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Is there a relationship between the presence of the binary toxin genes in Clostridium difficile strains and the severity of Clostridium difficile infection (CDI)?

Claire E Berry[1], Kerrie A Davies[1], David W Owens[2], Mark H Wilcox[1]

[1]Department of Microbiology, Leeds Teaching Hospitals NHS Trust and Healthcare Associated Infections Research Group, The University of Leeds, Old Medical School, Leeds. UK. [2]Leeds Institute of Health Sciences, The University of Leeds, Leeds. UK

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Corresponding author: K. A. Davies. Department of Microbiology, Leeds Teaching Hospitals NHS Trust, Leeds General Infirmary, Old Medical School, Leeds LS1 3EX, UK. kerrie.davies@nhs.net Tel: +44113 3928664 Fax: +44 113 3922696

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Data availability statement

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Abstract (232)

Purpose: Some strains of C. difficile produce a binary toxin, in addition to the main C. difficile virulence factors (toxins A and B). There have been conflicting reports regarding the role of binary toxin and its relationship to the severity of Clostridium difficile infection (CDI).

Methods: Samples, isolates and clinical data were collected as part of a prospective multicentre diagnostic study. C. difficile isolates (n = 1259) were tested by polymerase chain reaction (PCR) assay to detect binary toxin genes cdtA and cdtB. PCR binary toxin gene results were compared with clinical severity and outcome data, including 30-day all-cause mortality.

Results: The 1259 isolates corresponded to 1083 different patients (October 2010 to September 2011). The prevalence of binary toxin positive strains was significantly higher in faecal samples with detectable toxin A/B than in those without toxin but that were positive by cytotoxigenic culture (26.3% vs. 10.3%, $p<0.001$). The presence of binary toxin correlated moderately with markers of CDI severity (white cell count, serum albumin concentration and serum creatinine concentration). However, the risk ratio for all-cause mortality was 1.68 for binary toxin positive patients and patients were significantly less likely to survive if they had CDI caused by a binary toxin gene positive strain, even after adjusting for age ($p<0.001$).

Conclusions: The presence of binary toxin genes does not predict the clinical severity of CDI, but it is significantly associated with the risk of all-cause mortality.

Background

Clostridium difficile is a key nosocomial and community pathogen [1]. In order to manage C. difficile infection (CDI) effectively, timely and accurate diagnosis is of utmost importance [2]. As treatment options increase, predictors of increased CDI severity and poor outcome have increasing relevance as they may permit targeted interventions. The major virulence determinants of C. difficile are toxins A and B, although there remains uncertainty about their relative importance [3, 4]. Some C. difficile strains additionally produce a binary toxin (CDT) [5]. This comprises two distinct proteins: an enzymatic (CdtA) protein (463 amino acids, molecular mass 53kDa), and a cell binding (CdtB) protein (876 amino acids, molecular mass 98.8 kDa) [6, 7, 8]. The European prevalence of binary toxin in C. difficile strains, which is influenced by the clonality of particular strains, has been reported at between 17 and 23% [9, 10, 11].

C. difficile binary toxin was first reported in 1997 [12], and its pathogenic potential is supported by evidence that the highly related binary toxin in Clostridium spiroforme has been implicated as a virulence factor and is involved in the pathogenesis of intestinal disease in rabbits [13]. There are limited data on the function of C. difficile binary toxin due to difficulty isolating it in vitro [13, 14]. This difficulty plus the absence of a commercial assay, presumably explains why so much of the published work has utilised molecular detection rather than detection of the protein itself. The CdtLoc (the detection target), also known as the Cdt locus, is a 6.2kb region that includes the cdtA and cdtB toxin genes and a regulatory gene (cdtR) [7].

Binary toxin is not an absolute requirement for the virulence of C. difficile, although it may contribute as an independent factor to the pathogenicity of some strains [15]. It may play a complementary role in bacterial enterotoxicity, in addition to the cytotoxicity of toxin B [16]. We aimed to determine the relationship, if there is one, between binary toxin genes and the severity and outcome of CDI.

Methods

Study population

Samples and clinical data were collected over 12 months (October 2010 to September 2011) as part of a prospective multicentre diagnostic validation study [17]. The study involved four UK diagnostic laboratories, Leeds Teaching Hospitals NHS Trust, St George's Healthcare NHS Trust, Oxford University Hospitals NHS Trust, and University College London Hospitals NHS Foundation Trust.

Inclusion criteria

All diarrhoeal faecal samples (<7 days from collection) submitted for routine C. difficile screening were eligible for inclusion. All samples were tested for the presence of faecal toxin, determined by cell cytotoxin neutralisation assay (CCNA). In addition all samples were cultured on Brazier's agar and isolates were tested by cytotoxigenic culture (CT). CCNA, culture and CT were performed as previously described (17). Isolates were ribotyped [3] and stored in glycerol broth at -20^{o}C. To maintain patient confidentiality, study numbers were the only unique identifiers used.

Clinical data collection

A predefined clinical dataset was collected for all patients. Blood test results were gathered for routinely taken samples within three days of the faecal specimen to examine CDI severity markers: white cell count (WCC) ($x10^9$/L), serum creatinine (µmol/L) and serum albumin (g/L) concentration. The baseline serum creatinine (µmol/L) concentration within six months before faecal sampling was also recorded. Age, gender, length of stay and outcome (alive or deceased) at day 30 after CDI diagnosis were also collected.

Assay development and ribotyping

An in-house multiplex PCR for the detection of the gluD, cdtA and cdtB genes was developed using primers described previously [18,19]. GluD, a C. difficile housekeeping gene, was incorporated into the assay to act as an internal control for each isolate. Isolates were ribotyped in the Leeds Ribotyping Reference Laboratory, using an established method, and were identified via the UK C. difficile Ribotype Reference collection [20].

All C. difficile isolates were retrieved from frozen storage (-20^{o}C) by inoculating on to Columbia horse blood agar (E&O Laboratories Ltd., UK) and incubating anaerobically for 48 hours (A95 workstation, Don Whitley Scientific Limited, UK). Colonies were emulsified into 200µl purified water for automated nucleic acid extraction using the DX DNA extraction kit on the QIAxtractor (Qiagen Ltd., UK).

Statistical analysis

Mirroring the previous prospective multicentre diagnostic validation study (19), the faecal samples from which isolates were obtained were categorised into three groups: group 1, CCNA positive; group 2, CTA positive and CCNA negative; and group 3, CCNA and CTA negative.

Results were manually inputted into Excel (Microsoft) before export into Stata v12.1 (Statacorp LP) for statistical analysis. The 2-sample t-test was used for normally distributed metric data; the Mann Whitney test was used for skewed and for categorical data. Survival estimates were calculated by generating life tables and Kaplan Meier survival plots.

Results

From the 12,051 samples submitted for the multicentre diagnostic validation study (ref 17) 1275 isolates of C. difficile were recovered and stored; of these 1259/1275 (98.7%) were recovered from freezer stocks and PCR analysis for binary toxin genes was successfully performed on all 1259 isolates (corresponding to 1259 separate faecal samples from 1083 different patients). Overall ribotyping identified 79 different ribotypes, the most common being ribotypes 015 (n=128, 10.2% of all isolates), 014 (n=126, 10.0%), 027 (n=99, 7.9%) and 078 (n=65, 5.2%).

Of the 1259 isolates tested, 1046 (83.1%) were binary toxin negative, 428 (41.0%) of which were from male patients; 213 isolates (16.9%) were binary toxin positive, 107 (50.5%) of which were from male patients. 99/213 (46.5%) binary toxin positive strains were ribotype 027. All binary toxin positive isolates were positive for both the cdtA and cdtB genes. When categorised into the three groups for analysis, group 1

comprised 650 (51.6%) isolates, group 2, 339 (26.9%) isolates and group 3, 270 (21.4%) isolates. Notably the prevalence of binary toxin positive strains differed significantly across Groups 1, 2 and 3 (26.3%, 10.3% and 2.6%, respectively, all comparisons $p<0.001$).

Severity of infection

Serum creatinine concentration data were initially analysed by gender in addition to binary toxin status to take account of the different reference ranges for this analyte in males and females. Overall, there was a significant difference in the median serum creatinine concentration between patients carrying binary toxin positive and negative isolates for females only ($p=0.04$; Table 1). Further analysis of groups 1, 2 and 3 showed no significant differences between males or females (Tables 2, 3, 4). An alternative analysis was undertaken for 197 (25.3% of total) patients with an increase in serum creatinine concentration of $\geq$50% above their baseline value. There was however, no significant difference in the proportion of patients with an increase in serum creatinine between those with binary toxin positive isolates versus binary toxin negative isolates (20.9 vs 26.0%, $p=0.24$; Table 3).

WCCs were significantly elevated in patients infected with binary toxin positive strains, although median counts remained within the reference range (4-12 $x10^9$/L) ($p=0.008$; Table 3). Group 1 patients infected with a binary toxin positive isolate had significantly higher WCC verses binary toxin negative isolate ($p=0.008$); however, no significant differences were identified in either group 2 or 3 (Tables 2, 3, 4).

Overall, patients with binary toxin positive strains had significantly lower serum albumin levels than those infected with binary toxin negative strains ($p<0.001$; Table 3). This pattern was also seen in Groups 1 and 2 ($p=0.04$, $p=0.02$, respectively; Tables 2 and 3), although no such difference was seen for patients in group 3 (Table 4).

Length of admission

Overall, the median duration of stay was similar for patients with binary toxin positive isolates and binary toxin negative isolates (25 vs. 23 days, $p=0.90$; Table 3). Analysis of patients within groups 1, 2 or 3 also showed no significant differences in length of stay with respect to prevalence of binary toxin positivity (Tables 2, 3, 4).

All-cause Mortality

Mortality data were available for 880/1259 (69.9%) patients; overall, 752/880 (85.5%) survived and 128 (14.5%) died by day 30. All-cause mortality was significantly associated with detection of the binary toxin gene in C. difficile strains isolated from patient samples ($p=0.005$) (Table 3). To investigate whether mortality was influenced by age (as binary toxin positive patients were significantly older than binary toxin negative patients (median age 77 vs. 72 years respectively, $p=0.002$; Table 3), mortality rates were reanalysed after adjusting for age; confirming that a patient was less likely to survive if they were binary toxin positive regardless of age ($p<0.001$). The relative risk of mortality for patients with CDI caused by a binary toxin positive strain was 1.68 (95% CI: 1.18-2.40) compared with that for patients with a binary toxin negative strain (Table 5). This increased risk did not appear to be

significantly associated with the presence of 027 (RR 027 vs other binary toxin positive strains = 1.0)

All-cause mortality was also significantly associated with binary toxin status in group 1 patients (p=0.016); this association was further demonstrated by the Kaplan Meier plot, which shows significantly different survival estimates according to binary toxin status (p=0.040; Figure 1). Although more binary toxin positive patients than binary toxin negative patients died in group 2, the difference in mortality rates was not significant (Table 3). In addition, mortality rates in patients with binary toxin positive isolates in group 1 versus group 2 did not differ significantly (p = 0.444; Table 6). The risk of dying for group 1 patients with a binary toxin positive strain was very similar to that seen overall (RR 1.67); there was a non-significant increased risk of death in group 2 (RR 1.28; 95% CI: 0.48-3.40) and no increased risk in group 3 (no deaths) (Table 5).

The data were analysed to establish what effect deaths had on the median length of hospital stay. Overall, regardless of binary toxin status, patients who died had shorter median hospital stays; the median length of stay in those who died was 20 days compared with 24 days for patients who survived. Patients in group 1 and 2 who died had shorter stays than patients who survived.

Discussion

In this large cohort of C. difficile positive cases from four institutions, with clearly defined subgroups according to faecal toxin and CTA status, we detected binary

toxin genes in 20.8% (206/989) of toxigenic isolates, which is a comparable rate to previous studies [9, 10, 11]. It was notable that binary toxin positive strains were 2.5 fold as common among group 1 patients, that is those with detectable faecal toxin, than in samples from group 2 patients who were toxigenic culture positive (but did not contain free toxin) ($p<0.001$). This observation suggests that binary toxin-positive strains are truly associated with clinical disease, as the presence of detectable faecal toxin has been clearly associated with poorer outcomes, including all-cause mortality [17, 22]. Although non-toxigenic strains have recently been reported to express binary toxin [15], we failed to identify such examples in this large cohort.

Initial data analysis showed that patients with C. difficile isolates positive for the binary toxin gene had significant differences for age, gender, white cell count, serum albumin concentrations, and all-cause mortality when compared with subjects who had binary toxin negative isolates. Further analysis of only those patients who had CCNA positive diarrhoeal faecal samples (group 1) confirmed that gender, WCC, serum albumin concentrations, and all-cause mortality were significantly associated with binary toxin positive isolates. For patients with CTA positive isolates, but CCNA negative stool samples (group 2), only serum albumin concentrations were significantly different between those with binary toxin positive versus negative isolates. There were no significant findings according to binary toxin gene status for patients with toxin- and CTA-negative faecal samples (group 3).

The relationship between binary toxin positive C. difficile isolates and the severity of CDI has been unclear; our findings suggest that binary toxin may be associated with

some severity markers but not others. The presence of binary toxin gene was found to be significantly associated with all-cause mortality, with a relative risk of 1.68. This is a potentially important finding given that 30-day (all cause) mortality associated with CDI has been reported to occur in 6.5-16.6% of cases from large case series (albeit using different diagnostic approaches to define cases, and with varying rates of 'hypervirulent' strains) [1, 17, 23]. We found that mortality was higher in those patients with binary toxin positive isolates (22%) compared with mortality in those patients with binary toxin negative C. difficile isolates (13%). Interestingly, this excess risk of dying was not greater in patients with ribotype 027 versus those with other binary toxin positive ribotypes. A recent study in a non-ribotype 027 setting in Spain found that most binary toxin positive isolates (predominantly comprising ribotypes 078/126) were not associated with poor outcome [24].

Patients with binary toxin positive isolates had longer hospital stays, which suggests that these cases have greater morbidity. Patients who died, irrespective of their binary toxin status, had shorter lengths of hospital stay compared with those who survived (median 20 vs. 24 days). Therefore, severe infection that results in death is unlikely to be an explanation of prolonged hospital admission. An alternative explanation may be that older patients (possibly with more co-morbidities) tend to be infected with binary toxin positive strains (77 versus 72 years).

The presence of the binary toxin gene in isolates of group 3 patients was unexpected, as binary toxin has only been reported in toxigenic strains of C. difficile; at first glance, group 3 isolates are all non-toxigenic [9, 10, 11]. However, further investigation showed that within group 3 there were 7 isolates from stools that tested

negative in the CCNA that have previously been reported to be toxigenic ribotypes of C. difficile (023 n=3, 027 n=1, 045 n=1 and 078 n=2) [25, 26]. These isolates were found to be toxin negative by CTA culture, demonstrating that these isolates were not producing toxin at the time of testing, which presumably reflects the variation possible with such in vitro tests.

A limitation of our study is that we detected binary toxin gene as opposed to functional toxin. Carman et al. demonstrated that only 19/36 (53%) of stools that were binary toxin positive by polymerase chain reaction had the CdtB protein detected in the samples [8]. Thus, detection of a binary toxin gene might exaggerate the clinical significance of binary toxin. Additionally, we measured 30-day crude as opposed to CDI-attributable mortality. It is clearly possible that co-morbidities part-confounded the risk of dying, but it is very difficult to measure confidently true CDI attributable mortality. Notably, however, in this study cohort the risk of 30-day all-cause mortality in patients defined as having CDI (group 1) was approximately double that for patients who had diarrhoea but no evidence of toxigenic C. difficile (group 3) [17]. The nature of this study, a retrospective analysis of an existing dataset, did not allow full investigation of some potential confounders, such as recurrence.

It is not current recommended practice to include binary toxin gene testing in diagnostic testing strategies [2, 4, 26], mainly because no interventions have been described that could improve outcomes in binary toxin positive patients. However, it is plausible that more effective CDI treatment options could be targeted at binary toxin positive patients. There are some commercial binary toxin gene detection

assays available, however, if binary toxin detection were to be added to routine diagnostic practice, the incorporation of an assay (such as an enzyme immunoassay) to detect actual binary toxin would seem likely to be better than gene detection. As discussed previously, an enzyme immunoassay would need to detect CdtA and CdtB as both are required for functional binary toxin. Further work is needed to establish the clinical predictive value of the detection of binary toxin gene or toxin. However, given the association between binary toxin positive strains and mortality, there is a challenge to identify effective therapies in CDI caused by these strains.

Author contributions

The study was designed by CB, MHW and KD. CB analysed the data and wrote the manuscript, in conjunction with KD, DO and MHW. All authors reviewed drafts of the manuscript.

Compliance with ethical standards

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Potential conflicts of interest C.E.B has received research funding from bioMérieux and SUMMIT plc. K.A.D has received research funding from Astellas Pharma Europe Ltd**,** bioMérieux and Sanofi-Pasteur and has received honorarium from Astellas Pharma Europe Ltd and Summit. M.H.W has received research support from Abbott, Actelion, Alere, Astellas, Biomerieux, Cerexa, Cubist, Da

Volterra, European Tissue Symposium, Merck, Sanofi-Pasteur, Summit, The Medicines Company and Qiagen**,** has consultancies and/or lecture honoraria in the past 2 years from Actelion, Alere, Astellas, Astra-Zeneca, Basilea, Bayer, Cubist, Durata, European Tissue Symposium, J&J, Merck, Nabriva, Novacta, Novartis, Optimer, Pfizer, Roche, Sanofi-Pasteur and Seres and is on the speakers' bureau for Pfizer. DWO had no conflicts of interest to declare.

Ethical approval

The study was approved by the National Research Ethics Service (reference number 10/H0715/34).

Informed consent

Informed consent was not required for this study, as approved by the National Research Ethics Service.

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Table 1. Baseline characteristics and outcomes of C. difficile toxigenic positive patients according to binary toxin gene status

		Binary toxin positive patients			Binary toxin negative patients			
	Number in sample	Number (%)	Median (interquartile range)	Mean (standard deviation)	Number (%)	Median (interquartile range)	Mean (standard deviation)	P value
Demographics								
Total	1259	213 (16.9)			1046 (83.1)			
Gender								
Male		107 (50.5)			428 (41.0)			0.011
Female		105 (49.5)			616 (59.0)			
Age (years)	1256	212	77 (64-85)		1044	72 (57-84)		0.0022
Markers of CDI severity								
Rise in serum creatinine	780							
≥50% above baseline		24 (20.9)			173 (26.0)			0.241
<50% above baseline		91 (79.1)			492 (74.0)			
Male serum creatinine (umol/L)	429	92	94 (72 - 136)		337	89 (63 - 125)		0.1673
Female serum creatinine (umol/L)	519	69	78 (67 - 114)		450	71 (55 - 97)		0.0410
White cell count ($x10^9$/L)	942	160	10.4 (7.2 - 16.1)		782	9.5 (6.7-13.2)		0.0075
Serum albumin (g/L)	942	131		30 (7.1)	674		32.0 (7.9)	<0.001
30 day all-cause mortality	880							
Died		33 (21.9)			95 (13.0)			0.005
Survived		118 (78.1)			634 (87.0)			
Length of hospital stay (days)	787	128	25 (10-46)		659	23 (10-48)		0.9043
Length of hospital stay resulting in death (days)	117	29	30 (3-61)		88	18 (12-33)		0.4171
Length of hospital stay to discharge (days)	661	97	22 (10-45)		564	24 (10-51)		0.5617

Table 2. Baseline characteristics and outcomes of patients categorised by C. difficile testing results according to binary toxin gene status

Group1 Cell cytotoxin assay positive

		Group1 binary toxin positive patients			Group 1 binary toxin negative patients			
	Number in sample	Number (%)	Median (interquartile range)	Mean (standard deviation)	Number (%)	Median (interquartile range)	Mean (standard deviation)	P value
Demographics								
Total	650	171 (26.3)			479 (73.7)			
Gender								0.017
Male	262	82 (48.2)			180 (37.7)			
Female	385	88(51.8)			297 (62.3)			
Age (years)	647	170	78 (66 - 85)		477	75 (60 - 85)		0.0879
Markers of CDI severity								
Rise in serum creatinine								0.257
>50% above baseline	94	18 (19.6)			76 (25.3)			
<50% above baseline	298	74 (80.4)			224 (74.7)			
Male serum creatinine (umol/L)	213	72	95 (74 - 136)	116 (72.4)	141	93 (64 - 117)	136 (153.1)	0.2869 (MW 0.1616)
Female serum creatinine (umol/L)	276	59	78 (67 - 114)	113 (115.6)	217	71 (52 - 97)	95 (94.5)	0.2357 (MW 0.0089)
White cell count ($x10^9$/L)	483	128	11 (11 - 17)	14 (11.4)	355	10 (7 - 15)	12 (7.0)	0.0076 (MW 0.1737)
Serum albumin (g/L)	412	107		30 (7.2)	305		32 (7.3)	0.0434
30 day all-cause mortality								0.016
Died	77	29 (24.2)			48 (14.5)			
Survived	374	91 (75.8)			283 (85.5)			
Length of hospital stay (days)	382	101	24 (9 - 45)		281	25 (11 - 52)		0.1479
Length of hospital stay resulting in death (days)	66	25	30 (8 - 44)		41	34 (10 - 43)		0.8169
Length of hospital stay to discharge (days)	310	74	20 (9 - 45)		236	27 (12.5 - 53.5)		0.0537

MW - Mann Whitney

Table 3. The number of binary toxin positive strains of Clostridium difficile available for analysis in each category and the results

Group2 Cytotoxigenic culture positive only

		Group 2 binary toxin positive patients			Group 2 binary toxin negative patients			
	Number in sample	Number (%)	Median (interquartile range)	Mean (standard deviation)	Number (%)	Median (interquartile range)	Mean (standard deviation)	P value
Demographics								
Total	339	35 (10.3)			304 (89.7)			
Gender								0.097
Male	149	20 (57.1)			129 (42.4)			
Female	190	15 (42.9)			175 (57.6)			
Age (years)	339	35	73 (58 - 86)		304	73 (61 - 83)		0.7142
Markers of CDI severity								
Rise in serum creatinine								0.946
>50% above baseline	64	5 (29.4)			59 (28.6)			
<50% above baseline	159	12 (70.6)			147 (71.4)			
Male serum creatinine (umol/L)	119	16	86 (68 - 148)	122 (89.1)	103	85 (64 - 128)	146 (181.7)	0.6116 (0.8092)
Female serum creatinine (umol/L)	136	9	73 (44 - 90)	86 (71.7)	127	73 (55 - 107)	114 (94.5)	0.5475 (MW 0.3861)
White cell count ($x10^9$/L)	257	27	9 (7 - 11)	10 (5.0)	230	9 (6 - 13)	10 (5.0)	0.6615 (MW 0.6059)
Serum albumin (g/L)	212	19		28 (6.7)	193		32 (8.2)	0.0272
30 day all-cause mortality								0.624
Died	29	4 (14.8)			25 (11.6)			
Survived	214	23 (85.2)			191 (88.4)			
Length of hospital stay (days)	226	23	33 (18 - 42)		203	23 (9 - 47)		0.5729
Length of hospital stay resulting in death (days)	29	4	34 (11 - 73)		25	15 (12 - 28)		0.3585
Length of hospital stay to discharge (days)	194	19	33 (18 - 42)		175	24 (9 - 56)		0.8600

MW - Mann Whitney

Table 4. The number of binary toxin positive strains of Clostridium difficile available for analysis in each category and the results

Group3 Cell cytotoxin assay and cytotoxigenic culture negative

		Group 3 binary toxin positive patients			Group 3 binary toxin negative patients			
	Number in sample	Number (%)	Median (interquartile range)	Mean (standard deviation)	Number (%)	Median (interquartile range)	Mean (standard deviation)	P value
Demographics								
Total	270	7 (2.6)			263 (97.4)			
Gender								0.170
Male	124	5 (71.4)			119 (45.2)			
Female	146	2 (28.6)			144 (54.8)			
Age (years)	270	7	67 (50 - 77)		263	67 (50 - 80)		0.8253
Markers in CDI severity								
Rise in serum creatinine								0.682
>50% above baseline	39	1 (16.7)			38 (23.9)			
<50% above baseline	126	5 (83.3)			121 (76.1)			
Male serum creatinine (umol/L)	97	4	64 (59 - 86)	73 (23.7)	93	88 (60 - 127)	114 (96.0)	0.3896 (MW 0.3362)
Female serum creatinine (umol/L)	107	1	152 (na)	152 (na)	106	71 (56 - 96)	110 (133.6)	na (MW 0.1738)
White cell count ($x10^9$/L)	202	5	10 (8 - 10)	9 (6.4)	197	8 (5 - 12)	9 (6.8)	0.9689 (MW 0.8072)
Serum albumin (g/L)	181	5		32 (6.8)	176		32 (8.5)	0.8772
30 day all-cause mortality								0.459
Died	22	0 (0.0)			22 (12.1)			
Survived	164	4 (100.0)			160 (87.9)			
Length of hospital stay (days)	179	4	51 (33 - 78)		175	20 (8 - 39)		0.0840
Length of hospital stay resulting in death (days)	22	0	na		22	21 (16 - 34)		na
Length of hospital stay to discharge (days)	157	4	51 (33 - 78)		153	20 (8 - 39)		0.0802

MW - Mann Whitney

Table 5: The outcomes risk ratios according to the presence of binary toxin gene in all strains and toxigenic strains of Clostridium difficile

	Risk ratio (95% CI)	
	30 day all-cause mortality	Number (%) of patients with 30 day all-cause mortality data available
Binary toxin positive in all strains of C. difficile	1.68 (1.18-2.40)	880 (69.9)
Binary toxin positive in Group 1 strains of C. difficile	1.67 (1.10-2.51)	451 (69.4)
Binary toxin positive in Group 2 strains of C. difficile	1.28 (0.48-3.40)	243 (71.7)
Binary toxin positive Group 3 strains of C. difficile	0 (0-0)	186 (68.9)

Table 6.: The number of binary toxin positive strains of Clostridium difficile available for analysis in each category and the results

		Group1 binary toxin positive patients			Group 2 binary toxin positive patients			Group 3 binary toxin positive patients					
	Number in sample	Number (%)	Median (interquartile range)	Mean (standard deviation)	Number (%)	Median (interquartile range)	Mean (standard deviation)	Number (%)	Median (interquartile range)	Mean (standard deviation)	1 vs 2 p value	1 vs 3 p value	2 vs 3 p value
Demographics													
Total	213												
Gender													
Male	107	82 (48.2)			20 (57.1)			5 (71.4)			0.359	0.272	0.681
Female	105	88 (51.8)			15 (42.9)			2 (28.6)					
Age (years)	212	170	78 (66 - 85)		35	73 (58 - 86)		7	67 (50 - 77)		0.2893	0.0656	0.2872
Markers in CDI severity													
Rise in serum creatinine													
>50% above baseline	24	18 (19.6)			5 (29.4)			1 (16.7)			0.349	1.000	1.000
<50% above baseline	91	74 (80.4)			12 (70.6)			5 (83.3)					
Male serum creatinine (umol/L)	92	72		116 (72.4)	16		122 (89.1)	4		73 (23.7)	0.7582	0.2388	0.2913
Female serum creatinine (umol/L)	69	59		113 (115.6)	9		86 (71.7)	1		152 (na)	0.4964	na	na
White cell count ($x10^9$/L)	160	128		14 (11.4)	27		10 (5.0)	5		9 (6.4)	0.0845	0.3649	0.7618
Serum albumin (g/L)	131	107		30 (7.2)	19		28 (6.7)	5		32 (6.8)	0.1910	0.5164	0.2029
30 day all-cause mortality													
Died	33	29 (24.2)			4 (14.8)			0 (0.0)			0.444	0.572	1.000
Survived	118	91 (75.8)			23 (85.2)			4 (100.0)					
Length of hospital stay (days)	128	101	24 (9 - 45)		23	33 (18 - 42)		4	51 (33 - 78)		0.4615	0.0759	0.1416
Length of hospital stay resulting in death (days)	29	25	30 (8 - 44)		4	34 (11 - 73)		0	na		0.7516	na	na
Length of hospital stay to discharge (days)	97	74	20 (9 - 45)		19	33 (18 - 42)		4	51 (33 - 78)		0.3988	0.0698	0.1039

Figure 1: Kaplan Meier plots showing survival estimates in group 1 binary toxin positive and binary toxin negative patients (p=0.040)