Diagnostic Microbiology and Infectious Disease 84 (2016) 343–346

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Clinical Studies

Organism burden, toxin concentration, and lactoferrin concentration do not distinguish between clinically significant and nonsignificant diarrhea in patients with *Clostridium difficile*

Victoria Emma Anikst [a], Rajiv Lochan Gaur [a], Lee Frederick Schroeder [b], Niaz Banaei [a,c,d,*]

[a] Department of Pathology, Stanford University School of Medicine, CA, USA
[b] Department of Pathology, University of Michigan School of Medicine, Ann Arbor, MI, USA
[c] Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University School of Medicine, CA, USA
[d] Clinical Microbiology Laboratory, Stanford Health Care, CA, USA

ARTICLE INFO

Article history:
Received 1 October 2015
Received in revised form 24 November 2015
Accepted 27 November 2015
Available online 30 November 2015

Keywords:
Clostridium difficile
Biomarker
Infection
Diarrhea
Toxin
Lactoferrin

ABSTRACT

Clostridium difficile infection is often overdiagnosed in patients with mild diarrhea. We evaluated 4 biomarkers as surrogates for clinically significant diarrhea (≥3 episodes in 24 hours) in 59 PCR-positive patients with and 59 PCR-positive patients without clinically significant diarrhea. Organism burden (median *tcdB* cycle threshold value, 26.9 versus 27.1, $P = 0.25$) and toxin A and B concentrations (toxin A, median, 0 versus 0 ng/mL, $P = 0.42$; toxin B, median, 0 versus 0 ng/mL, $P = 0.25$) were not significantly different between patients with and without clinically significant diarrhea. Fecal lactoferrin concentrations were significantly increased in patients with clinically significant diarrhea (median, 99.0 versus 55.1 μg/mL, $P = 0.05$); however, lactoferrin could not sufficiently classify patients into those with and without clinically significant diarrhea. Interventions that limit *C. difficile* testing to patients with clinically significant diarrhea are needed to improve the positive predictive value of *C. difficile* diagnostics.

1. Introduction

Accurate diagnosis of *Clostridium difficile* infection (CDI) is critical for the treatment interventions and the prevention of transmission to susceptible hosts. However, accurate diagnosis of CDI is challenging because *C. difficile* colonizes up to 20% of hospitalized patients and detection of toxigenic *C. difficile* in stool does not constitute disease (Alasmari et al., 2014; Clabots et al., 1992; Johnson et al., 1990; Leekha et al., 2013; Samore et al., 1994). In the absence of a definitive laboratory gold standard, CDI case definition per published guidelines by the Infectious Diseases Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA) is based on "the presence of diarrhea, defined as passage of 3 or more unformed stools in 24 or fewer consecutive hours" and "a stool test result positive for the presence of toxigenic *C. difficile* or its toxins" (Cohen et al., 2010). We recently showed that 66.6% (418/628) of positive patient samples sent for routine CDI PCR testing did not have clinically significant diarrhea (Banaei et al., 2015). At 4 other academic institutions, 55% (22/40) and 8.3% (11/132) of patients diagnosed with CDI did not have clinically significant diarrhea (Buckel et al., 2015; Guerrero et al., 2011), and 36% (54/150) and 39% (142/365) of patients undergoing CDI screening were found to not have clinically significant diarrhea (Dubberke et al., 2011; Peterson et al., 2007). These findings underscore the need for disease-specific biomarkers to improve the accuracy of *C. difficile* diagnostics. Given that pathogenesis of CDI requires organism expansion, toxin production, and inflammation (Kuehne et al., 2010), we determined whether organism burden, toxin A and B concentration, and/or fecal lactoferrin concentration could serve as laboratory surrogates for clinically significant diarrhea.

2. Materials and methods

2.1. Study subjects and stool specimens

Between 07/1/12 and 01/4/14, electronic chart review was performed on patients with stool samples submitted to the laboratory for *C. difficile* testing and who tested positive by PCR using the GeneXpert C.diff Epi assay (Cepheid, Sunnyvale, CA, USA). Chart review included determination of number of loose stools in the 24-hour period prior to specimen collection based on physician's admission and progress notes, nursing documentation of stool frequency and consistency, clinic notes, and transcripts of phone calls and emails from patients and their caregivers. Using leftover stool samples, a nested case–control study of 59 PCR-positive patients with (experimental group) and 59 without (control group) clinically significant diarrhea was conducted. Stool

* Corresponding author. Tel.: +1-650-736-8052; fax: +1-650-725-5671.
E-mail address: nbanaei@stanford.edu (N. Banaei).

http://dx.doi.org/10.1016/j.diagmicrobio.2015.11.022
0732-8893/

samples were held in the refrigerator up to 24 hours after routine testing, and then aliquots were measured using a digital scale and stored at −80 °C. Clinically significant diarrhea definition was adopted from the IDSA and SHEA guidelines (Cohen et al., 2010). Patients in this study were randomly selected from a larger cohort (Banaei et al., 2015), although matched for laxatives if they were administered in the 48 hours preceding stool collection (49 untreated and 10 treated). Laxatives, which included the stool softener docusate, were classified under "laxatives" or "laxatives and cathartics" drug classes in Epic electronic health record software. The demographic and clinical data are summarized in Table 1. This study was approved by the Stanford University Internal Review Board.

2.2. C. difficile burden

To determine the organism burden, 0.3 grams of frozen stool sample was emulsified in 1.0 mL of sample reagent and centrifuged at 240 × *g* to remove particulate matter (validation data not shown). 0.8 mL of supernatant was added to 1.0 mL of sample reagent and tested with the GeneXpert C.diff Epi *tcdB* PCR assay according to the package insert. The cycle threshold (CT) value for the *tcdB* target, which inversely correlates with *C. difficile* genomic equivalents and colony-forming units (Dionne et al., 2013), was used as a proxy for organism burden. In addition, 027/NAP1/BI strain result was recorded to calculate positivity rate.

2.3. TcdA and TcdB enzyme-linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA)

Quantitative ELISA for toxin TcdA and TcdB were performed on 0.05 grams of frozen stool using the tgcBIOMICS ELISA kit (tgcBIOMICS, Bingen, Germany) according to the manufacturer's instruction. Qualitative toxin testing with the C. DIFF QUIK CHEK COMPLETE EIA (TechLab, Blacksburg, VA, USA) was performed according to the manufacturer's instruction.

Table 1
Demographic and clinical data for randomly selected laxative-matched groups of patients with and without clinically significant diarrhea.

	Significant diarrhea (n = 59)	No significant diarrhea (n = 59)	*P* value
Demographics			
Age, median	66	61	0.85
Female sex, % (n)	56 (33)	44 (26)	0.2
Inpatient, % (n)	79.7 (47)	76.3 (45)	0.66
Antibiotics prescribed, % (n)			
In 60 days prior	100 (59)	100 (59)	1.0
Empiric CDI therapy in 2 days prior	10 (6)	5 (3)	0.18
CDI treatment	100 (59)	100 (59)	1.0
Vancomycin	41 (24)	36 (21)	0.57
Metronidazole	66 (39)	61 (36)	0.57
Fidaxomicin	2 (1)	3 (2)	0.56
Underlying disease, % (n)			
Cardiovascular	27 (16)	27 (16)	1.0
Neurological	3 (2)	8 (5)	0.24
Neoplasm	42 (25)	41 (24)	0.85
Diabetes	14 (8)	22 (13)	0.23
Hepatic	10 (6)	7 (4)	0.51
Renal	24 (14)	25 (15)	0.83
Pulmonary	7 (4)	7 (4)	1.0
Other	2 (1)	5 (3)	0.31
Laboratory results (n)			
WBC × 10^9/L, median	9.35 (48)	9 (49)	0.30
Albumin g/dL, median	2.6 (45)	2.5 (36)	0.12
Creatinine mg/dL, median	1 (48)	1.1 (48)	0.40
027/NAP1/BI strain, %	23.7 (14)	18.6 (11)	0.38

WBC = white blood cell.

2.4. Fecal lactoferrin ELISA

Quantitative ELISA for lactoferrin was performed on 0.05 grams of frozen stool using the IBD-SCAN® ELISA kit (Tech Lab), according to the manufacturer's instruction.

2.5. Statistical analysis

This study was designed at a statistical power level of 80% and probability level of 5% (1-tailed hypothesis) to detect an effect size between the experimental and control groups as estimated by Cohen's *d* population parameter of at least 0.46 (Cohen, 1988). A Cohen's *d* of 0.46 is equivalent to an area under the receiver operator characteristic curve of 63% (McGraw and Wong, 1992). The Mann–Whitney *U* test was used to compare differences in continuous results, and the chi-squared test was used to compare differences in proportions. A 1-sided type I error rate of 5% was used in statistical tests for CT, toxin A and B, and lactoferrin. All other statistical tests were computed for a 2-sided type I error.

3. Results

As shown in Fig. 1A, there was no difference in organism burden between groups with and without clinically significant diarrhea (median CT, 26.9 [interquartile range {IQR}, 23.9–32.2] versus 27.1 [IQR, 23.4–30.7]; $P = 0.25$; mean CT 27.9 versus 27.4). Quantitative toxin measurement by ELISA for either TcdA or TcdB showed no difference in toxin concentrations between groups (TcdA, median 0 ng/mL [IQR, 0–26.7] versus 0 ng/mL [IQR, 0–41.1], $P = 0.42$; mean 54.8 ng/mL versus 55.6 ng/mL; TcdB, median 0 ng/mL [IQR, 0–0] versus 0 ng/mL [IQR, 0–0], $P = 0.25$; mean 6.7 ng/mL versus 10.0 ng/mL) (Fig. 1B and C). Detectable TcdA and TcdB rates were 39.0% (23) and 16.9% (10) in patients with clinically significant diarrhea and 35.6% (21) and 20.3% (12) in patients without clinically significant diarrhea. A subgroup analysis of patients with detectable toxins A or B also did not show a difference in toxin concentrations between groups ($P = 0.28$ and 0.31, respectively). Consistent with quantitative toxin results, qualitative toxin testing with the C. DIFF QUIK CHEK COMPLETE EIA (Tech Lab) did not show an enrichment of positive results in patients with clinically significant diarrhea (49% versus 47.5%, $P > 0.05$). Quantitative fecal lactoferrin measurement by ELISA showed a statistically significant difference in lactoferrin concentrations between groups with and without clinically significant diarrhea (median 102.4 μg/mL [IQR, 24.0–163.4] versus 55.1 μg/mL [IQR, 11.7–115.7]; $P = 0.05$; mean 97.6 μg/mL versus 75.9 μg/mL) (Fig. 1D). Given that lactoferrin concentrations were significantly different between the groups, we performed a receiver operator characteristic curve analysis to determine how well lactoferrin concentrations could distinguish between patients with and without clinically significant diarrhea. As shown in Fig. 2, lactoferrin could not sufficiently classify patients with and without clinically significant diarrhea (area under the curve = 0.58).

4. Discussion

Contrary to what we hypothesized based on the pathogenesis model for CDI, we did not find a biomarker that could be used as a surrogate for stool frequency, a factor that is intrinsic to IDSA and SHEA guideline CDI case definition (Cohen et al., 2010). We did not find a significant difference in organism burden and toxin A and B concentrations in stools of patients with and without clinically significant diarrhea. We did find statistically significant increase in lactoferrin concentrations in patients with clinically significantly diarrhea, but no cutoff could be used to adequately discriminate between the two groups. Our findings are consistent with several studies showing a lack of or a weak association between toxin positivity or organism burden and disease severity in patients with CDI (El Feghaly et al., 2013a; El Feghaly et al., 2013b; Guerrero et al., 2011; Humphries et al., 2013; Thabit and Nicolau, 2015).