ARTICLE

Elevated lactoferrin is associated with moderate to severe *Clostridium difficile* disease, stool toxin, and 027 infection

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Abstract We evaluated blood and fecal biomarkers as indicators of severity in symptomatic patients with confirmed *Clostridium difficile* infection (CDI). Recruitment included patients with CDI based on clinical symptoms and supporting laboratory findings. Disease severity was defined by physician's assessment and blood and fecal biomarkers were measured. Toxigenic culture done using spore enrichment and toxin B detected by tissue culture were done as confirmatory tests. Polymerase chain reaction (PCR) ribotyping was performed on each isolate. There were 98 patients recruited, with 85 (87 %) confirmed cases of toxigenic CDI (21 severe, 57 moderate, and seven mild), of which 68 (80 %) were also stool toxin-positive. Elevated lactoferrin ($p=0.01$), increased white blood cell (WBC) count ($p=0.08$), and low serum albumin ($p=0.03$) were all associated with the more severe cases of CDI. Ribotype 027 infection accounted for 71 % of severe cases ($p<0.01$) and patients with stool toxin had significantly higher lactoferrin levels and WBC counts ($p<0.05$). Our findings show that elevated fecal lactoferrin, along with increased WBC count and low serum albumin, were associated with more severe CDI. In addition, patients infected with ribotype 027 and those with stool toxin had significantly higher fecal lactoferrin and WBC counts.

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Introduction

The incidence of *Clostridium difficile* infection (CDI) is increasing and estimates are now around 500,000 cases per year in the U.S., with a yearly cost of 3.2 billion dollars [2, 8, 23, 24]. An epidemic strain, ribotype 027, has been implicated in a number of outbreaks in Europe, Canada, and the U.S. [2, 6, 10, 12, 13, 36]. This strain has toxin A and toxin B genes (*tcd*A and *tcd*B, respectively), genes for a binary toxin, 18-base-pair *tcd*C deletion (down regulator for the expression of toxin), and fluoroquinolone resistance. Studies have shown that the ribotype 027 strain expresses increased levels of toxin in vitro, more spores, and is associated with severe CDI [10, 21, 22, 26].

Identifying actual cases of CDI and determining disease severity are important factors when recommending a proper course of treatment, including choosing the antibiotic therapy; metronidazole for mild CDI and vancomycin for severe CDI [5, 15, 18, 40]. In general, patients with *C. difficile* disease often present with fever, have slightly raised white blood cell (WBC) counts (leukocytosis), and experience mild to moderate abdominal pain. Most cases of CDI require discontinuation of inciting antibiotic therapy when possible and the initiation of *C. difficile*-specific antimicrobial therapy [5, 18]. Defined laboratory parameters for fulminant *C. difficile* colitis are WBC count $\geq 15,000/\mu L$, a rising serum creatinine (50 % increase and levels ≥ 2.0 mg/dL), and albumin levels dropping below 2.5 mg/dL. Clinical complications may involve pseudomembrane formation, severe abdominal pain and/or cramping, and colonic thickening observed by computed tomography (CT) scan. Toxic megacolon stemming from the ileus may occur, causing nausea, vomiting, severe dehydration, and extreme lethargy [5, 7, 12, 13, 35]. Currently, no single laboratory parameter or combination of clinical symptoms is routinely used to stratify patients based on the severity of CDI for directing therapy.

Intestinal inflammation is a hallmark of patients with CDI [13, 35]. The severe stage of CDI, pseudomembranous colitis (PMC), results from the excessive tissue damage and inflammation. Biomarkers that help determine the "inflammatory status" in the intestine of a CDI patient potentially help predict the severity of CDI and possibly help direct the choice of antibiotic therapy [16]. Fecal lactoferrin is a biomarker for intestinal inflammation and has been evaluated in both infectious diarrhea and inflammatory bowel disease [9, 11, 16, 30–32, 34, 38]. Previous studies have demonstrated that elevated lactoferrin occurs in patients with *C. difficile* disease [1, 17, 25, 39]. Although these studies identified increased fecal lactoferrin in patients with CDI, none correlated the significance of levels detected in patients with defined CDI characteristics in combination with blood biomarkers for assessing disease severity. Defining a diagnostic role for blood and fecal biomarkers of inflammation for stratifying CDI patients according to disease severity would offer a clinical index for optimizing treatment and improving patient outcomes.

In this study, we evaluated blood and fecal biomarkers as indicators of severity in symptomatic patients with confirmed CDI by toxigenic culture. Patients were stratified by disease severity according to a physician's assessment, presence of ribotype 027 infection, and the detection of stool toxin.

Materials and methods

Study design and fecal specimens

The study protocol and recruiting method was approved by the institutional review boards (IRBs) of the Summa Health System (Akron, Ohio) and TechLab, Inc. (Blacksburg, Virginia). Recruitment was done between February 2010 and September 2012 using informed consent and included adult hospitalized patients and outpatients that were identified as having CDI based on a combination of symptoms, including ≥3 stools per day and in vitro diagnostic testing for glutamate dehydrogenase (GDH) and stool toxin performed by the clinical microbiology laboratory at Summa Health System. Patients with documented enteric infection other than *C. difficile*, inflammatory bowel disease, irritable bowel syndrome, and celiac disease were excluded. Each patient provided a single stool specimen for clinical diagnosis and, if CDI-positive, a portion of this specimen was then shipped to TechLab, Inc. for tissue culture testing, toxigenic culture, ribotyping of isolates, and fecal lactoferrin analysis. Stool testing was done within 72 h of collection by the clinical laboratory or after a single freeze–thaw at TechLab. Patient information and stool specimens were coded to maintain anonymity. Specimen consistency was defined according to the Bristol Stool Chart as follows: liquid specimens were defined as type 7; partially formed and soft specimens were defined as types 4–6; formed specimens were defined as types 1–3 [19, 22].

Chart reviews and disease assessment

Patient chart reviews were performed to record patient age, gender, co-morbidities, antibiotic use, treatment outcomes, reported symptoms, blood analysis for WBC count, and serum albumin level. Disease severity for CDI was done by physician's assessment using the following guidelines: (i) mild CDI cases included patients of any age, but usually ≤65 years, WBC <15 × 10⁹/L, stool <5 per day, no abdominal, peritoneal, or radiographic signs of disease, no co-morbid conditions, and able to tolerate oral intake; (ii) moderate CDI cases were of any age, WBC <15 × 10⁹/L, stool ≥5 per day, usually able to tolerate oral intake, reported abdominal complaints, had radiographic or peritoneal signs, existing co-morbid conditions including but not limited to renal failure and immunosuppression; (iii) severe CDI cases involved patients of any age, but was automatically classified as severe if age ≥65 years, WBC >15 × 10⁹/L, stool ≥10 per day, not able to tolerate oral intake, usually abdominal complaints, radiographic or peritoneal signs, multiple co-morbidities including but not limited to renal failure and immunosuppression.

Tissue culture for toxin B

The *C. DIFFICILE* TOX-B Test (TechLab® Inc., Blacksburg, VA) was performed according to the manufacturer's package insert with modification to include a neutral goat serum control. Briefly, a total of 50 µl of fecal supernatant was added to each of three wells containing a confluent layer of human foreskin cells and 50 µl of one of the following: PBS, *C. difficile* antitoxin, and neutral goat serum. A positive cytotoxic effect was defined as greater than 50 % rounding in the wells containing neutral goat serum and PBS but neutralized with no rounding in wells containing antitoxin.

Qualitative detection of GDH and toxin by immunoassay

The *C.DIFF QUIK CHEK COMPLETE*™ membrane-based enzyme-linked immunosorbent assay (ELISA) test (TechLab® Inc., Blacksburg, VA) was performed according to the manufacturer's package insert.

Quantitative fecal lactoferrin

Fecal lactoferrin concentrations were determined by immunoassay using the *IBD-SCAN*® test (TechLab®, Inc., Blacksburg, VA). A clinical cut-off of ≥7.25 µg/g stool was used to

define an elevated level of fecal lactoferrin as an indicator of intestinal inflammation [11].

Toxigenic culture

The isolation of *C. difficile* was done using the method previously described by Boone et al. [4].

PCR analysis and ribotyping

C. difficile isolates were polymerase chain reaction (PCR) ribotyped using the procedure developed by Stubbs et al. [33].

Statistical analysis

Normal distribution was confirmed prior to analysis. Median, standard deviation, standard error, and significance were generated using the JMP 9 (Cary, NC) statistical program by analysis of variance (ANOVA), Student's *t*-test, or the Chi-square likelihood ratio test, depending upon the data type examined. Student's *t*- test was used when comparing two independent sets of continuous data with assumed equal variances. Chi-square was employed to determine the significance between two groups of categorical data within a contingency table of a large sample size having only one degree of freedom. The clinical significance of severe CDI was determined using one-way ANOVA and expressed as *p*-values. A multilinear regression model that included combined patient variables was used to investigate predictors of severe CDI. For this study, the mean results were reported with standard error (SE) and significance was defined as having a $p < 0.05$. The range of levels for each biomarker is provided as a minimum and maximum (min, max).

Results

A total of 98 patients identified as having CDI based on a combination of symptoms, history of antibiotic use, stool GDH, and toxin were enrolled into the study. The mean age was 67 years and 64 % were female (Table 1). There were 85 (87 %) confirmed cases of toxigenic CDI using toxigenic culture, of which 68 (80 %) had stool toxin detected by the tissue culture assay. Of these, 38 (45 %) were infected with ribotype 027. One patient had a mixed infection of toxigenic and nontoxigenic *C. difficile* based on the isolation of a nontoxigenic ribotype (009) in combination with the detection of stool toxin by the tissue culture assay. This patient was admitted directly from an extended-care facility and had previous antibiotic therapy. Fecal lactoferrin (15 μg/g) and WBC count (11.9×10^9/L) were slightly elevated and the serum albumin level was low (2.4 mg/dL). Repeat bacterial culture never produced a toxigenic isolate.

Six patients (6 %) were negative for stool toxin and colonized with nontoxigenic isolates, as demonstrated by the isolation of nontoxigenic ribotypes, including 009 (1), 010 (1), 038 (2), and 039 (2). Repeated attempts to isolate a toxigenic ribotype were unsuccessful. The remaining six patients were negative for *C. difficile* by both bacterial culture and tissue culture. Additional patient characteristics including reported symptoms, co-morbidity, and treatment history are listed in Table 1.

Patients infected with toxigenic *C. difficile* were stratified according to physician's assessment for disease severity, resulting in 21 severe cases, 57 moderate cases, and seven mild cases. Age, reported abdominal pain, number of stools per day, blood, and fecal biomarkers were evaluated between severity groups and the results are shown in Table 2. Patients who suffered with clinically severe CDI had significantly elevated lactoferrin, higher WBC counts, and lower serum albumin when compared to those cases with moderate and mild clinical disease. In addition, we evaluated the clinical impact for blood and fecal biomarkers with severe CDI using one-way ANOVA. We identified a significant impact ($p < 0.05$) for increased lactoferrin, high WBC count, low serum albumin, and ribotype 027 infection for severe CDI (Table 2). In addition, we utilized a multilinear regression model that included age, presence of pain, stool toxin, lactoferrin, serum albumin, WBC count, and stools per day to investigate predictors of severe CDI. The model showed a significant association between these variables and severe CDI ($p = 0.0175$). The effect weight for each variable in the model expressed as a *p*-value is shown Table 3. Of the biomarkers included in the model, lactoferrin and serum albumin levels were highly significant ($p < 0.05$) and the WBC count trended close to being significant ($p = 0.0802$).

In our population, we had an overall 027 infection rate of 45 %, with the next four most common non-027 toxigenic ribotypes being 014 (8 %), 106 (5 %), 002 (5 %), and 126 (4 %). There were six non-027 isolates that were nontoxigenic and these patients were not included in the analysis for evaluating disease severity. There was a significant difference for elevated lactoferrin ($p = 0.012$) and for lower serum albumin levels ($p = 0.003$) between 027-infected patients compared to other toxigenic non-027-infected patients. Age, WBC count, number of stools per day, and reported abdominal pain were similar between the ribotype groups.

Testing for stool toxin by tissue culture showed that 80 % of patients infected with a toxigenic ribotype had detectable toxin. When patients were stratified based on the presence of stool toxin, toxin-positive patients had significantly higher lactoferrin ($p = 0.019$) and WBC count ($p = 0.029$), and the serum albumin levels trended lower (Table 4). The number of toxin-positive patients reporting abdominal pain trended towards significance ($p = 0.054$) and there was a significantly higher number of toxin-positive patients with ribotype 027

Table 1 Patient demographics and culture results for the study population. The percentage of patients for each criterion is shown, along with the number of patients presented in parentheses

Patient characteristics (N=98)		Percentage of total (n)
Gender	Female	64 % (63)
Age	>64 years	62 % (61)
	Mean age	67 years
Co-morbidity	Pulmonary disease	42 % (41)
	Diabetes	33 % (32)
	Coronary disease	31 % (30)
	Kidney disease	25 % (24)
	Cancer	24 % (23)
Symptoms	Abdominal pain	60 % (59)
	Fever	32 % (31)
	Stool mucus	23 % (22)
	Stool blood	12 % (12)
Stool consistency at assessment	Bristol 6–7 (unformed)	62 % (61)
	Bristol 4–5 (soft formed)	36 % (35)
	Bristol 1–3 (hard formed)	2 % (2)
Overall clinical condition	Severe	25 % (24)
	Moderate	67 % (66)
	Mild	8 % (8)
Patient history	Antibiotics	75 % (73)
	Antibiotics and CDI	14 % (14)
	No prior antibiotics	10 % (10)
	CDI with no antibiotics	1 % (1)
Culture	Toxigenic CDI	87 % (85)
	Nontoxigenic CDI	6 % (6)
	Mixed	1 % (1)
	No growth	6 % (6)
Ribotype	027	45 % (38)
	Non-027	55 % (47)

Table 2 Age, clinical symptoms, blood, and fecal biomarkers for patients with toxigenic *Clostridium difficile* infection (CDI). The ranges for each biomarker are included in parentheses (min, max) and a significant difference ($p<0.05$) between the mean ± standard error (SE) and percentages is noted with an asterisk (*) when compared to the moderate and mild groups and with a double asterisk (**) when compared to the mild group. Comparisons of severe CDI versus moderate/mild cases based on a one-way analysis of variance (ANOVA) are noted with **bold** p-values

Disease severity	Mean age (years)	Mean±SE (µg/g) fecal lactoferrin (min, max)	Mean±SE (10⁹/L) WBC (min, max)	Mean±SE (mg/dL) serum albumin (min, max)	Reported pain (%)	Mean stools (per day)	Tissue culture positive (%)	027 infection (%)
Severe	66	961±303* (2, 4,672)	19±3.2* (7.5, 66.1)	2.2±0.2* (0, 3.2)	62 %	9	91 %**	71 %*
Moderate	67	292±42 (1, 1,391)	13±0.9 (1.6, 28.6)	2.7±0.1 (1.8, 3.6)	65 %	8	83 %**	37 %
Mild	69	73±52 (3, 376)	9±1.3 (4.3, 14.2)	2.5±0.2 (1.8, 3.1)	29 %	5	29 %	29 %
Severe versus moderate/mild CDI	p=0.8443	**p=0.0003**	**p=0.0048**	**p=0.0083**	p=0.9370	p=0.4298	p=0.1414	**p=0.0043**

infection ($p=0.044$). There were a total of 19 patients that required additional antibiotics for their CDI episode and 90 % of these patients had stool toxin and 68 % of them were infected with ribotype 027. For this group, the mean±SE for lactoferrin, WBC count, and serum albumin was 566±261, 17.1±3.8, and 2.3±0.2, respectively. No difference was observed between groups for age and the number of stools per day. When stratified by gender, male patients were significantly more likely to be stool toxin-positive compared to female patients ($p=0.002$). Of the patients with detectable stool toxin, the levels of lactoferrin ranged from 1 to 4,672 µg/g feces, and only 3 (4 %) patients had normal fecal lactoferrin levels (<7.25 µg/g feces). For patients that were negative for stool toxin, 41 % had normal lactoferrin and all but a single patient had levels below 85 µg/g feces.

Table 3 Multilinear regression model for combined patient variables in predicting severe CDI. p-Values for association with severe CDI for each patient variable are listed (N=85). Significant effects based on the model are shown with an asterisk (*)

Variable	p-Values
Age	0.6857
Abdominal pain	0.6521
Stool toxin	0.3308
Lactoferrin	0.0141*
Serum albumin	0.0310*
WBC	0.0802
Stools/day	0.2196

Discussion

In this study, we examined blood and fecal biomarkers for assessing disease severity in patients with CDI. We measured two commonly used blood parameters for assessing disease severity in CDI, WBC count and serum albumin level, for comparison with fecal lactoferrin levels as an indicator of intestinal inflammation. *C. difficile*-specific diagnostic biomarkers including GDH, toxin B, and ribotype 027 isolates were also measured. Our results showed significantly more inflammation as determined by increased WBC counts (peripheral) and elevated fecal lactoferrin (intestinal) for patients with clinically assessed moderate to severe CDI. Serum albumin was lower in patients with more severe disease, indicating a damaged mucosa, resulting in the loss of serum protein into the bowel. A multilinear regression model examining the relationship of clinical symptoms and biomarkers on disease severity indicated a strong association for severe CDI ($p=0.0175$). In addition, the fecal lactoferrin and blood biomarkers, WBC count and low serum albumin level showed a significant association within severe CDI ($p<0.05$). Further studies are needed for evaluating the combination of fecal lactoferrin and blood biomarkers with symptoms and patient characteristics for developing a sensitive and specific disease severity index for stratifying CDI patients for optimal treatment and disease management.

Another important finding of our study is the clinical importance of determining stool toxin as an indicator of disease. Our results showed that elevated biomarkers of inflammation, WBC count and lactoferrin, were associated with the presence of stool toxin. Patients with clinically assessed severe and moderate CDI were more often likely to have a positive stool toxin (80 %). The combination of stool toxin and increased intestinal inflammation as shown by increased lactoferrin is not an unexpected finding because: (i) both toxins A and B are strong chemotactic proteins that cause the release of IL-8 and the infiltration of activated neutrophils into the intestinal mucosa [29]; (ii) toxin A also stimulates other pro-inflammatory cytokines, including IL-1β and tumor necrosis factor alpha (TNF), while toxin B is a cytotoxin that causes both tissue damage and inflammation [14, 20, 28, 29]; and (iii) the combined effects of the tissue

Table 4 Blood and fecal biomarkers for stool toxin-positive versus stool toxin-negative patients. Mean ± standard error (SE) for each biomarker was compared between the toxin-positive and toxin-negative groups, and a significant difference ($p<0.05$) is noted with an asterisk (*). The corresponding range (min, max) for each biomarker is in parentheses and the number of patients (n) included is provided for each group

Tissue culture for toxin B (N=85)	Mean age (years)	Mean±SE (µg/g) fecal lactoferrin (min, max)	Mean±SE (10^9/L) WBC (min, max)	Mean±SE (mg/dL) serum albumin (min, max)	Reported pain (%)	Mean stools (per day)	027 infection (%)
Stool toxin-Positive (68)	68	539±93* (1, 4,672)	15.0±1.2* (1.6, 66.1)	2.5±0.1 (0, 3.6)	66 %	8	50 %*
Stool toxin-negative (17)	64	41±22 (2, 376)	9.3±2.3 (3.6, 16.3)	2.7±0.2 (1.8, 3.2)	41 %	6	24 %

damaging and chemotactic activities of toxins A and B contribute to the severity of CDI [29]. Recently, Planche et al. analyzed 12,420 specimens from 10,691 patients using the reference assays toxigenic culture and tissue culture assay for stool toxin. Patients with stool toxin had a significantly higher mortality rate (16.6 %) compared to the toxigenic culture-positive only (9.7 %; $p=0.022$) and *C. difficile*-negative groups (8.6 %; $p=0.001$). In addition, the stool toxin-positive patients had significantly higher ($p=0.001$) WBC counts compared to the other two groups. Based on these results, Planche et al. concluded that patients having a positive stool toxin have *C. difficile* disease with an increased risk of mortality [27]. Considering our findings along with the Planche et al. study, a method for determining the presence of intestinal inflammation in combination with the presence of toxin in stool identifies patients with severe CDI. In addition, even though quantifying the amount of toxin in the stool was beyond the initial scope of this study, future work should consider measuring the level of toxins A and B and the correlation of amounts to biomarkers of inflammation and severity of disease.

A new finding from this study is the correlation of increasing fecal lactoferrin levels with increasing severity of clinically assessed CDI. Lactoferrin levels showed over a 5-fold increase in patients classified as severe CDI compared to moderate disease. Multiple studies have demonstrated increased WBC count as a useful indicator of severity in CDI; however, it is worth noting that these counts are not specific for intestinal inflammation and may be increased by inflammatory co-morbidities. Fecal lactoferrin is a glycoprotein that is present in most mucosal secretions and a primary component of the granules of activated neutrophils; thus, it is a specific marker of intestinal inflammation. During the onset of intestinal inflammation caused by *C. difficile*, activated neutrophils infiltrate the intestinal lumen, causing an increase in fecal lactoferrin [9, 11]. Our results support a role for assessing patients with CDI using fecal lactoferrin as an indicator of intestinal inflammation and then using the amount as an aid for stratifying patients based on disease severity. In addition, a biomarker like fecal lactoferrin may offer the utility for monitoring disease activity in patients with CDI as an indicator of response to medical treatment and for predicting a relapse.

The emergence of ribotype 027, which has been shown to express in vitro, more toxin, and production of increased spores has been linked to numerous outbreaks involving more severe *C. difficile* disease and a greater chance of relapse compared to other known ribotypes [10, 12, 21, 22, 26]. However, some recent studies have shown no link between the infecting ribotype and more severe clinical disease and worse outcomes, raising questions on the clinical significance, in terms of severity, of an 027 infection [3, 23, 37]. In our study population, we had 45 % of patients with toxigenic *C. difficile* infected with ribotype 027. These patients were more likely to be assessed as severe by the physician and had significantly higher lactoferrin indicating more intestinal inflammation, lower serum albumin levels, and a higher frequency of positive stool toxin (92 %). More research is needed in order to determine the in vivo production of increased stool toxin in patients infected with 027 as a virulence factor causing more inflammation, resulting in severe CDI.

In conclusion, our results show increased fecal lactoferrin, higher WBC counts, and lower serum albumin levels in CDI patients who have severe clinical disease. The presence of stool toxin was associated with increased inflammation for moderate and severe cases. In addition, we confirmed that older, sicker patients with ribotype 027 CDI are more likely to be stool toxin-positive, resulting in increased intestinal and peripheral inflammation, as indicated by higher levels of fecal lactoferrin and WBC counts, respectively. Future studies are needed in order to evaluate the optimization of treatment based on a clinical index for severity that combines patient characteristics with fecal and blood biomarkers in patients infected with *C. difficile*.

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