Validation of a point-of-care desk top device to quantitate fecal calprotectin and distinguish inflammatory bowel disease from irritable bowel syndrome☆

Michael J. Sydora, Beate C. Sydora, Richard N. Fedorak*

Center of Excellence for Gastrointestinal Inflammation and Immunity Research, Division of Gastroenterology, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

Received 22 June 2011; accepted 12 August 2011

KEYWORDS
Calprotectin; Inflammatory bowel diseases; Ulcerative colitis; Crohn's disease; Irritable bowel syndrome; ELISA; Diagnosis

Abstract

Background and aims: The neutrophil protein calprotectin has been investigated as a surrogate marker for intestinal inflammation. This study was designed to contrast fecal calprotectin levels in patients with inflammatory and non-inflammatory intestinal diseases and to compare the results obtained from the standard ELISA-based method with those obtained from a novel desk-top device.

Methods: Soluble proteins were extracted from stool samples of 50 participating patients, including those diagnosed with Ulcerative Colitis, Crohn's Disease or IBS, and volunteers with no known intestinal problems. Calprotectin was assessed in the extracted material using the "desk top" Bühlmann Quantum Blue Reader® or by standard ELISA techniques.

Results: The mean concentration of calprotectin in the IBD patients group was significantly higher than the mean concentration found in IBS patients and healthy controls (p=0.01). Calprotectin concentrations in IBS patients and controls were indistinguishable. IBD patients that had undergone recent surgery displayed scores similar to controls and IBS patients. Excluding these patients yielded a specificity of 100% for results from both CD and UC patients and an accuracy rate of 1 for CD and 0.89 for UC patients in ROC analysis. Quantum Blue Reader® calprotectin levels were available within 30 min and correlated well with results derived from standard ELISA assays, which took over 8 h to complete.

☆ Conference presentation: Canadian Digestive Disease Week (CDDW), Vancouver, Canada February 26 to March 01, 2011.
* Corresponding author at: Center of Excellence for Gastrointestinal Inflammation and Immunity Research, Division of Gastroenterology, Zeidler Leducor Center, University of Alberta, Edmonton, Alberta, Canada T6G 2X8. Tel.: +1 780 492 6941; fax: +1 780 492 8121.
E-mail addresses: msydora@ualberta.ca (M.J. Sydora), bsydora@ualberta.ca (B.C. Sydora), richard.fedorak@ualberta.ca (R.N. Fedorak).
1. Introduction

Physicians often face the diagnostic difficulty of differentiating patients with inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn’s disease, from those with non-inflammatory diseases such as irritable bowel syndrome (IBS), because many symptoms are common to both conditions, including abdominal pain, bloating, excessive flatus, and altered bowel habits. Therefore, during their diagnostic investigation patients with IBS are often subject to the same invasive and costly diagnostic procedures as patients with IBD. There is thus a need for the development of a fast and effective method to distinguish patients with inflammatory gastrointestinal diseases from those with non-inflammatory gastrointestinal diseases to assist in advancing diagnosis effectively and avoiding costly and unnecessary diagnostic procedures.

Current prevalence estimates for IBS indicate that approximately 15% of women and 12% of men are affected by this condition. These estimates have generally been based on patients fitting a number of symptom-based diagnostic criteria that have been developed to identify patients with IBS.1–11 A number of investigators have recommended a straightforward approach to the evaluation and treatment of patients with IBS based on the use of the Rome criteria as a means of cost-effective management, avoiding the costly workup to sort through a confusing array of gastrointestinal symptoms.1–3 The stepwise assessment of patients with suspected IBS based on positive Rome criteria includes a full blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), serum chemistry, thyroid function tests and stool examination for parasites and ova to exclude organic diseases. However, as shown by Tolliver et al., these parameters have a disappointing diagnostic yield and are thus rarely used in clinical practice.12

The most striking difference between IBS and IBD is that the former is non-inflammatory in nature. Therefore, one possibility is to measure surrogate markers of intestinal inflammation to differentiate between the two. Assessment of serologic markers of inflammation (ESR, CRP) has in general been disappointing, probably because of their lack of sensitivity and specificity, as they are indirect measures of inflammation and can be affected by a number of non-intestinal diseases.13–15 The direct assay of feces for inflammatory markers has the potential to improve the discriminate value of the serologic markers.

Among candidate markers, fecal calprotectin has been investigated with promising results showing consistency in defining patients with IBD, colorectal carcinoma, and non-steroidal enteropathy.16–21 Calprotectin is a 36 kDa calcium and zinc binding protein found throughout the body in blood cells and plasma, indeed, it accounts for about 60% of the cytosol in neutrophils, one of the major players in gut intestinal inflammation.22,23 Calprotectin exhibits bacteriostatic and fungistatic properties in vitro, inhibiting the growth of various microorganisms by a mechanism including extra-cellular zinc exclusion.24–27 Calprotectin is not actively secreted from the neutrophils, but is released following cell death or cell disruption.28 Once released Calprotectin induces apoptosis in other cells. As a result, levels of calprotectin from the apoptotic neutrophils rise during cell activation and turnover under inflammatory conditions. As the calprotectin sits in the intestine, it is absorbed by fecal material passing through. This accounts for the existence of calprotectin in stool without being attached to the neutrophil. Furthermore, calprotectin is fairly unaffected by medications and enzymatic degradation and is stable outside the body at room temperature for up to 7 days.17 These characteristics make calprotectin a useful component for reliable ELISA testing.

Therefore, calprotectin is an ideal marker to determine if a patient is suffering from inflammatory intestinal diseases as opposed to non-inflammatory functional diseases.29,30 Unfortunately, standard ELISA methods for measuring calprotectin are time consuming and labor intensive, taking more than 8 h to complete one test. Recently, a point-of-care desk-top device to measure fecal calprotectin in a fast and efficient manner has been developed. This device is based on lateral flow technology and results can be obtained in less than 30 min including protein extraction. While test results obtained with standard ELISA technique are often difficult to compare and are subject to careful standardization, the point-of-care desk-top device uses internal standards within a range of 30–300 μg/g and a sensitivity of <10 μg/g, thus, guarantying consistency in results. Another major advantage is the simplicity of sample preparation and analysis. No more than 80 mg stool sample is required for the assessment and sample preparation and analysis is user friendly and does not involve the need of special equipment, which makes it ideally suited for every lab.

This study was designed to contrast fecal calprotectin levels in patients with inflammatory and non-inflammatory intestinal diseases and to compare the results obtained from the standard ELISA-based method with those obtained from the point-of-care desk-top device.

2. Materials and methods

2.1. Patients

This study was performed at the University of Alberta with patients seen in consultation by the University of Alberta hospital IBD program. A total of 50 patients participated. Of the enrolled patient group, 16 were diagnosed with IBD including 9 with UC and 7 with CD. 26 served as non-inflammatory control group and included 7 IBS patients and...
19 volunteers with no known intestinal problems. 8 stool samples had to be excluded for late or improper handling of stool samples before delivery. All patients were informed about the purpose of the experiment and a written confirmed consent was obtained from all participants. A Mayo clinic or Harvey Bradshaw Index (HBI) disease activity score for patients with ulcerative colitis or Crohn’s disease, respectively, and their medical history was obtained at the time of stool collection. The study was approved by the University of Alberta Health Research Ethics committee.

2.2. Collection of fecal samples

Participants were provided a tube equipped with a spatula to collect fecal material and delivered the fecal samples to the hospital within 24 h. Samples were then refrigerated and processed for analysis within 6 days. Prolonged, as well as improper storage of fecal samples before processing can result in inaccurate concentration measurements. Thus samples that were not delivered within 24 h were discarded from the cohort. Protein extraction for standard ELISA and for Quantum Blue Reader® analysis was performed on the same day in parallel.

2.3. Preparation of fecal samples for standard ELISA analysis

The assessment of calprotectin by standard ELISA techniques involves several time consuming steps of weighing samples, incubation, centrifugation and plate washing. In addition, the generation of a reproducible standard curve is vital for the assessment of calprotectin levels in stool samples.

Briefly, sample preparation was performed according to the protocol provided in the Calprotectin ELISA kit (Alpco Immunoassays, Salem, NH). An amount between 50 and 100 mg by weight of faeces was placed into tubes and topped with an amount equalling 49 times the weight in volume of an extraction buffer provided in the kit. The samples were homogenized by vigorous vortexing for 30 min. Larger particles were sedimented as described above. The quantitative assessment of Calprotectin in the extracted material, a 60 μl aliquot was titrated into the loading port of a test cartridge. The point-of-care Quantum Blue Reader® was adjusted to the test kit and cartridge via a chip card supplied with the test kit. After a set time frame of 12 min the cartridge was read and the calculated results were shown on the POC reader display. The time to complete this process was less than 30 min.

2.5. Statistical analysis

Results using the Bühlmann Quantum Blue Reader® were compared to the amount of calprotectin in these same stool aliquots quantified by standard ELISA techniques. Data were collected from patient groups according to their diagnosed diseases and expressed as means±SEM. Differences between mean values were evaluated using t-tests or analysis of variance (Mann–Whitney Rank Sum Test or Kruskal–Wallis One Way Analysis of Variance on Ranks), where appropriate (SigmaStat, Jandel Corporation, San Rafael, CA). Receiver operator analysis were performed to determine sensitivity and specificity values for distinguishing organic (UC or CD) from non organic (IBS) disease and to calculate the positive (PPV) and negative (NPV) predictive value for calprotectin levels.

3. Disclosure

The authors declare no conflict of interest; however, reagents and materials used to perform the ELISA as well as the Quantum Blue Reader® analysis were provided to us from Alpco Immunoassays (Salem, NH) at no cost.

4. Results

4.1. Patients’ demographic profile

Patient demographics are shown in Table 1. While age differences were not significantly reflected in the calprotectin scores of control and CD patients, levels of calprotectin in IBS patients were slightly higher in participants above the

<table>
<thead>
<tr>
<th>Patients</th>
<th>N</th>
<th>age (average)</th>
<th>male/female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>19</td>
<td>21–81 (52)</td>
<td>5/14</td>
</tr>
<tr>
<td>IBS</td>
<td>7</td>
<td>34–84 (57)</td>
<td>0/7</td>
</tr>
<tr>
<td>CD</td>
<td>7</td>
<td>32–76 (50)</td>
<td>0/7</td>
</tr>
<tr>
<td>UC</td>
<td>9</td>
<td>19–61 (33)</td>
<td>4/5</td>
</tr>
</tbody>
</table>

Table 1 Patients demographic profile.
age of 60 (Fig. 1). Three out of 4 IBS patients that scored above average were older than 60 while all 3 patients that scored below average were age 51 and less, suggesting that an increase in age in this patient group can lead to a slight raise in calprotectin concentration. However, none of the IBS or control patients scored higher than the cutoff level of 150 \( \mu g/g \) which is a level previously determined as the cutoff level for non-inflammatory calprotectin levels and which we were able to verify in our study by calculations from the receiver operating characteristics (ROC) plot analysis (see below). The age-related increase in calprotectin levels in IBS patients was also obvious when calprotectin levels were measured with standard ELISA techniques (data not shown).

In our enrolment group, two patients with CD and one with UC had undergone recent surgery (marked with open circles in Fig. 1). Despite high disease activity indices (HBI scores of 8 and 6 and a Mayo score of 6), fecal samples from all of these three patients displayed calprotectin levels below the calculated cutoff line suggesting reduced calprotectin release in the luminal contents due to decrease in inflammatory environment. Except for the two patients with surgery all CD patients presented with calprotectin levels exceeding 200 \( \mu g/g \) with no obvious correlation with regard to HBI score. Besides the one patient that had undergone recent surgery, there were three other patients with low calprotectin levels in the UC patient group. Two of these patients were on NSAID (Ibuprofen and Nabumetone) which might explain the low levels of fecal calprotectin due to a reducing effect of NSAID on inflammatory mediators. Similar to the HBI score in CD patients we were not able to make a correlation between calprotectin levels and differences in Mayo scores in UC patients.

### 4.2. Calprotectin levels are significantly elevated in both UC and CD patients but not in IBS patients; a comparison of standard ELISA analysis and point-of-care analysis

Calprotectin levels were measured in identical stool samples using two methods: standard ELISA method and the commercialized point-of-care Quantum Blue Reader® technique (see methods above). The standard ELISA method of analysis required a minimum time commitment of 8 h (480 min) while the point-of-care Quantum Blue Reader® technique could be

![Figure 1](image1.png) Correlation of calprotectin concentrations with age and clinical activity as determined by HBI score (CD) and Mayo score (UC). Each dot represents the concentration of fecal calprotectin as determined with the Bühlmann Quantum Blue Reader® in individual UC, CD, and IBS patients and control subjects without intestinal diseases. Numbers represent clinical activity scores and open circles represent patients with recent surgery. The dotted line denotes the mean value in each group.

![Figure 2](image2.png) Comparison of calprotectin levels measured with the point-of-care desk-top Bühlmann Quantum Blue Reader® (A) with those measured by standard ELISA techniques (B). Data represent the mean±SEM from samples of controls (n =19), IBS (n =7), CD (n =7), and UC (n =9) patients. \( **p<0.05 \) compared with both IBS and controls, \( *p<0.05 \) compared with controls.
performed in approximately 30 min. Both methods produced an identical outcome with regards to calprotectin levels (Fig. 2A and B). Similarly low concentrations of calprotectin were measured with both methods in control subjects and IBS patients without intestinal inflammation. A significant increase in average Calprotectin concentrations were found in both UC and CD patients when compared to controls (p=0.01 and 0.001 for UC and CD patients respectively when measured with the Quantum Blue Reader®, and p=0.05 and 0.002 for UC and CD patients respectively when measured with standard ELISA techniques); however, only CD patients reached calprotectin levels that were, on average, significantly different from IBS patients (p=0.008 when measured with Quantum Blue Reader and p=0.05 when measured with standard ELISA techniques).

4.3. Calprotectin levels measured with the Quantum Blue Reader® correlate well with those measured by standard ELISA

While the assessment of calprotectin by standard ELISA methodology is well documented it is not clear whether the assessment with the point-of-care Quantum Blue Reader® will suffer in accuracy and specificity due to the simplicity and ease in handling and the minimal time commitment.

A direct comparison of the two methods is shown in Fig. 3. Each line connects the level of calprotectin for an individual patient measured with the point-of-care Quantum Blue Reader® to the level measured with standard ELISA technique. The calprotectin measurements with the point-of-care Quantum Blue Reader® are limited between a minimum measurement value of 30 μg/g and a maximum measurement value of 300 μg/g. In contrast, the standard ELISA method does not have a lower or upper limit of measurement (Table 2). Nevertheless, the magnitude of calprotectin levels determined by point-of-care Quantum Blue Reader® technique correspond well to the calprotectin amounts computed with the standard ELISA method (median in Table 2 and mean in Fig. 2). Using the point-of-care Quantum Blue Reader®, there is a clear cut at the 150 μg/g level that distinguishes control subjects and IBS patients from those

<table>
<thead>
<tr>
<th>Patients</th>
<th>n</th>
<th>Fecal calprotectin concentration [μg/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Quantum Blue Reader®</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>Controls</td>
<td>19</td>
<td>35</td>
</tr>
<tr>
<td>IBS</td>
<td>7</td>
<td>66</td>
</tr>
<tr>
<td>CD</td>
<td>7</td>
<td>275</td>
</tr>
<tr>
<td>UC</td>
<td>9</td>
<td>208</td>
</tr>
</tbody>
</table>

5. Discussion

In this study we have quantified calprotectin levels in stool of patients with intestinal inflammatory diseases and in patients with non-inflammatory intestinal disease by two methods: A) standard ELISA techniques and B) with the point-of-care desk-top Bühlmann Quantum Blue Reader®, an ELISA-based instrument with greatly reduced calprotectin assessment time.

Our results validate the use of calprotectin as a bio marker for intestinal inflammatory diseases. Using both the
standard ELISA method and the desk-top device we have shown that calprotectin levels are only elevated above a control-determined threshold in patients with intestinal inflammation such as Ulcerative colitis and Crohn’s disease, but not in patients with non-inflammatory IBS or controls without any gastrointestinal symptoms. Similar results have been reported by others. While all of these studies show a high specificity of calprotectin in inflammatory diseases compared to functional diseases and controls, it is also clear that calprotectin cannot be used to distinguish individual inflammatory diseases. Calprotectin levels cannot be used to distinguish UC from CD or other infectious or ischaemic forms of colitis, and are similarly elevated in various forms of colorectal cancer.

Recent surgery among our patients resulted in calprotectin levels below the cutoff level, possibly as a result of the removal of the immediate inflammation origin. Due to the small patient number in our study, we could not establish a clear correlation between patients in remission (as determined by a Mayo score below 2 for UC and an HBI score below 5 for CD) and low calprotectin levels. Elevated calprotectin levels in IBD patients during remission have been reported by Roseth et al. In addition, high calprotectin levels in patients in remission have been proposed as predictors of relapse; however the results of these investigations are controversial with regard to the usefulness of relapse prediction for both diseases UC and CD. While some investigators claim calprotectin levels are equally reliable predictors for relapse in UC and CD, others report a high accuracy in prediction of relapse only for UC patients.

A cutoff level of 150 μg/g for calprotectin levels in CD patients was previously suggested by Tipple et al. with ELISA techniques. While this cutoff is at a higher concentration than the one previously suggested for healthy subjects, it was better suited with higher sensitivity in a comparison between IBD and IBS patients. Using the Bühlmann Quantum Blue Reader® we computed the same cutoff level. Excluding the patients with recent surgery from the analysis, a cutoff at 150 μg/g resulted in an accuracy rate of 1 for CD patients and 0.8 for UC patients with 100% test specificity for both conditions when compared with IBS patients. In order to achieve the same accuracy and specificity with standard ELISA methodology in our analysis, the cutoff line had to be raised to 230 μg/g.

In contrast to standard ELISA techniques, the Quantum Blue Reader® has a limited range from 30 to 300. However, there is a clear separation between data from control and IBS patients without inflammation and data derived from samples of patients with active inflammation. Results from standard ELISA tests, as seen in Table 2 and Fig. 3, have a larger range for IBD, IBS and controls. The Bühlmann Quantum Blue Reader®, however, has a smaller range, and thus is more specific in differentiating IBD and non-IBD.

While both methodologies are based on ELISA techniques they differ substantially in the ease of handling and input of time and effort. Standard ELISA technique requires skilled laboratory personnel for the execution of the test. Accuracy in pipetting and preparing dilutions of standard curves is vital for the reproducibility and valet comparison of data. Standard ELISAs are customarily performed in duplicates in 96 well plates. Efficient testing with regard to time commitment and reagents usage, therefore, is only achieved when a full plate is assayed, which would require the testing of about 40 samples at the same time. However, more often than not, the result of an individual patient’s test is needed at a given time. Standard ELISA technique involves several incubation periods ranging from about 15 min (the quantitative enzyme-supported reaction of the fluorochrome) to 2 h (binding between substrates and antibodies). The total of 4 incubation times together with several required washing steps and the pipetting of individual reagents and standards, inclusive the preparation of samples from faecal aliquots lengthens the assessment of calprotectin by standard ELISA assay to over 8 h. In comparison, the assessment of calprotectin levels with the desk-top Quantum Blue Reader® does not require specific skills and can be performed by untrained operators. The elaborate steps involved in standard ELISA techniques are reduced to one simple step of loading 60 μl sample on a small designated well in the cartridge. Incubation times are replaced by lateral flow chromatography. Each sample is measured individually in a short 12-minute time frame and an internal standard allows for quantitative assessment and guarantees comparable results between tests.

In summary, our data supports previous investigations showing that calprotectin is a useful diagnostic tool to distinguish inflammatory from non-inflammatory intestinal diseases. The point-of-care desk-top Quantum Blue Reader® achieves the same accuracy of fecal calprotectin measurement as standard ELISA methodology with a clear discrimination between patients with inflammatory and non-inflammatory intestinal disorders. Nevertheless, the point-of-care desk-top Quantum Blue Reader® permits accurate calprotectin determinations in less than 30 min compared to over 8 h for the standard ELISA method. The point-of-care desk-top Quantum Blue Reader® is the instrument of choice for fast and reliable determination of fecal calprotectin levels.

Acknowledgements

Statement of authorship:
MJS carried out the studies, enrolled patients, collected and analyzed samples, was involved in data analysis, and drafted the manuscript.

BCS participated in sample analysis and data interpretation, performed statistical analysis and was involved in manuscript writing.

Table 3 Receiver operating characteristic (ROC) analysis.

<table>
<thead>
<tr>
<th>Quantum Blue Reader®, cutoff at 150 μg/g</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC versus IBS</td>
<td>55.50%</td>
<td>100%</td>
<td>0.75</td>
</tr>
<tr>
<td>CD versus IBS</td>
<td>71.43%</td>
<td>100%</td>
<td>0.86</td>
</tr>
<tr>
<td>UC (-S) versus IBS</td>
<td>62.50%</td>
<td>100%</td>
<td>0.8</td>
</tr>
<tr>
<td>CD (-S) versus IBS</td>
<td>100%</td>
<td>100%</td>
<td>1</td>
</tr>
</tbody>
</table>

In contrast to standard ELISA techniques, the Quantum Blue Reader® has a limited range from 30 to 300. However, there is a clear separation between data from control and IBS patients without inflammation and data derived from samples of patients with active inflammation. Results from standard ELISA tests, as seen in Table 2 and Fig. 3, have a larger range for IBD, IBS and controls. The Bühlmann Quantum Blue Reader®, however, has a smaller range, and thus is more specific in differentiating IBD and non-IBD.
References

1. Drossman DA, Whitehead WE, Camilleri M. Irritable bowel syn-
10. Camilleri M, Prather CM. The irritable bowel syndrome: mecha-
14. Nielsen OH, Vainier B, Madsen SM, Seidelin JB, Heegaard NH. Established and emerging biological activity markers of inflam-
24. Steinbakk M, Naess-Andresen CF, Lingaaas E, Dale I, Brandtzæg P, Fagerhol MK. Antimicrobial actions of calcium binding leuco-
32. Summerton CB, Longlands MG, Wiener K, Shreeve DR. Faecal calprotectin: a marker of inflammation throughout the intesti-
35. Hoff G, Grotmol T, Thils-Evensen B, Bredthauer M, Gondal G, Vatn MH. Testing for faecal calprotectin (PhiCaI) in the Norwe-
gian Colorectal Cancer Prevention trial on flexible sigmoidosco-
36. Tibble JA, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. Gastroen-


