Supplementary Online Content

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This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods. Additional Methods for Laboratory Testing, Clinical Data Collection, and Statistical Analysis

Laboratory Testing

The Meridian Premier *C. difficile* toxins A & B immunoassay was performed clinically on all samples. Two FDA-approved molecular assays were used during the study, the Cepheid Xpert *C.difficile*/Epi real-time PCR assay and the Meridian Illumigene *C. difficile* loop-mediated isothermal amplification assay. The cell cytotoxin assay (TechLab *C. difficile* Tox-B test) was performed following the manufacturer's package insert with MHRF cells from Diagnostic Hybrids, Inc. Stool toxin quantitation was performed with the Acea Biosciences xCELLigence Real Time Cell Analysis System version 2.[1] The alcohol-shock culture procedure was performed as follows: A 0.5 mL aliquot of feces was mixed with an equal amount of 95% ethanol (1:1) and allowed to sit for 10 minutes. A cotton-tipped swab was dipped in the alcohol-treated feces and used to inoculate a pre-reduced cycloserine-cefoxitin-fructose agar supplemented with taurocholate (CCFA-ST; Remel Catalog# R01269). The inoculated CCFA-ST plate was incubated anaerobically and examined daily for colonies consistent with *C. difficile* for 72 hours. Suspicious colonies were identified by a combination of colony appearance, yellow color change of surrounding agar, Gram stain, horse barn odor, chartreuse fluorescence under long-wave UV light, and positive L-Proline aminopeptidase test (PRO disc). Confirmed *C. difficile* isolates were tested for *in vitro* toxin production using supernatant from a 48 hour chopped meat broth culture and the cell cytotoxin assay described above. *C. difficile* isolates were ribotyped using capillary gel-electrophoresis and comparison of band patterns with a large international collection of *C. difficile* isolates.[2] *C. difficile* ribotype 027 and 078 strains were classified as hypervirulent. For lactoferrin, samples were screened using the TechLab Leuko EZ Vue test and confirmed with quantitation using the TechLab IBD-Scan kit according to the manufacturer's package insert. Discordant positive toxin immunoassay results (e.g., positive toxin immunoassay with negative PCR and culture, positive toxin immunoassay with negative toxin quantitation and low *C. difficile* DNA load) were tested by the cell cytotoxin assay and interpreted as false positive immunoassay results if cell cytotoxin assay was negative.

Clinical Data Collection

The following clinical data were collected retrospectively from EHR and administrative databases for the index inpatient encounter, preceding outpatient and inpatient encounters within 90 days prior to admission, and subsequent inpatient and outpatient encounters within 30 days after admission: demographics; location prior to admission; inpatient and outpatient medications; hospital and intensive care unit dates and lengths of stay; inpatient nurse recorded stool counts and consistencies; and colostomy and rectal tube outputs; results of relevant laboratory tests, pathology, abdominal radiologic studies, and lower gastrointestinal endoscopy procedures; Medicare diagnosis related group (MS-DRG), 3M™ All Patient Refined Diagnosis Related Group, and Severity of Illness and Risk of Mortality subclasses (APR-DRG, SOI, ROM); all present on admission (POA) and acquired in-hospital International Classification of Diseases, 9th Edition, Clinical Modification (ICD-9-CM) diagnosis and procedure codes; current procedural terminology (CPT) codes; and discharge disposition. Pre-existing comorbidities were enumerated using software from the Agency for Healthcare Research and Quality (AHRQ).[3] Non-*C. difficile* diagnoses in Table 1 and eTable 2 were identified using the ICD-9 codes listed in eTable 1. Gastrointestinal and abdominal procedures used in the evaluation and diagnosis or treatment of patients of patients with suspected or confirmed CDI (e.g., colonoscopy, abdominal computed tomography scan, colectomy) were identified from administrative data using the ICD-9 codes and CPT codes listed in eTable 1 and during the manual chart review process. Repeat toxin tests were identified from laboratory data. Antibiotic treatment was determined from inpatient medication administration and outpatient prescription data.

Statistical Analysis

A log rank test was used to compare the Kaplan-Meier estimated time to resolution of diarrhea curves for statistical significance. Age, number of pre-existing comorbidities, ICU status on Day 1 (±1 day), antibiotic days pre-Day 1, metronidazole/oral vancomycin exposure ≤48 hours pre-Day 1, maximum white blood cell

count (WBC) on Day 1 (±1 day), *C. difficile* ribotype, and fecal lactoferrin were evaluated as potentially relevant covariates in the Cox proportional hazards model with ribotype nested within the *C. difficile* test group. All variables had the proportional hazard assumption tested prior to analysis. The final multivariable model was derived by a stepwise selection procedure using patients with at least one day of diarrhea and the Tox-/PCR- Group as the reference group. The final model included Tox+/PCR+ status, age, WBC, and lactoferrin as significant predictors of the duration of diarrhea relative to Tox-/PCR- patients. For all analyses, a 2-tailed *P*-value <.05 was considered significant. All statistics were performed in SAS, version 9.3.

References

1. Huang B, Jin D, Zhang J, et al. Real-time cellular analysis coupled with a specimen enrichment accurately detects and quantifies Clostridium difficile toxins in stool. *J Clin Microbiol.* 2014;52(4):1105-1111.
2. Tickler IA, Goering RV, Whitmore JD, et al. Strain types and antimicrobial resistance patterns of Clostridium difficile isolates from the United States, 2011 to 2013. *Antimicrob Agents Chemother.* 2014;58(7):4214-4218.
3. Elixhauser A, Steiner C, Harris DR, Coffey RM. Comorbidity measures for use with administrative data. *Med Care.* 1998;36(1):8-27.

eTable 1. Codes Used in Clinical Data Collection and Analysis

Diagnosis	**ICD-9 codes used to indicate diagnosis**
Ulcerative Colitis	556, 556.0, 556.1, 556.2, 556.3, 556.5, 556.6, 556.8, 556.9
Crohns / Regional Enteritis	555, 555.0, 555.1, 555.2, 555.9
Diverticulitis	562.11, 562.13
Appendicitis	540, 540.0, 540.1, 540.9, 541
Vascular Insufficiency/Ischemic Colitis	557, 557.0, 557.1, 557.9
Peritonitis, Retroperitoneal and Mesenteric Infections	567, 567.0, 567.21, 567.22, 567.23, 567.29, 567.3, 567.31, 567.38, 567.39, 567.82
Other Infectious Colitis/Enterocolitis	003.0, 004, 006.0, 006.1, 006.2, 007, 008.0, 008.41, 008.42, 008.43, 008.44, 008.46
Viral Enteritis	008.6, 008.61, 008.62, 008.63, 008.64, 008.65, 008.66, 008.67
Ill-Defined Intestinal Infections	009, 009.0, 009.1, 009.2, 009.3
Graft vs. Host Disease NOS	279.50
Cytomegaloviral disease	078.5
Other Non-Infectious Colitis/Enterocolitis NOS	558, 558.1, 558.2, 558.3, 558.4, 558.9
Functional Diarrheal Disorder	564.1, 564.2, 564.5
Diarrhea NOS	787.91
C. difficile Colitis	008.45
Megacolon, Not Hirschprungs	564.7
Diabetes	250, 250.1, 250.2, 250.3, 250.4, 250.5, 250.6, 250.7, 250.8, 250.9
Procedure	**ICD-9/CPT codes used to screen for procedures**
Colectomy	45.71, 45.72, 45.73, 45.74, 45.75, 45.76, 45.79, 45.81, 45.82, 45.83, 48.69, 17.32, 17.33, 17.34, 17.35, 17.36
Abdominal Computed Tomography (CT)	74150, 74160, 74170
Abdominal X-Ray	74000, 74020, 74022
Abdominal Ultrasound	76700, 76705
Colonoscopy/Sigmoidoscopy/ Proctosigmoidoscopy/Stomal Endoscopy	45.12, 45.22, 45.23, 45.24, 45.25, 48.23, 48.22, 48.24, 44380, 44382, 44383, 44385, 44386, 44388, 44389, 44390, 44391, 44392, 44393, 44394, 44397, 45300, 45303, 45305, 45307, 45308, 45309, 45315, 45317, 45320, 45321, 45327, 45330, 45331, 45332, 45333, 45334, 45335, 45337, 45338, 45339, 45340, 45341, 45342, 45345, 45355, 45378, 45379, 45380, 45381, 45382, 45383, 45384, 45385, 45386, 45387

eTable 2. Additional Baseline Characteristics of the Study Population by *C. difficile* Test Group

Characteristic	No. (%)			*P* Value[d]
	C. difficile-positive		*C. difficile*-negative	
	Tox+/PCR+[a] (n = 131)	Tox-/PCR+[a,b] (n = 162)	Tox-/PCR-[a,c] (n = 1123)	
Antibiotic days ≤30D pre-Day 1, median (IQR)	11 (5-18)	7.5 (4-18)	7 (4-14)	<.001
Received antibiotics ≤90D pre-Day 1	128 (98)	157 (97)	1067 (95)	.24
Received antibiotics ≤30D pre-Day 1	126 (96)	151 (93)	1055 (94)	.52
Laxative or enema ≤48 hrs. pre-Day 1	74 (57)	79 (49)	646 (58)	.11
Proton pump inhibitor or Histamine H2-receptor blocker ≤30D pre-Day 1	110 (84)	130 (80)	926 (83)	.69
Antineoplastic (cytotoxic or antibody) ≤30D pre-Day 1	5 (4)	11 (7)	54 (5)	.46
Immunosuppressant or glucocorticoid ≤30D pre-Day 1	44 (34)	60 (37)	400 (36)	.83
NSAIDS ≤30D pre-Day 1	21 (16)	35 (22)	217 (19)	.48
Ulcerative colitis	1 (1)	4 (3)	21 (2)	.39
Crohn's disease	1 (1)	3 (2)	23 (2)	.72
Ischemic colitis/vascular insufficiency	2 (2)	6 (4)	25 (2)	.42
Functional diarrheal disorder	1 (1)	2 (1)	9 (1)	.76
Diverticulitis	0 (0)	1 (1)	10 (1)	.86
Appendicitis	0 (0)	0 (0)	11 (1)	.45
Peritonitis, retroperitoneal, mesenteric infection	3 (2)	5 (3)	55 (5)	.32
Graft vs. host disease	0 (0)	0 (0)	2 (0)	1
Viral enteritis	0 (0)	0 (0)	2 (0)	1
Ill-defined intestinal infection	0 (0)	1 (1)	1 (0)	.37
Other infectious enterocolitis	0 (0)	0 (0)	1 (0)	1
Other non-infectious enterocolitis	0 (0)	7 (4)	18 (2)	.02

[a] *C. difficile* test group based on FDA-approved toxin immunoassay and PCR results
[b] Tox-/PCR+ group includes 1 patient with positive toxin immunoassay, negative cell culture toxin test, and low *C. difficile* DNA concentration interpreted as a false positive toxin immunoassay
[c] Tox-/PCR- group includes 20 patients with positive toxin immunoassay and negative results for all other *C. difficile* tests interpreted as false positive toxin immunoassay results.
[d] *P* value for significance across three groups

eTable 3. Clinical History and Follow Up for Tox-/PCR+ Patients with High Fecal Lactoferrin (>89.05 ug/mL)

Positive cell cytotoxin assay patients[a]							
Patient ID	**Log_{10} *C. difficile* DNA (cpy/mL)**	***C. difficile* Toxin B[b] (ng/mL)**	**Lactoferrin (ug/mL)**	**Clinical history**	**Metro./oral vanc. exposure ≤48 hrs. pre-Day 1**	**Metro./oral vanc. ≤14 days after Day 1 (days)**	**CDI-related complication or mortality ≤30 days**
310	6.3	2.3	229.8	54 yr. old F with lupus, viral encephalitis, renal disease on peritoneal dialysis and leukocytosis; treated empirically for CDI, later diagnosed with bacterial peritonitis	No	14	No
560	7.6	129.4	714.1	60 yr. old M admitted with abdominal distension and obstructive symptoms after ileostomy reversal following LAR partial colectomy for colon CA; normal WBC and creatinine; treated with steroid enemas for diversion colitis, transitioned from obstruction to diarrhea to resolution on steroids with increased PO intake and no antibiotics	No	0	No
642	6.8	31.9	246.3	52 yr. old M with severe, refractory ulcerative colitis (UC)	No	13	No
1283	6.9	640.8	428.5	76 yr. old M with methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia and toxin+ *C. difficile* colitis on oral vancomycin x 8 days	Yes	5	No
1534	4.0	0.7	103.4	51 yr. old F with ovarian cancer, ascites, small bowel obstruction, sepsis on ceftriaxone and metronidazole x 6 days	Yes	0	No
1641	6.6	165.6	201.7	90 yr. old F in hospital with MRSA pneumonia, bacteremia, congestive heart failure, chronic kidney disease, recurrent CDI develops diarrhea and leukocytosis; negative D0 *C. difficile* toxin test, treated empirically with IV metronidazole, retested toxin+ on D1, vancomycin retention enemas added; diarrhea resolved on D8; medical support withdrawn on D8 due to poor overall life quality and prognosis; patient expired on D18	No	8	Yes
1714	5.0	2.0	92.9	43 yo female with subarachnoid hemorrhage and urinary tract infection	No	0	No

[a]Fecal toxin status based on *C. difficile* Tox-B assay (TECHLAB, Inc.) using MHRF cells (Diagnostic Hybrids, Inc.)
[b]Fecal toxin concentration measured by xCELLigence Real Time Cell Analysis System, version 2 (ACEA Biosciences, Inc.)

eTable 3. Clinical History and Follow Up for Tox-/PCR+ Patients with High Fecal Lactoferrin (>89.05 ug/mL) (continued)

Negative cell cytotoxin assay patients[a]							
Patient ID	**Log_{10} *C. difficile* DNA (cpy/mL)**	***C. difficile* Toxin B[b] (ng/mL)**	**Lactoferrin (ug/mL)**	**Clinical history**	**Metro./oral vanc. exposure ≤48 hrs. pre-Day 1**	**Metro./oral vanc. ≤14 days after Day 1 (days)**	**CDI-related complication or mortality ≤30 days**
179	3.5	1.9	109.7	40 yr. old F with cirrhotic liver disease and urosepsis on lactulose for hepatic encephalopathy	No	0	No
320	4.6	0.0	96.7	57 yr. old F with multisystem autoimmune disease and pancolitis, treated empirically for CDI but colonoscopy suggested viral or autoimmune colitis, blood CMV PCR+	Yes	9	No
363	5.1	0.0	122.3	41 yr. old F with rectovaginal fistula and colostomy after cervical cancer surgery	No	0	No
675	6.9	1.8	102.0	74 yr. old M with myasthenia gravis flare due to urinary tract infection	No	0	No
718	6.5	2.0	529.5	62 yr. old F with abdominal abscess and colocutaneous fistula after multiple surgeries	No	9	No
793	5.8	1.7	119.8	52 yr. old F with colon cancer, sigmoid colon perforation, gangrenous leg, UTI	Yes	3	No
867	3.4	0.7	118.8	59 yr. old M with Crohn's disease and small bowel obstruction; normal colonoscopy	No	1	No
947	6.8	0.4	108.5	57 yr. old F with bloody diarrhea and colon ulcers after kidney transplant; no pseudomembranes on endoscopy	No	5	No
1054	5.9	0.6	623.6	64 yr. old M with multiple injuries and ileus after motor vehicle accident; received laxatives, enemas, neostigmine	No	0	No
1189	3.4	ND	98.6	53 yr. old M with atherosclerotic vascular disease, bloody diarrhea, ischemic colitis	Yes	0	No
1484	3.3	0.4	192.6	81 yr. old M with COPD, CVA, sepsis, and recurrent CDI on oral vancomycin x 8 days	Yes	7	No
1784	6.4	2.2	965.3	30 yr. old F with polymyositis and recurrent CDI on oral vancomycin x ≥2 days	Yes	7	No

[a]Fecal toxin status based on *C. difficile* Tox-B assay (TECHLAB, Inc.) using MHRF cells (Diagnostic Hybrids, Inc.)
[b]Fecal toxin concentration measured by xCELLigence Real Time Cell Analysis System, version 2 (ACEA Biosciences, Inc.)

eTable 4. Significant Predictors of Resolution of Diarrhea in the Final Cox Proportional Hazards Model

Parameter	DF	Estimate	Standard Error	Chi-square	*P* value	Hazard ratio [95% C.I.]
Tox+/PCR+	1	-0.464	0.142	10.60	.001	0.63 [0.48, 0.83]
Age	1	-0.008	0.003	5.20	.02	0.99 [0.99, 1.00]
WBC	1	0.019	0.006	10.77	.001	1.02 [1.01, 1.03]
Lactoferrin	1	-0.0008	0.0004	4.04	.04	0.99 [0.99,1.00]