Clinical significance of *Clostridium difficile* and its toxins in faeces of immunocompromised children

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Abstract

In this study, clinical and laboratory findings were tested for correlation with the presence of *Clostridium difficile*. The toxigenicity of the isolated strains and the toxins were determined in faecal samples of immunocompromised children admitted to a single room for protective isolation. Using the toxin assay as the gold standard, the culture sensitivity of toxigenic *C. difficile* was 94.1%, the specificity 93.8%, the positive predictive value 62.8%, and the negative predictive value 99.3%. Correction for stools with a positive culture of toxigenic *C. difficile* preceding detection of toxin, resulted in a positive prediction value of 78.4%. A statistically significant association was found between a positive faecal toxin assay and fever, and between a positive culture of toxigenic *C. difficile* and abdominal pain: 42% of the patients with positive toxin assays had fever versus 21% with negative toxin assays, and 66% of the patients with a positive culture for toxigenic *C. difficile* had abdominal pain, versus 22% with negative cultures. Further analysis of the cultures and toxin assays showed no statistically significant association with diarrhoea, fever, white blood cell count, C reactive protein concentrations, or abdominal pain. Based on these findings, it is suggested that immunocompromised children should be treated when toxigenic *C. difficile* is cultured or when toxin is detected in stool samples.

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colonised with *C. difficile* than bottle fed children,^{7,9} although this has been contradicted by others.^{5,10} Fetal intestinal cells are less sensitive to the toxins than adult intestinal cells in vitro.¹¹ Infants may lack toxin A receptors.¹ Numerous cases of antibiotic associated diarrhoea and colitis have, however, been described in this age group, especially in compromised patients.^{7,12-19} Disease resulting from *C. difficile* toxins has complicated not only antibiotic use, but also cancer chemotherapy.²⁰⁻²³ Invasive diagnostic procedures are clearly difficult to use in this age group. Presumptive diagnosis is dependent on the isolation of toxigenic *C. difficile* or the finding of toxin A or toxin B, or both, in the stools. Children with haematological malignancies are a special group with respect to the clinical significance of colonisation by *C. difficile*. On the one hand they are at risk for developing antibiotic associated diarrhoea because they are often admitted to hospital because of the underlying disease or cancer chemotherapy, while on the other hand they are treated with multiple courses of antimicrobial agents. Furthermore, these children can develop diarrhoea for a number of other reasons, for example, chemotherapy.

In a previous study we showed a high incidence of *C. difficile* in this infant subgroup. In this study we looked at the clinical significance of the presence of *C. difficile*.

Patients and methods

In the Wilhelmina Children's Hospital in Utrecht, The Netherlands, the nursing of children who are very ill or are severely immunocompromised, or both, and therefore need reverse isolation is concentrated in one ward with a capacity of eight isolation rooms. From November 1990 to May 1992 faecal samples of all children admitted to this ward were cultured once weekly for the presence of *C. difficile* and checked for the presence of toxins using a cytotoxin tissue culture assay. The results of these weekly tests were correlated with diarrhoea, fever, abdominal pain, white blood cell count, and the concentration of C reactive protein using the χ^2 test; $\alpha < 0.05$ denoted statistical significance.²⁴ One way analysis of variance (ANOVA) was used to analyse age and admission period; the significance of differences between groups was calculated by separate *t* tests.²⁴ The following definitions were used: diarrhoea, either more than five stools a day, more than three loose stools a day or more than one liquid stool a day; fever, either body temperature of 38.0°C for at least 12 hours or at least one peak over

Since 1978 *Clostridium difficile* has been recognised as an important cause of antibiotic associated colitis. This disease is caused by the two toxins produced by *C. difficile*, toxin A and toxin B (for review see Lyster *et al*).¹ Non-toxigenic strains of *C. difficile* cannot produce any toxin because they lack the genes encoding for both toxin A and toxin B.²⁻⁴

The role of *C. difficile* as a cause of diarrhoea in neonates and infants has been the subject of many studies. There is a high incidence (up to 25-50%) of asymptomatic colonisation of both toxigenic and non-toxigenic *C. difficile* strains in neonates and infants.⁵⁻⁷ It seems that, especially during the first six months of life, the gut is insensitive to the toxins produced by *C. difficile*. Several factors are considered to contribute to this phenomenon. For example colostrum neutralises both toxin A and toxin B.⁸ Some researchers have found that breast fed children were significantly less often

38.5°C. Abdominal pain was only checked for children over two years old. Each culture result was related to the clinical signs and symptoms for the two days before and after the day of culture. Only when it was suspected that *C. difficile* was the cause of disease, were the patients treated with vancomycin or metronidazole.

CULTURE OF FAECAL SAMPLES

Faecal samples were transported to the laboratory and processed within 12 hours. The specimens were inoculated in modified cycloserine-cefoxitin-fructose-agar (CCFA) (Oxoid, Basingstoke, United Kingdom) containing 250 mg/l cycloserine and 8 mg/l cefoxitin (Oxoid),²⁵ as well as in *C. difficile* agar (Oxoid) containing 500 mg/l cycloserine and 16 mg/l cefoxitin (Oxoid), both prepared according to the manufacturer's instructions. The cultures were incubated anaerobically and observed for 72 hours. Presumptive identification of *C. difficile* was based on colonies consisting of Gram positive rods, producing a horse-stable odour, having a yellow, ground glass appearance with a filamentous edge on CCFA, or having a white-grey, non-haemolytic, ground glass appearance with a filamentous edge on *C. difficile* agar. Definitive identification was obtained by prereduced anaerobically sterilised biochemical testing as described by Holdeman *et al.*²⁶

CYTOTOXIN TISSUE CULTURE ASSAY

The cytotoxin tissue culture assay was performed on all faecal specimens as described by Allen²⁷ with some modifications. For the tissue culture assay, Vero cells were grown in 96-well microtitre plates. Stool supernatant was tested in serial threefold dilutions and neutralisation was obtained with *C. sordellii* antitoxin (Wellcome, Basingstoke, UK). To determine the toxin titre, the highest dilution was taken that showed at least 50% cytopathic effect, providing the neutralisation assay did not show any cytopathic effect.

DETERMINATION OF TOXIGENICITY OF ISOLATED *C. DIFFICILE* STRAINS

The presence of the toxin B gene of the isolated *C. difficile* strains was determined by either polymerase chain reaction (PCR) as described by Fluit *et al.*² or colony blot hybridisation as described by Wolfhagen *et al.*²⁸ Briefly, the PCR reactions were performed in 50 mM KCl, 1.5 mM MgCl₂, 10 mM TRIS-HCl (pH 8.3), 0.01% (wt/vol) gelatin, 100 µM of each of the dNTPs, 50 pmol of each appropriate primer, and 2.5 U Taq polymerase (Cetus, Emeryville, CA, USA). The sequence of the primers used for amplification of the toxin B gene were 5'-TAATAGAAAACAGTTAGAAA and 5'-TCCAATCCAAACAAAATGTA, which produced a 301 bp fragment.²⁹ Samples were subjected to 30 cycles of amplification, with each cycle consisting of one minute at 94°C, one minute at 50°C, and two minutes at 72°C. For determination of toxigenicity by colony

blot hybridisation, *C. difficile* was cultured for 24 hours on *C. difficile* agar. The colonies were replica plated onto a nylon filter (Zeta-probe, Biorad, USA). The colonies on the filter were then lysed with 10 mg/ml lysozyme in TRIS-EDTA (10 mM TRIS-HCl (pH 7.5), 1 mM EDTA) for 30 minutes. DNA was denatured twice in 0.5 N NaOH and 1.5 M NaCl for five minutes. This was followed by neutralisation with 0.2 M TRIS-HCl (pH 7.5) and 2×SSC (1×SSC equals 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), twice for five minutes; 2×SSC, twice for five minutes; 0.5 N NaOH and 1.5 M NaCl once for 10 minutes; and 2×SSC, twice for 10 minutes. Finally, the bacterial remnants were removed by pressing the filters between dry Whatmann 3 MM paper. Hybridisation was performed with a digoxigenin labelled probe of 89 nucleotides specific for toxin B in 50% formamide, 0.1% N-lauroylsarcosine, 0.2% SDS, and 2.5% blocking reagent (Boehringer, Mannheim, Germany) overnight with 50–100 ng probe per ml at 37°C. The filter was washed twice with 2×SSC and 0.1% SDS for 15 minutes at 37°C. Hybridisation was detected using sheep anti-digoxigenin polyvalent Fab-alkaline phosphatase conjugate (Boehringer) with X-phosphate and 4-nitroblue tetrazolium chloride as substrate, according to the instructions of the manufacturer.

Results

In total we studied 78 admissions of 62 patients. Nine patients were admitted twice to the same ward, two patients three times, and one patient four times. The patients were admitted to hospital because of haematological malignancies (n=22), other haematological diseases (n=11), exacerbations of autoimmune diseases (n=12), AIDS (n=3), Kawasaki syndrome (n=2), severe infections (n=4), or other reasons (n=8).

In total 343 faecal samples were collected; toxigenic *C. difficile* was cultured from 55 samples, while in 39 cases non-toxigenic *C. difficile* was cultured and 249 samples were negative. The toxin assay was evaluable for 339 of 343 samples (in four samples with a positive culture of toxigenic *C. difficile*, the toxin assay was not performed); 34 samples were positive. The toxin assay was positive in 62% (32 of 51) of the samples from which toxigenic *C. difficile* could be isolated and in only 0.8% (2 of 288) of stools from which no toxigenic *C. difficile* could be isolated. When the toxin assay was used as the gold standard and compared with the culture of toxigenic isolates, the sensitivity of culture was 94.1%, the specificity 93.8%, the positive predictive value 62.8%, the negative predictive value 99.3%, the positive likelihood ratio 15.1, and the negative likelihood ratio 0.06. A comparison of the toxin assay with the culture of both toxigenic and non-toxigenic *C. difficile* resulted in a sensitivity of 94.1%, a specificity of 80.9%, a positive predictive value of 35.6%, a negative predictive value of 99.2%, a positive likelihood ratio of 4.9, and a negative likelihood ratio of 0.07.

TABLE I Admissions data subdivided according to the results of faecal sample analysis; Clostridium difficile culture positive includes toxigenic as well as non-toxigenic Clostridium difficile strains

	C difficile culture negative and cytotoxin negative stools	C difficile culture positive* but cytotoxin negative stools	C difficile cytotoxin positive stools†	All stools
Admissions (n)	49	16	13	78
Total (wk)	122	64	166	352
Mean (SD)	2.5 (2.3)	4 (5.8)	12.8 (15.9)	4.5 (8.1)
Range	1–12	1–23	1–56	1–56
Patients (n)				
Male	17	11	7	35
Female	32	5	6	43
Age (y)				
Mean (SD)	8.4 (5.3)	4.3 (4.6)	6.5 (4.6)	7.2 (5.3)
Range	0.1–18.4	0–15.2	0.1–14.9	0–18.4
Antimicrobial treatment (%) (n)	80 (39)	81 (13)	92 (12)	82 (64)
Selective gut decontamination (%) (n)	49 (24)	38 (6)	85 (11)	53 (41)
Cancer chemotherapy (%) (n)	41 (20)	44 (7)	62 (8)	45 (35)
Immunocompromised (%) (n)	71 (35)	63 (10)	85 (11)	72 (56)
Treatment for antibiotic associated diarrhoea (%) (n)	0 (0)	19 (3)	69 (9)	15 (12)

*At least one culture had to be positive during the observation period; †only two patients were cytotoxin positive and culture negative, the others were both culture and toxin positive.

As Table I shows, during 29 of a total of 78 admissions (37%), there was evidence for the presence of *C difficile* by culture or cytotoxin assay, or both. These comprised 16 patients with at least one positive culture and 13 patients with at least one positive toxin assay, of whom 11 were also culture positive. During 66 admissions to this ward antimicrobial treatment was given and during 35 admissions cancer chemotherapy. During eight admissions neither antimicrobial treatment nor cancer chemotherapy was given; however, seven of these eight patients received antibiotics or chemotherapy in the preceding period. One way ANOVA showed a significant group effect, as defined in Table I, on duration of admission to the ward ($F(2.75)=10.03$, $p<0.01$) and on age of the patients ($F(2.75)=4.06$, $p<0.05$). Separate *t* tests showed significant differences between the presence of toxin in stool samples and the absence of both toxins and *C difficile* for the duration of the admission ($t=2.24$, degrees of freedom=12.1, $p<0.05$); respectively the presence of *C difficile* but not its toxin, and the absence of both for age ($t=2.93$, degrees of

TABLE II Admissions data subdivided according to the results of faecal sample analysis; Clostridium difficile culture positive is restricted to only toxigenic Clostridium difficile strains

	C difficile culture negative and cytotoxin negative stools	C difficile culture positive* but cytotoxin negative stools	C difficile cytotoxin positive stools†	All stools
Admissions (n)	56	9	13	78
Total (wk)	143	43	166	352
Mean (SD)	2.6 (2.6)	4.8 (6.8)	12.8 (15.9)	4.5 (8.1)
Range	1–12	1–23	1–56	1–56
Patients (n)				
Male	24	4	7	35
Female	32	5	6	43
Age (y)				
Mean (SD)	7.6 (5.4)	5.6 (5.4)	6.5 (4.6)	7.2 (5.3)
Range	0–18.4	0.4–15.2	0.1–14.9	0–18.4
Antimicrobial treatment (%) (n)	82 (46)	67 (6)	92 (12)	82 (64)
Selective gut decontamination (%) (n)	48 (27)	33 (3)	85 (11)	53 (41)
Cancer chemotherapy (%) (n)	41 (23)	44 (4)	62 (8)	45 (35)
Immunocompromised (%) (n)	68 (38)	78 (7)	85 (11)	72 (56)
Treatment for antibiotic associated diarrhoea (%) (n)	2 (1)	22 (2)	69 (9)	15 (12)

*At least one culture had to be positive during the observation period; †only two patients were cytotoxin positive and culture negative, the others were both culture and toxin positive.

TABLE III Presence of both toxigenic and non-toxigenic Clostridium difficile in relation to diarrhoea, fever, abdominal pain, white blood cell count, and C reactive protein

	Culture		χ^2	<i>p</i> Value
	Positive	Negative		
Diarrhoea	56	169		
No diarrhoea	38	80	2.08	NS
Fever	24	53		
Normal temperature	67	191	0.81	NS
Decreased WBC	40	128		
Normal WBC	41	83		
Raised WBC	12	40	3.58	NS
CRP <10 mg/l	52	123		
CRP 10–20 mg/l	14	48		
CRP >20 mg/l	17	59	2.08	NS
Abdominal pain present*	10	30		
Abdominal pain absent	13	85	2.82	NS

WBC=white blood cell count; CRP=C reactive protein. *Not all patients could express the presence of abdominal pain.

freedom=28.4, $p<0.01$). Furthermore, an association, evidenced by significant χ^2 values, was found between the presence of toxin and selective gut decontamination ($\chi^2=7.06$, degrees of freedom=2, $p<0.05$), and, obviously, between the presence of toxin and specific treatment against *C difficile* ($\chi^2=40.69$, degrees of freedom=2, $p<0.001$).

In Table II the same demographic data are shown, however, non-toxigenic *C difficile* strains are categorised in the culture negative group. Statistical analysis of these groups showed the same statistically significant associations as mentioned in Table I, except that one way ANOVA showed no significant group effect on the age of patients.

Tables III, IV, and V show relations between the different groups and diarrhoea, fever, white blood cell count (WBC), C reactive protein values, and abdominal pain. The data are evaluated for the overall culture results, including toxigenic as well as non-toxigenic strains (Table III), culture of toxigenic *C difficile* (Table IV), and positive toxin assays (Table V). From these Tables it is clear that there are statistically significant associations between a positive faecal toxin assay and fever and a positive culture of toxigenic *C difficile* and abdominal pain: 42% of the patients with a positive toxin assay had fever versus 21% with a negative toxin assay, 66% of the patients with a positive culture for toxigenic *C difficile* had abdominal pain, while only 22% with negative cultures showed this symptom.

TABLE IV Presence of toxigenic Clostridium difficile in relation to diarrhoea, fever, abdominal pain, white blood cell count, and C reactive protein

	Culture		χ^2	<i>p</i> Value
	Positive	Negative		
Diarrhoea	34	191		
No diarrhoea	21	97	0.42	NS
Fever	16	61		
Normal temperature	37	221	1.85	NS
Decreased WBC	30	138		
Normal WBC	18	106		
Raised WBC	6	46	1.40	NS
CRP <10 mg/l	30	145		
CRP 10–20 mg/l	13	49		
CRP >20 mg/l	13	63	0.49	NS
Abdominal pain present*	10	30		
Abdominal pain absent	5	93	11.61	$p<0.001$

*Not all patients could express the presence of abdominal pain. Abbreviations as in Table III.

TABLE V Faecal Clostridium difficile toxin assay in relation to diarrhoea, fever, abdominal pain, white blood cell count, and C reactive protein

	Toxin assay		χ^2	P Value
	Positive	Negative		
Diarrhoea	26	195	2.12	NS
No diarrhoea	8	110		
Fever	14	63	7.75	p<0.01
Normal temperature	19	238		
Decreased WBC	16	148	2.49	NS
Normal WBC	14	107		
Raised WBC	2	50	2.53	NS
CRP <10 mg/l	20	155		
CRP 10–20 mg/l	3	57	2.45	NS
CRP >20 mg/l	10	68		
Abdominal pain present*	5	34		
Abdominal pain absent	5	93		

*Not all patients could express the presence of abdominal pain. Abbreviations as in Table III.

Discussion

In this study we investigated the significance of isolating both toxigenic and non-toxigenic *C difficile* isolates and using the toxin assay of stool samples in a population of severely diseased children. Most children were immunocompromised and treated with antimicrobial agents and chemotherapy. One important complicating factor in the analysis is that, because of the underlying diseases and related treatment, these children are especially at risk of developing diarrhoea and gastrointestinal symptoms. Overall diarrhoea was present 65% of the time (225 of 343 weeks). In 75% of these periods with diarrhoea (168 of 225 weeks), no relation with *C difficile* or its toxin could be found. With this high background incidence of diarrhoea, the classic symptom of antibiotic associated diarrhoea loses much of its value in this patient group. Furthermore, isolation of *C difficile* without determination of the toxigenicity of the strain is not a significant clue for the diagnosis of antibiotic associated diarrhoea; no significant association between the overall culture results and any of the signs and symptoms could be found. If toxigenic *C difficile* isolates are taken into account, however, there is a positive association with abdominal pain. For the presence of toxin B in stools, there is a positive association with fever. Nevertheless, neither symptom indicates antibiotic associated diarrhoea specifically, because they are also often present with culture and toxin negative samples. Because treatment of antibiotic associated diarrhoea with vancomycin or metronidazole is not especially risky for these patients, we think that stool samples with a positive toxigenic *C difficile* culture or a positive toxin assay should lead to treatment, to exclude this agent as a contributing factor to disease. As shown in a previous study³⁰ eradication of *C difficile* was possible in a high number of cases.

Culture of toxigenic *C difficile* had a positive predictive value of 62.8%, a negative predictive value of 99.3%, a positive likelihood ratio of 15.1, and a negative likelihood ratio of 0.06. Even though this culture scored very well, too many positive samples were found compared with the gold standard of the toxin assay. Nevertheless, several studies have shown that patients with negative toxin assays and positive

cultures are related to antibiotic associated diarrhoea.^{31 32} In this study a positive culture of toxigenic *C difficile* preceded detection of toxin in three of 13 patients (23%). If these stools are considered toxin positive, the outcome of the positive prediction value can be raised to 78.4% and the positive likelihood ratio to 25.8.

The significant group effect on age found in Table I was lost in Table II. Stools from which non-toxigenic *C difficile* strains were isolated, were considered culture negative in Table II, showing that non-toxigenic *C difficile* strains are predominantly isolated in the younger age groups. This phenomenon has been confirmed by numerous other studies.^{33–36} Group effects of the presence of toxin on admission time and the use of selective gut decontamination (Tables I and II) can be explained because children who are admitted for a longer time, are at risk for a longer time and are probably sicker and in need of selective gut decontamination because of severe immunosuppression.

In conclusion, we established a statistically significant association between a positive faecal toxin assay and fever, and between a positive culture of toxigenic *C difficile* and abdominal pain. This clearly suggests that toxigenic *C difficile* is a cause of disease in immunocompromised children.

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