Characterization of the Immune Response in Patients Infected with Clostridioides Difficile Due to Serum Biomarkers' Level with Correlation to Clinical Characteristics and Bacterial Toxin Production

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Abstract

**Background:** *Clostridioides difficile* infection (CDI) have a high risk for complications up to death which requires identifying patients with severe disease and treating them accordingly. We examined the serum level of 6 cytokines and chemokines (IL-6, IL-21, IL-23, IL-33, BCA-1, TRAIL) and we checked the correlation between them to the patients' clinical characteristics and the bacterial strain.

**Methods:** Concentrations of 6 cytokines and chemokines were measured using the MILLIPLEX®MAP kit (Billerica, USA) based on the Luminex xMAP® technology, in serum samples, attained from 54 CDI patients within a median time of 24-48 hours after laboratory confirmation of *C. difficile* presence. The demographic and clinical data were retrospectively collected from medical records. Disease severity score was determined according to the guidelines of the "Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America" (SHEA-IDSA).

**Results:** Out of 54 patients (mean age, 76.6 years, 61.1% female), 20 (37%) had mild to moderate disease and 34 (63%) had severe disease. Two immunological markers were associated with a more severe disease: IL-16 ($p = 0.005$) and BCA-1 ($p = 0.012$). The study didn't show a correlation between the immunological markers to the gender, the type of toxin which produced by the bacteria, in hospital mortality and infection acquisition.

**Conclusions:** cytokines and chemokines may serve as a biomarker for early prediction of CDI severity in the future. Improved and more accessible assessment of CDI severity will contribute to adjustment of the medical treatment which will lead to a better patient outcome and hopefully will reduce the patient's mortality.

Introduction

*Clostridioides difficile* (*C. difficile*) is a gram positive, spore-forming, toxin producing, obligate-anaerobic rod, belonging to the Firmicutes phylum [1]. *C. difficile* was identified as the primary cause of antibiotic-associated diarrhea and pseudomembranous colitis [2]. In the last two decades, *C. difficile* infection (CDI) has become the most common nosocomial infection acquired in hospitals and institutions for prolonged hospitalization, especially in developing countries [3].

The pathogenesis of CDI depends on several virulence factors that contribute to disease development and severity; the main cause is the production of two toxins, toxin A and toxin B with glycosyltransferase activity. Rho proteins, the toxins' target, are located in the cytosol and are involved in numerous signal processes including actin cytoskeleton regulation, cell cycle progression, and gene transcription, as well as control of kinases activity [4]. In addition, these toxins cause an increase in intestinal permeability and fluid accumulation that leads to diarrhea, which is one of the hallmarks of CDI [5].

Antibiotic exposure, advanced age, and hospitalization are three major risk factors for CDI. Prior exposure to antimicrobial agents is the most widely recognized and modifiable risk factor for CDI. Patients with CDI
in a hospital setting are generally exposed to an antibiotic prior to acquiring the infection. Antibiotics cause a severe disruption of the natural gut microbiome, imbalance of the intestinal bacterial population, and a decrease of bacterial species richness, which leads to \textit{C. difficile} colonization \cite{6}. CDI symptoms can range from mild to severe and potentially life-threatening disease. Symptoms include diarrhea lasting several days accompanied by abdominal pain, fever, and diminished appetite leading to weight loss. In severe cases, intestinal obstruction and toxic megacolon with digestive tract perforation may lead to sepsis and death. An increased level of leukocytes and IgG in the blood and serum, respectively, can be observed \cite{7}.

During the appearance of bacterial infections, such as toxin producing \textit{C. difficile}, the innate immune system is activated, primarily in the intestinal mucus. This reaction leads to secretion of a variety of pro-inflammatory cytokines, which are used as messengers that transmit messages between different cells, including cells of the immune system. This leads to the secretion of other inflammatory mediators that propagate the inflammatory process.

Cytokine is a general term for a diverse group of soluble proteins and peptides, which are used as regulators in normal conditions. In pathological conditions, these proteins and peptides regulate the functional activities of cells and tissues.

Several studies have shown the involvement of cytokines in CDI. For example, the cytokine IL-8 activates the chemotaxis mechanism for neutrophils recruitment and activation of innate lymphoid cells \cite{8}. Previous studies have shown that fecal IL-8 levels were high in samples of CDI patients and was positively correlated with disease severity \cite{9,10}. Another work has reported that the concentration ratios of IL-1\textbeta/IL-receptor \alpha (IL-ra) were significantly increased in patients with severe CDI, compared to patients with mild CDI \cite{9}. Similar to IL-8, IL-1\textbeta is essential for host responses and provides protection following infection and injury \cite{11}. It is produced and released by a wide variety of cells such as intestinal epithelial cells, dendritic cells, and macrophages. In addition to direct neutrophil recruitment, IL-1\textbeta also promotes the production of cytokine IL-8, thus further increasing the levels of recruited neutrophils in the inflamed site \cite{12}. A recent study by Abt et al. has suggested a critical role for IFN-\gamma-producing type I innate lymphoid cells in mediating host survival during \textit{C. difficile} colitis \cite{13}.

In this study, we want to focus on cytokines that were recently identified as associated with CDI and on less studied cytokines in relation to CDI - IL-16, IL-21, IL-23, IL-33, BCA- 1 and TRAIL. Therefore, we essay the immunological response by correlation between the patients' serum level of the previously mentioned cytokines and chemokines to the severity of their disease.

**Methods**

**2.1 Study population**

The study population consists patients above the ages 18 years who were diagnosed with infection caused by \textit{C. difficile} at the Poriya Baruch Padeh Medical Center, between the years 2015–2019 By stool
examination using the GeneXpert all CDI cases were confirmed for toxigenic \textit{C. difficile} polymerase chain reaction (PCR) assay (Cepheid, Sunnyvale, CA, USA), identifying three targets: Toxin B, Binary Toxin, and presence of \textit{tcdC} deletion.

Patients who were recruited to the study signed a consent form or had a legal guardian sign in their place. Pregnant women and patients suffering from mental illness were excluded from this study in addition patients with pneumonia, sepsis due to causes other than CDI, and bacteremia were excluded from the analysis.

Serum and stool samples were collected from each patient. \textit{C. difficile} was isolated from each stool sample and its characteristics were evaluated by several assays including toxin detection.

This study received the approval of the Institutional Committee of Helsinki of the Medical Center, approval No. POR-0085-15.

\textbf{2.2 Measurement of cytokine concentrations – IL16, IL-21, IL-23, IL-33, BCA-1, TRAIL}

Cytokine and chemokine concentrations will be measured using the MILLIPLEX®MAP kit (Billerica, USA) based on the Luminex xMAP® technology. This technology is based on fluorescent-coded magnetic beads that are coated with a specific capture antibody for each cytokine. First, 25 ml serum is added into each well in a-96 wells plate. Then, 25 µl magnetic beads coated with specific monoclonal antibodies will be introduced to each well. Whenever there is a presence of cytokines, they will bound to the specific antibody on the beads. After overnight incubation and washing steps, 25 µl of Detection Antibodies is added to each well. These are secondary antibodies chemically conjugated to Biotin. After one-hour incubation, 25 µL of Streptavidin-Phycoerythrin is added. After short incubation and washing steps, the Fluorescence of the plate was read using the Luminex 200 ™ reader. Each cytokine has a different color.

\textbf{2.3 Disease severity scoring and demographic data collection}

\textit{C. difficile} severity score index was calculated using a score to measure the severity of the infection. For this end, the following demographic data were collected from the medical records: age, gender, functional status, community versus nosocomial acquired CDI, and death during hospitalization. According to this score, CDI patients were divided into groups of mild to moderate disease and severe disease using a severity score index according to the guidelines of the “Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America” (SHEA-IDSA) as follows: mild to moderate disease ≤ leukocytosis ≤ 15,000 cells/ml and creatinine ≤ 1.5 times the premorbid level; severe disease leukocytosis ≥ 15,000 cells/ml or serum creatinine ≥ 1.5 times the premorbid level. Severe disease was defined also as a serum albumin level of ≤ 3 g/dl at the time of active infection [22, 23].

\textbf{2.4 Toxin detection}
Two toxin, A and B were detected using the CerTest *Clostridioides difficile* GDH + Toxin A + B kit according to the manufacturer's instructions (Certest Biotec, S.L, San Mateo de Gállego, Zaragoza, Spain). The kit is a colored chromatographic assay for qualitative detection of *C. difficile* antigen glutamate dehydrogenase (GDH) and Toxins A and B in stool samples. The sample was mixed with a test solution that contains mouse monoclonal antibodies anti-GDH/Toxin A/Toxin B conjugated to red polystyrene latex. When GDH antigen/Toxin A/Toxin B is present in the sample, the antigen/toxin reacts with its specific antibodies in the test solution and this complex is captured by the antibodies in the test strips, resulting in a visible red line.

### 2.5 Statistical analysis

Chi-square was applied for analyzing the differences in distribution of categorical parameters between mild and moderate disease severity. The Non-parametric Wilcoxon-Mann-Whitney Rank sum test for independent samples applied for analyzing the differences in continuous parameters between mild and moderate disease severity. The Non-parametric Wilcoxon-Mann-Whitney Rank sum test or Analysis of Variance (ANOVA) applied for analyzing the differences in continuous parameters between mild and moderate disease severity. All tests applied by two-tailed, and a p value of 5% or less was considered statistically significant. The data was analyzed using SAS® version 9.3 (SAS Institute, Cary, North Carolina).

### Results

The study included fifty-four patients aged 46–98 years (mean age, 76.6 years, 61.1% female) that were positive for *C. difficile*. Table 1 summarizes the clinical characteristics and demographic of the patients. The majority of CDI cases (37/54, 68.5%) were nosocomial, while the rest were acquired in the community. CDI severity was mild to moderate in 20 patients (37%) and severe in 34 patients (63%). The isolated bacterial strains that had both A+B toxin were the most common (34/54 patients, 63%). Six isolates (11.1%) had Toxin A and 14 (25.9%) had Toxin B.
Table 1
Clinical and demographic characteristics of patients with *Clostridioides difficile* infection (CDI)

| Parameter           | N (%)  |
|---------------------|--------|
| Gender              |        |
| Male                | 21 (38.9) |
| Female              | 33 (61.1) |
| Disease severity    |        |
| Mild to moderate    | 20 (37) |
| Moderate            | 34 (63) |
| Toxicity            |        |
| Toxin A             | 6 (11.1) |
| Toxin B             | 14 (25.9) |
| Toxins A + B        | 34 (63) |
| In-hospital mortality|      |
| Alive               | 39 (72.2) |
| Died                | 15 (27.8) |
| Infection acquisition|      |
| Nosocomial          | 37 (68.5) |
| Community           | 17 (31.5) |

Characterization of the immune response in CDI patients

With the intention of finding a biological marker that could correlate with disease severity, the concentrations of 6 cytokines and chemokines were measured in patients’ serum and compared between patients with mild to moderate and severe CDI (Table 1). Out of these 6 inflammatory mediators, significantly higher levels of 2 cytokines were associated with a more severe disease- IL-16 (*p* = 0.005) and BCA-1 (*p* = 0.012), using SHEA-IDSA criteria. (Table 2 and Fig. 1 and Fig. 2).

Table 2: Serum inflammatory mediator levels (cytokines and chemokines) in patients with *Clostridioides difficile* infection (CDI), by disease severity*
| Parameter                  | Mild to moderate (N = 20) mean (Range) | Severe (N = 34) mean (Range) | p - value |
|---------------------------|----------------------------------------|-----------------------------|-----------|
| IL-16                     | 250.3 (0-622.1)                        | 778 (0-6578.3)              | 0.005     |
| IL-21                     | 7.7 (0-110.9)                          | 2.2 (0-27.7)                | 0.956     |
| IL-23                     | 294.8 (0-3083.6)                       | 198.9 (0-2638.1)            | 0.740     |
| IL-33                     | 26 (0-408.8)                           | 12.9 (0-174.1)              | 0.758     |
| BCA-1                     | 344.7 (63.2–1000)                      | 712.8 (39.6–4266)           | 0.012     |
| TRAIL                     | 276 (16.9-881.1)                       | 190.8 (0-584.3)             | 0.333     |

* As determined using SHEA-IDSA criteria.

The study didn't show a correlation between the immunological markers to the gender, the type of toxin which produced by the bacteria, in hospital mortality and infection acquisition (Table 3).

### Table 3

Serum inflammatory mediator levels in patients CDI by patient characteristics

| Parameter                  | IL-16 | IL-21 | IL-23 | IL-33 | BCA-1 | TRAIL |
|---------------------------|-------|-------|-------|-------|-------|-------|
| Gender (male/female)      | 0.531 | 0.182 | 0.360 | 0.751 | 0.487 | 0.401 |
| Toxicity (toxin A/B/A + B)| 0.215 | 0.391 | 0.851 | 0.936 | 0.543 | 0.098 |
| In-hospital mortality (alive/dead) | 0.284 | 0.792 | 0.227 | 0.207 | 0.309 | 0.380 |
| Infection acquisition (nosocomial/community) | 0.635 | 0.474 | 1     | 0.600 | 0.322 | 0.402 |

**Discussion**
CDI is a bacterial gastrointestinal infection which lead to the hospitalization of many people each year worldwide.

The pathogenesis of CDI depends on several virulence factors that contribute to the disease development and the disease severity, those include the bacterial toxins.

The bacteria and its toxins causing an immune reaction which leads to the activation of many immunological mechanisms. In those we can see the production and elevation of several cytokines and chemokines that have a role in the course of the disease.

In this study we essay the correlation between CDI severity to the level of 6 cytokines and chemokines - IL-16, IL-21, IL-23, IL-33, BCA-1 and TRAIL which were taken from 54 patients' serum.

We perform this study with the intention of finding a biological marker that could correlate with disease severity. In addition, we checked the correlation with the gender, toxicity, in hospital mortality and infection acquisition to which we didn't see any correlation with these last parameters.

Our findings identified a correlation between the levels of 2 immunological markers to the disease severity – IL-16 and BCA-1. Our study results are consistent with the results of others who have shown as well that CDI stimulates the production of multiple cytokines and chemokines, which is considered a characteristic of CDI.

IL-16 is a pleiotropic cytokine that functions as a chemoattractant and a modulator of T cell activation. This cytokine signaling process is mediated by CD4. The function of the IL-16 is exclusively attributed to the C-terminal peptide in contrast to the N-terminal product which may play a role in cell's apoptosis. There is an involvement of caspase 3 in the proteolytic processing of this protein.

IL-16 is released by different kind of cells include lymphocytes and epithelial cells. Originally this interleukin described as an attractor of active T-cells [14] but studies have shown that IL-16 play a role in attraction and activation of verity of other cells which express the CD4 molecule. These cells include eosinophils, monocytes and dendritic cells [15].

We demonstrated statistical significance between the IL-16 level in the patients' serum to the patients' severity score of the disease. These findings suggest that IL-16 and T cells, contribute to immunopathology and the course of C. difficile-mediated diseases. These findings suit other studies that showed a positive correlation between IL-16 to the severity of CDI [16] and other studies which reported association in other intestinal diseases and inflammation include Crohn's disease and ulcerative colitis [17].

B cell-attracting chemokine 1 (BCA-1) also known as CXCL-13 is a chemokine who responsible for selectively chemotactic for B cells, both B-1 and B-2 subsets. This chemokine achieving its effect by interaction with the chemokine receptor CXCR5. Both BCA-1 and CXCR5 expressed in high numbers in the guts, lymph nodes, spleen and the liver [18, 19].
We demonstrated statistical significance between the BCA-1 level in the patients' serum to the patients' severity score of the disease. These findings suggest that also BCA-1 has a major role in the contribution to the immunopathology and the course of C. difficile-mediated diseases. To the best of our knowledge, our study is the first that shows that serum BCA-1 levels are associated with CDI severity. Previous studies have shown association in infected children with helicobacter pylori and in adults with gastric cancer [20]. Other studies reported a poor prognosis in a few types of cancer include gastric cancer and prostate cancer [21, 22].

Our results didn't yield a correlation between the other 4 cytokines that were measured – IL-21, IL23, IL-33, TRAIL. IL-21 is a cytokine with a potent regulatory effect on the NK cells and cytotoxic T cells. In addition, this cytokine can be found in activated CD4 t cells. IL-21 has a role in up-regulation in Th2 and Th17 subsets of T helper cells [23]. IL-23 is heterodimeric proinflammatory cytokine composed of an IL12B and IL23A subunits. IL-23 has a major role in the maintenance and the expansion of the Th17 cells.

We expected to find increased levels of IL-21 and IL-23 in more severe CDI, because both of those cytokines have a pro-inflammatory effect. Furthermore, previous studies have detected that in mice there was a relationship between Clostridioides difficile toxin B to the excretion of IL-21 by iNKT [24]. Other study that was preform as well in mice have shown that toxins alone wouldn't cause surge in the IL-23 levels although with the presence of E. coli's LPS the IL-23 levels were increased significantly. In addition, this study shown that mice that didn't have the ability to produce IL-23 were immune from mortality in CDI [25]. However, no statistically significant differences were noted in the level of these cytokines between mild to moderate and severe CDI. A possible explanation is that the patients' co-morbidities masked part of the immune response moreover it might be some differences between studies that were preform on mice to those who were on humans.

The study has several limitations who could influence our results as well. First, the study population was small. Second, although the serum sampling was done within 24–48 hours since receiving a positive results for C.difficile presence, the samples may not have been collected at the same stage in terms of the immune response. Third, the mortality was in low rates. Therefore, for further studies, we suggest including also patients with more sever disease. Also, we suggest collecting several blood samples on sequential days during hospitalization in order to detect the time point at which a change in cytokine levels occurs. Fourth, out study was performed on patients positive to C.difficile and no comparison was made to other intestinal diseases not caused by it.

Conclusions

Our results point to the involvement or two pro-inflammatory immunological markers in CDI pathogenesis, which is reflected in recruitment and activation of various cells from the humans' immunity system and by many different mechanisms. In line with our hypothesis, disease severity correlated with the enhancement of this immune response. Furthermore, the significant association between the disease severity and the increased levels of IL-16 and BCA-1 indicates that these cytokine and chemokine may
serve as an effective diagnostic tool to predict disease severity in the case of moderate CDI. These immunological markers are easy to measure, and the results are achieved quickly. Consequently, we suggest that in the same time to the initial identification if CDI, testing immunological markers as cytokines and chemokines levels in the blood as a routine part in the regular blood examinations, this may be effective for predicting the disease severity. Improved and more accessible assessment of CDI severity will contribute to adjustment of the medical treatment which will lead to a better patient care and hopefully will reduce the patient's mortality. Further prospective studies are needed to be done in order to investigate the efficacy of pro-inflammatory cytokines in the diagnosis and later phases of CDI, as an alternative or as a supplement to today's procedures.

List Of Abbreviations

*C. difficile* - Clostridium difficile

CDI - *C. difficile* infection

IL 6- Interleukin 6

IL-16- Interleukin 16

IL-21- Interleukin 21

IL-23- Interleukin 23

IL-33- Interleukin 33

Declarations

*Ethics approval and consent to participate*: This study received the approval of the Institutional Committee of Helsinki of the Medical Center, approval No. POR-0085-15.

*Consent for publication*: Not applicable

*Availability of data and materials*: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

*Competing interests*: The authors declare that they have no competing interests.

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*Authors’ contributions*: DG, MA, ZH and AP designed the study, analyzed, interpreted the data, and wrote the final manuscript. AP, MA and ON were involved in development of the protocols. All authors read and approved the final manuscript.
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Figures
Figure 1

Serum inflammatory mediators' levels correlated with disease severity of CDI patients. In BCA-1 and IL-16, severe CDI patients had higher inflammatory mediators' levels than mild patients. *p < 0.05, **p < 0.01.