

Retrospective assessment of fecal myeloperoxidase activity in *Clostridium difficile* associated diarrhea

Chetana Vaishnavi, Prashant Kapoor, Sukhminderjit Kaur, Ibrahim Masoodi, Rakesh Kochhar

Department of Gastroenterology,
Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

ABSTRACT

Background & Objective: Immune defense cells such as polymorphonuclear (PMN) leucocytes and monocytes are present in the colonic mucosa and aid in local inflammatory response. Myeloperoxidase (MPO) abundantly present in immune defense cells gets released upon neutrophil activation. *Clostridium difficile* is an anaerobic bacterium responsible for nosocomial diarrhea and severe colitis.

Methods: A retrospective study was undertaken to quantify the presence of colonic inflammation by evaluation of fecal MPO activity as an adjunct to *C. difficile* diarrhea. A total of 560 patients with nosocomial diarrhea and 123 healthy subjects with no diarrhea formed the basis of our investigation. *C. difficile* was investigated either by stool culture (n=351) or by *C. difficile* toxin (CDT) assay (n=209) using purified anti-toxin A and anti-toxin B. MPO activity was measured using dianisidine hydrogen peroxidase.

Results: MPO was positive in 76.8% of patient samples. Chi square test for MPO analysis showed that it was significantly distributed over positive and negative values. A total of 115 stool cultures were positive for various organisms, of which 91 were also MPO positive. There were 38 *C. difficile* culture positive of which 34 were also MPO positive. MPO activity in relation to CDT assay showed that 43% were positive for both CDT and MPO. When control samples were analyzed, MPO was positive in 11.7% with *C. difficile* growing in 4/30 (13%) of the cultured samples. CDT was negative in the remaining control samples.

Interpretation and Conclusion: High levels of MPO may signal the acuity of the disease and indicate inflammation. Fecal MPO is a simple, inexpensive and objective tool for assessing the degree of acute inflammation in the intestine.

Keywords: *C. difficile* culture, *C. difficile* toxin, fecal MPO

INTRODUCTION

Immune defense cells are present in the colonic mucosa and are a feature of the pathology of colonic inflammation.¹ The local inflammatory response cascade results in the recruitment and infiltration by polymorphonuclear leukocytes (PMNs) and monocytes which get activated by microorganisms. *Clostridium*

difficile is an anaerobic bacterium responsible for nosocomial diarrhea and severe colitis.² The detection and quantification of the extent of bowel inflammation is routinely done by endoscopic and histologic studies of the biopsy specimens which are the standard gold methods. However these are invasive and expensive procedures and therefore alternate non-invasive, inexpensive and patient-friendly laboratory testing techniques are required.

Corresponding Author :

Dr. C. Vaishnavi,
Professor (GE Microbiology)
Department of Gastroenterology
PGIMER, Chandigarh, India.
Tel: 91-172-2756609
Fax: 91-172-2744401; 2745078
E mail: cvaishnavi@rediffmail.com
chetanavaishnavi@gmail.com

Myeloperoxidase (MPO) is a hemoprotein abundantly present in lysosomes of neutrophils and to a much lesser extent of monocytes and tissue macrophages.³ MPO is found both in the intestinal mucosa and in gut lavage and its level is found to get elevated during the various gastrointestinal diseases inclusive of *C. difficile* associated diarrhea (CDAD) which are all linked with colonic inflammation.⁴ MPO forms a part of an antimicrobial system in the phagosome which gets

released during the degranulation process. Activation of neutrophils and of reactive species generating enzyme, acts in host defense by catalyzing the production of hypochloric acid.⁵ The greenish color in some of the fecal samples is imparted by the heme pigment present in the various secretions such as pus and mucus which are rich in neutrophils.

A specific neutrophil/monocyte marker can be useful to evaluate the presence and extent of colonic inflammation in normal mucosa. MPO has been studied as a biomarker and documented to be important for diagnosis of various gastrointestinal inflammations.^{6,7} High MPO levels may both signal the acuity of the disease process as well as indicate the oxidative potential at the site of inflammation. MPO levels can also be used for monitoring the outcome of treatment. The present study was undertaken to quantify the presence of inflammation by evaluation of fecal MPO activity as an adjunct to CDAD.

MATERIALS AND METHODS

- (i) **Study population and samples:** A total of 560 stool samples from patients with nosocomial diarrhea each collected in sterical vials with collection spoons attached to them (HiMedia, Mumbai, India) and submitted to the Gastroenterology, Microbiology Division for investigation of *C. difficile* were analyzed. Nosocomial diarrhea was defined as the passage of three or more unformed stools in 24 h from patients who developed diarrhea later than 48 h of hospital admission. Stool samples from non-diarrheic healthy subjects (n=123) comprising of attendants of the patients were also included as controls. *C. difficile* was investigated either by stool culture or by *C. difficile* toxin (CDT) assay. MPO activity was measured retrospectively in the stored fecal supernatants using dianisidine hydrogen peroxidase.
- (ii) ***C. difficile* culture:** For *C. difficile* culture fecal samples were plated onto selective media such as cefoxitin cycloserine fructose agar and Columbia blood agar, both directly and after alcohol shock treatment. The media plates were incubated anaerobically at 37°C for 72 h for the isolation of *C. difficile*. Identification of *C. difficile* was done by colony morphology, Gram staining and biochemical methods.
- (iii) ***C. difficile* toxin assay:** Fecal supernatants were used for toxins A and B assay as described earlier^{8,9} using purified antitoxin A and antitoxin B (kindly provided by Dr. M. Warny, USA). The fecal samples were subjected to 1 in 5 dilutions in phosphate buffer saline and supernatant prepared similarly by vortex mixing and centrifugation. In brief, 50 µl of the supernatant was taken on a clean glass slide to which ready-to-use *C. difficile* antitoxin A or B coated latex beads were added. The slide was gently rocked manually and checked for immediate macroscopic agglutination. The supernatant that agglutinated with latex beads coated with antitoxin A was taken to be positive for toxin A and that which agglutinated with antitoxin B as positive for toxin B. A known positive fecal sample obtained from a patient with antibiotic-associated diarrhea was the positive control. Two negative controls consisted of (i) an unreactive fecal sample from a healthy subject who had no antibiotic exposure for 6 weeks prior to testing and (ii) uncoated latex beads plus diluted test sample.
- (iv) **MPO activity:** MPO activity was evaluated by a modified method of Bradley *et al*¹⁰ using dianisidine hydrogen peroxidase assay in the stored fecal supernatants. Two milliliters hexadecyltrimethylammonium bromide buffer was added to tubes before adding the fecal samples. Samples were then homogenized on ice for 10 seconds. One ml homogenate was transferred to eppendorf tubes and three cycles of freezing and thawing were done. The samples were then subjected to centrifugation (10000 x g for 15 minutes at 4°C) and the supernatant was transferred to fresh tubes. Freshly prepared reactive buffer (O- dianisidine dihydrochloride) and 1% H₂O₂ was then added to the samples. Human MPO 0.1 unit/100 µl (Sigma, USA) was used as the standard. After development of color, MPO activity was measured using an ELISA Reader at 450 nm. The results were reported as MPO units/milliliters of fecal supernatant. A unit of MPO activity was defined as that converting 1 mole of hydrogen peroxide to water in 1 min at 25°C. MPO level >0.065 units/ml was considered to be positive.
- (v) **Statistical analysis:** Statistical analysis of data was done by using Fisher test (Chi-square test) using SPSS 15.0 version.

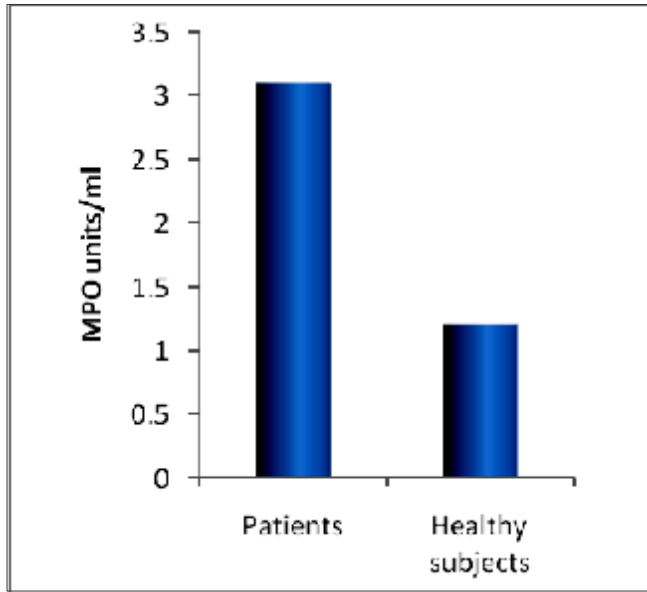


Figure I: Fecal MPO activity in patients and healthy subjects

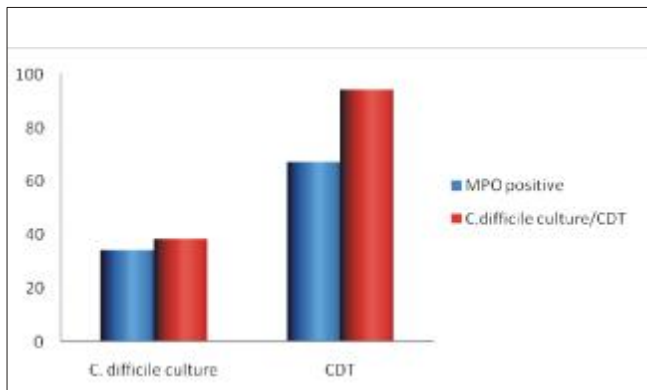


Figure II: MPO activity in relation to C. difficile culture and CDT

RESULTS

Retrospective assessment of fecal myeloperoxidase activity in relation to *C. difficile* was made in a total of 560 patients. *C. difficile* was investigated either by stool culture (n=351) or by CDT assay (n=209). MPO was positive in 76.8% of patient samples. The mean MPO value for patients was 3.10261 and that for healthy subjects was 1.19980 (Figure I). Chi-square test revealed that data was significantly distributed over positive and negative values (p= 0.000).

A total of 115 stool cultures were positive for various organisms, of which 91 (79%) were also MPO positive. There were 38 *C. difficile* culture positive of which 34 (89%) were also MPO positive. Of the 209 samples whose CDT assay was done, 45% (n=94) were found to be CDT positive. Samples positive for CDT assay had 71.3% (n=67) MPO positive. MPO activity in relation to

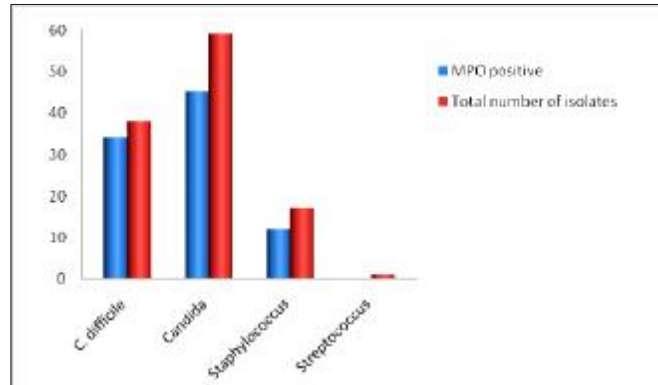


Figure III: MPO activity in relation to different isolates

CDT assay showed that 43% were positive for both CDT and MPO (Figure II). When healthy control samples were analyzed, MPO was positive in 11.7% with *C. difficile* growing in 4/30 (13%) of the cultured samples. CDT was negative in the remaining control samples. Retrospective data analysis for other organisms isolated was also made. Of 351 culture data available 32.8% were culture positive, with *C. difficile* in 33.0%, candida in 51.3%, staphylococci in 14.8% and streptococci in 0.9% (Figure III).

DISCUSSION

Toxin formation directed at host tissues is responsible for the pathogenicity of many microorganisms, inclusive of *C. difficile*. MPO is abundantly present in PMN leukocytes and monocytes and is one of the main enzymes released upon neutrophil activation. MPO is an important component of the neutrophil cytotoxic armament. MPO has both antimicrobial and cytotoxic properties.^{11, 12} It contributes to the bactericidal action of PMN^{13,14} by catalyzing the formation of hypochlorous acid, which is a potent oxidant with bacterial activity *in vitro*.¹⁵ Neutrophils move towards region of inflammation and liberate cellular contents and enzymes which act on the microorganism present there. Extracellular MPO activity results due to leakage before complete closure of the developing phagosome or in response to stimulation by an antibody/complement-coated surface too large to be ingested. MPO, when released, can be inactivated by products of the respiratory burst or be cleared from the extracellular fluid by uptake by macrophages through reaction with the mannose receptor.

Myeloperoxidase functions in the oxygen-dependent killing of microorganisms. It is released from the primary granules of neutrophils during acute inflammation and

its concentration is proportional to the number of neutrophils within that region. Peterson *et al*¹⁶ found a relationship between fecal MPO levels and the histological indices of disease activity in ulcerative colitis. Similarly, Wagner *et al*¹⁷ showed that normalized MPO levels predicted a complete response to treatment in 100% of the patients. However, elevated MPO levels predicted an incomplete response in 23% patients. In this respect, MPO might potentially be used as a surrogate marker for a successful treatment outcome in inflammatory bowel disease (IBD) patients, similar to calprotectin.

The intestinal mucosa gets inflated by neutrophils in response to infectious stimuli. Castagliuolo *et al*¹⁸ demonstrated that *C. difficile* toxin A induces macrophage inflammatory protein-2 (MIP-2) release from intestinal epithelial cells and that MIP-2 contributes to neutrophil mucosal influx during toxin A enteritis. The pathogenesis of toxin A mediated enteritis involves interactions between sensory nerves, enterocytes and inflammatory cells of the intestinal epithelial and lamina propria cells.^{19,20} Administration of toxin A into rat ileum increased mucosal levels of potent PMN chemo-attractant MIP-2.¹⁸

In the present study high levels of fecal MPO was found in patients with CDAD which may signal the acuity of the disease and indicate inflammation.^{21,22} Fecal samples from which other organisms were isolated were also positive for MPO. Fecal MPO is thus a simple, inexpensive and objective tool for assessing the degree of acute inflammation in the intestine. It could be used as an adjunct to aid the diagnosis of nosocomial diarrhea due to *C. difficile*. Assessment of fecal MPO can also prove to be a reliable test to differentiate transient carriage of pathogenic organisms from true infections of the colon and can be a valuable tool in nosocomial diarrhea.

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