had not received antibiotics, it is safe to assume that the carriage of *C difficile* in patients with cystic fibrosis in their study was also clearly associated with antibiotics.

It is valid, therefore, to compare the carriage rate of *C difficile* in patients with cystic fibrosis, who are receiving antibiotics in addition to or other than in the nebulised form and who do not have diarrhoea, with that seen in patients with other disorders who are receiving antibiotics. Our carriage rate of 43% in patients with cystic fibrosis receiving antibiotics (other than just nebulised antibiotics) and values of 50%[2] and 22%[4] noted by others compare favourably with the asymptomatic carriage rates of 21% and 46% recorded by Viscidi *et al*[12] and George *et al*,[13] respectively, and the 17% seen in this study for other patients receiving antibiotics. The range of colonisation rates seen is probably a reflection of differences in the degree of exposure to *C difficile* at different centres, as well as differences in the sample sizes examined, and it is not dissimilar to the large differences in colonisation rates seen in neonates,[14] a group susceptible to colonisation.

The higher rate of carriage in the patients with cystic fibrosis—compared with that of controls seen in our study—could be a reflection of the longer period of hospital stay (a mean of 12·8 days compared with 8·9 days) but it more probably reflects the increased number of times that patients with cystic fibrosis are admitted.

Stools from patients with cystic fibrosis receiving antibiotics but who were not colonised with *C difficile* were analysed for their ability to inhibit the growth of *C difficile* in vitro showing that the faecal flora was, in general, non-inhibitory to *C difficile*. There were some cases in which the faecal emulsion was grossly inhibitory, but this inhibition was also present in the sterile filtrate of the one faecal emulsion in which sufficient material was available for study, implying that the inhibition was unrelated to the presence of viable bacteria. This inhibition was not due to pH and was most likely due to inhibitory titres of antibiotic present in the gut. The in vitro findings in this group are very similar to those for adults without diarrhoea, receiving antibiotics, and who do not have cystic fibrosis.[11] In contrast, faecal emulsions prepared from the stools of healthy adults inhibited the growth of *C difficile*, though sterile filtrates did not, highlighting the importance of the presence of bacteria in inhibition.[11]

Although 75% of our isolates of *C difficile* from patients with cystic fibrosis were cytotoxigenic, toxin B was rarely detected in their faeces. The absence of the acid labile toxin B was not due to faecal pH as all of the stools analysed had a pH value greater than 5·5, a value at which toxin B is stable at 37°C.[15] In the study by Wu *et al*[2] only 27% of the *C difficile* isolates were cytotoxigenic, and none of their patients had toxin B present in the faeces. In contrast, Welkon *et al*,[4] found most of their isolates to be cytotoxigenic and toxin B present in 62% of their patients.

The lack of diarrhoea in the patients excreting toxin B may be due to the absence of toxin A. In support of this hypothesis is our study showing that the three patients with cystic fibrosis were asymptomatically excreting toxin B and had undetectable amounts of toxin A present. In infants, however, it is possible to have asymptomatic carriage of *C difficile*, even with high titres of both toxins A and B in the faeces[16 17]: a similar situation could, therefore, exist in patients with cystic fibrosis. Asymptomatic carriage of toxigenic *C difficile* in any group is difficult to explain. The lack of toxin production in vivo explains some cases, but why the environmental conditions are not conducive to toxin production in these cases remains unexplained, as do the reasons why, in our work, only one toxin was detected in the faeces. Although this could be a reflection of the differing sensitivities of the toxin detection systems used, the one for detection of toxin B being the most sensitive, there is evidence that available nutrients can determine the relative amounts of the two toxins produced.[18]

The fact that specific mucosal receptors for the toxins may be absent in patients with cystic fibrosis must also be considered. Despite the fact that it has recently been shown that not all toxigenic strains of *C difficile* are equally virulent[19] this did not explain the asymptomatic carriage in our study, as the isolates tested were virulent in hamsters. In some patients there might have been sufficient concentrations of antibiotics to inhibit full expression of virulence. Whatever the reasons for the observation of asymptomatic carriage, it is evident that the environment of the gastrointestinal tract inhibits full expression of virulence.

Finally, it is now evident that patients with cystic fibrosis should be recognised as a possible reservoir of infection and that they represent a cross infection risk, especially as they are often nursed in large medical wards.

We thank the patients and nursing staff in this study and consultant physicians Dr DM Geddes, Dr SJ Steel, Dr J Warner, and Dr J Batten. We also thank Dr S Haslam for faecal enterotoxin analyses.

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