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ORIGINAL ARTICLE

Turbidimetric Determination of Fecal Calprotectin Using Two Table Top Chemistry Analyzers: Mindray BS-200E and Cobas® c111

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SUMMARY

Background: **Fecal calprotectin assays are widely used in diagnosis and monitoring of inflammatory bowel disease (IBD) in patients with suspected IBD. The most frequently used technique is ELISA and microtiter plates. Turbidimetric assays for analysis of fecal calprotectin can significantly reduce turnaround time. Many laboratories may be reluctant to run fecal samples on their large chemistry analyzers. The aim of this study was to evaluate fecal calprotectin particle enhanced turbidimetric immunoassay (PETIA) on smaller chemistry analyzers that could be dedicated for fecal samples.**
Methods: **The BÜHLMANN fCAL® turbo assay was validated on two table top chemistry analyzers, Mindray BS-200E and cobas® c111.**
Results: **The assay was linear in the range between 20 and 1,900 µg/g with a limit of quantification around 20 µg/g on both instruments. The total coefficient of variation was < 7% in the range between 50 and 1,300 µg/g on both instruments. No antigen excess hook effect was observed up to 18,000 µg/g on the Mindray BS-200E and up to 20,000 µg/g on cobas® c111. The BÜHLMANN fCAL® turbo assay showed a high correlation with the BÜHLMANN fCAL® ELISA.**
Conclusions: **Running the BÜHLMANN fCAL® turbo on Mindray BS-200E or cobas® c111 chemistry analyzers can provide rapid test results without exposing large routine chemistry analyzers to stool samples.**
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KEY WORDS

biological markers, calprotectin, feces, inflammatory bowel diseases, humans, method validation

INTRODUCTION

Accurate diagnosis and evaluation of disease activity is essential for optimal treatment and follow-up of patients with inflammatory bowel disease (IBD). The two major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC) [1]. Both CD and UC are chronic diseases that have episodes of remissions and relapses [2]. Especially early in the course it is difficult to distinguish IBD patients from patients with irritable bowel syndrome (IBS). Another problem is that clinical symp-

toms do not always correlate with endoscopic or histological examinations. Active enteric inflammation may also be present in patients that do not have any symptoms [3].
Endoscopic examinations are costly, burdensome for the patient, and may worsen the disease condition [4]. Therefore, non-invasive methods have been developed as a complement to the invasive methods [5]. The most widely used blood marker for IBD has been C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) but leukocyte count, albumin, and α1 acid glycoprotein have also been used [6]. Fecal calprotectin has been extensively studied as a marker for IBD [7-9] and has, over the years, been shown to have a higher sensitivity and specificity for IBD than ESR and CRP [10-12]. Calprotectin is present in high concentrations in the cytoplasm of neutrophils where it represents approximately 30 - 40% of the protein content [13]. Mucosal inflammation leads to infiltration of granulocytes in the inflamed areas of the gut and may also cause bleeding. Both mechanisms increase the levels of calprotectin in stool [14].
Traditional microtiter plate tests for fecal calprotectin are usually performed as batch testing and the samples are stored at the laboratory until there are sufficient samples for a full microtiter plate. In contrast, modern chemistry analyzers usually are running in a random access mode analyzing the samples continuously. The development of particle enhanced turbidimetric immunoassays (PETIA) for fecal calprotectin can significantly reduce turnaround time and would permit more laboratories to perform these tests. We believe that some laboratories may be reluctant to run fecal samples on their main chemistry analyzers together with their serum or plasma samples. A dedicated instrument could thus be an alternative for these laboratories eliminating the risk of contaminating their main analyzers with extracts from fecal samples.
The aim of our study was to evaluate a new fecal calprotectin PETIA on two bench-top analyzers that could be used as dedicated instruments for fecal samples.

MATERIALS AND METHODS

The study was approved by the local ethical board (01-367) and complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research.

Samples

Fecal samples for the study were collected from routine fecal calprotectin requests that were anonymized and used for the method validation. Frozen samples were thawed weighed and extracted according to manufacturer´s instruction for use. The weighed fecal samples were diluted 1:500 with an extraction buffer provided by the manufacturer. After the samples were dissolved in the buffer, the samples were centrifuged at 1500 x g for 10 minutes at room temperature to remove any solids in the samples that could interfere with the pipetting of the samples.

Reaction buffer and immunoparticles

The BÜHLMANN fCAL® turbo assay (BÜHLMANN Laboratories AG, Schönenbuch. Switzerland) was evaluated. The reagent kit contains reaction buffer (R1) and immunoparticles (R2).

Calibrators

Human calprotectin from granulocyte extracts in BÜHLMANN B-CAL-EX extraction buffer was used to achieve six calibrator levels: 0, 50, 192, 468, 934, and 1,862 µg/g. The calprotectin concentrations of the calibrators were assigned via a value transfer protocol against the BÜHLMANN fCAL® ELISA (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland). Calibrators were provided by the manufacturer and were ready for use.

Controls

Controls were prepared by adding human calprotectin from granulocyte extracts to the BÜHLMANN B-CAL-EX extraction buffer. Two levels, 75 and 250 µg/g, provided by the manufacturer were used in the evaluation. Controls are provided as ready to use material.

Instruments

Method parameter settings were optimized for the BÜHLMANN fCAL® turbo on the Mindray BS-200E (Mindray Medical International, Shenzhen, China) and the cobas® c111 (Roche Diagnostics, Mannheim, Germany). The parameter settings are listed in Table 1. For the method comparison, the samples were measured on the BÜHLMANN fCAL® turbo and the BÜHLMANN fCAL® ELISA.

Limit of quantitation (LOQ)

Four samples in the measuring range of 10 to 25 µg/g were prepared by diluting fecal sample extracts (assigned using the BÜHLMANN fCAL® ELISA) with extraction buffer supplied from the manufacturer. The theoretical concentration of the diluted samples was calculated using the dilution factor and the sample values were assigned. The diluted samples were aliquoted and frozen at -20°C. Aliquots were thawed and measured in 12 replicates during 3 days. In total, 36 measurements for each concentration level were performed. CVs and biases from theoretical values were calculated and the total error was determined by the root mean square method [15]. LOQ was defined as the concentration where the total error was below 20%.

Antigen excess

A calprotectin stem solution was prepared by extracting calprotectin from human granulocytes. The high-level antigen excess samples were prepared by adding this stem solution into BÜHLMANN B-CAL-EX extraction

buffer to a calprotectin concentration of approximately 24,000 µg/g.
From the stem solution, a dilution series of 8 samples were prepared from the high concentration antigen excess sample ranging from 2.5% to 100% of the original sample. All samples were measured in duplicate. The point of antigen excess was defined as the concentration where the turbidimetric result of the measured sample was both lower than the expected value and below the value of the highest calibrator.

Linearity
The samples for the determination of the linearity were prepared by diluting a high fecal calprotectin sample with a low fecal calprotectin sample at different concentrations creating eleven levels ranging from 100% to 0%. The expected value of each level in the series was calculated from the assigned value of the high and low fecal calprotectin sample and the percentages of high and low sample at each level. All samples were measured in duplicates and the deviation from the expected value was calculated. The acceptance criterion is 80% to 110% recovery from baseline.

Precision
Four samples in the range between 40 to 1,400 µg/g and two controls (Low Control and High Control) were aliquoted and stored at -20°C. Before analysis, the samples were thawed and immediately measured in duplicate. After a minimum of two hours a new run of duplicate measurements was performed. This procedure was repeated for 5 days on the cobas® c111 and 10 days on the Mindray BS-200E. A new calibration curve was established after five days. Within-run, between-run, and between-day CVs were calculated. The acceptance criteria for total imprecision are CV values < 10% for samples ≥ 40 µg/g and CV values < 20% for samples < 40 µg/g.

On-board stability
The samples used for the calibration curve stability study were prepared and stored in the same way as the samples for the precision study.
The mean values of each sample on day 0 were used as the baseline value. For each week, a set of samples was thawed and measured in triplicate. The mean values were compared to the baseline value and the recoveries were calculated. The acceptance criterion is 80% to 100% recovery from baseline.

Sample Carry Over
The design of the sample carry over experiment follows the recommendations in the National Committee for Clinical Laboratory Standards (NCCLS) document EP10-A2, Vol. 22, No 29. Preliminary Evaluation of Quantitative Clinical Laboratory Methods.
Three samples at different levels - low, medium and high - ranging from 40 to 1,100 µg/g were aliquoted and stored at -20°C. Samples were thawed and 4 medium, 3 low, and 3 high samples were analyzed in a special sequence during 5 days. A sample carry-over effect of less than 0.6% was accepted.

Method comparison
Fifty-six samples in the range of 35 to 10,408 µg/g (according to the fCAL ELISA) were measured with the BÜHLMANN fCAL® turbo on Mindray BS-200E and the BÜHLMANN fCAL® ELISA. The results were compared according to CLSI EP09-A2 guideline by applying an ordinary least squares (OLS) analysis on the results. The coefficient of determination and slope was investigated.
For the Passing-Bablok linear regression analysis test the Method Validator 1.19 (Metz, France) was used. The BÜHLMANN fCAL® ELISA was the comparative method. Method comparison between the BÜHLMANN fCAL® turbo on Mindray BS-200E and cobas® c111 was performed by analyzing 28 samples in the range 8 to 1,690 µg/g.

RESULTS

Limit of quantitation (LOQ)
Samples at different concentration levels (10 - 25 µg/g) were analyzed during 3 days in 12 replicates. LOQ was determined as the lowest concentration where the total error was below 20%. The total error for cobas® c111 was 14.6% at 20 µg/g and the total error on the Mindray BS-200E was 19.1% at 18.8 µg/g.

Antigen excess
The effects of very high concentrations of fecal calprotectin were analyzed to determine if a high dose hook effect occurs. No antigen excess issues were observed up to fecal calprotectin concentrations of 18,000 µg/g on the Mindray BS-200E and up to 20,000 µg/g on the cobas® c111.

Linearity
Dilution series were prepared on the two instruments. The high samples and low samples for each instrument were assigned and the theoretical value for all levels in the series was calculated. The results listed in Tables 2A (cobas® c111) and 2B (Mindray BS-200E) show that the assay is linear according to the acceptance criterion within the range between LOQ and the highest calibrator.

Precision
The 5-day precision study resulted in a total imprecision of less than 7% for samples between 43 and 1,383 µg/g on the cobas® c111. On the Mindray BS-200E the total imprecision of the 10-day study was observed to be less than 5.5% for all samples ranging from 43 to 1,269 µg/g. For more information about the observed precision see Tables 3A and 3B.

Table 1. Parameter settings Mindray BS-200E and cobas® c111.

Parameter	Mindray BS-200E	cobas® c111
Sample volume (µL)	14	15
R1 volume (µL)	150	100
R2 volume (µL)	25	20
Wavelength (nm)	546	552
Reading time ([cycles]/sec)	1 - 10 [37 - 58]/252	12 - 28 [36 - 55]/152

Table 2A. Linearity study of fecal calprotectin method on cobas® c111.

Fraction of high - %	Observed value - µg/g	Expected value - µg/g	Recovery (%)
100	1964.75	1961	100.2
80	1737.95	1570	110.7
60	1177.00	1179	99.9
40	763.70	787	97.0
20	360.45	396	91.0
10	212.65	201	105.9
5	107.85	103	104.7
2.5	56.45	54	104.4
1.25	29.75	30	100.3
0.625	18.60	17	106.8
0	4.10	5	below LOQ

A high sample was diluted with a low sample to achieve different fecal calprotectin concentrations. The observed values were compared with the expected values, and the recovery was calculated. Acceptance criteria for deviation: < 10%. The linearity is acceptable according to the preset criteria for the cobas® c111 in the entire tested range. LOQ - limit of quantification.

Table 2B. Linearity testing of the fecal calprotectin method on Mindray BS-200E.

Fraction of high - %	Observed value - µg/g	Expected value - µg/g	Recovery (%)
100	1514	1514	100.0
80	1198	1211	98.9
60	864	908	95.1
40	586	606	96.8
20	298	303	98.5
10	153	151	101.1
5	78.5	76	103.3
2.5	40.0	38	105.7
1.25	24.5	19	below LOQ
0.625	19.5	9	below LOQ
0	0.5	0	below LOQ

A high sample was diluted with a low sample to achieve different fecal calprotectin concentrations. The observed values were compared with the expected values and the recovery was calculated. Acceptance criteria for deviation: < 10%. The linearity is acceptable according to the acceptance criteria for the Mindray BS-200E in the entire tested range. LOQ - limit of quantification.

Table 3A. Precision data for the fecal calprotectin method on cobas® c111.

cobas® c501	Sample 1	Sample 2	Sample 3	Sample 4	Low control	High control
n	20	20	20	20	20	20
Mean (µg/g)	43.7	92.8	273.0	1382.6	71.4	252.4
Within run CV (%)	3.86	1.30	0.57	0.98	2.14	0.93
Between day CV (%)	5.27	6.25	1.67	2.26	1.07	0.70
Between run CV (%)	1.99	1.60	0.75	0.78	0.1	1.63
Total CV (%)	6.83	6.58	1.92	2.59	2.39	2.00

CV was calculated for four different sample concentrations and two control levels.

Table 3B. Precision data for the fecal calprotectin method on Mindray BS-200E.

Mindray BS-200E	Sample 1	Sample 2	Sample 3	Sample 4	Low control	High control
n	20	20	20	20	20	20
Mean (µg/g)	43.5	67.5	433	1271	75.0	267.7
Within run CV (%)	4.1	5.8	1.53	0.6	2.9	1.1
Between day CV (%)	1.8	2.0	1.42	3.0	6.3	0.8
Between run CV (%)	2.9	0.5	2.1	2.2	2.0	2.2
Total CV (%)	5.50	3.89	2.92	3.82	7.2	2.6

CV was calculated for four different sample concentrations and two control levels.

On-board stability

The samples measured had an acceptable recovery to baseline after 25 days suggesting an on-board calibration curve stability of at least 25 days for the turbidimetric fecal calprotectin assay on the cobas® c111. Recovery from baseline in the range 99 - 105% implies an on-board stability significantly longer than 25 days. The samples measured at week 10 of the calibration curve stability study on the Mindray BS-200E showed a recovery between 89% and 101%. The observed calibration curve stability duration was 10 weeks in this study.

Sample carry over

No carry over was detected either on the cobas® c111 nor on the Mindray BS-200E.

Method comparison

Method comparison was performed between the BÜHLMANN fCAL® turbo on the Mindray BS-200E and BÜHLMANN fCAL® ELISA. The equation from the Passing Bablok linear regression analysis was: BS-200E = 0.9886 x fCAL ELISA values - 15.5 for samples between 35 µg/g to 10,408 µg/g (Figure 1). We also performed a method comparison between the BÜHLMANN fCAL® turbo on Mindray BS-200E and cobas® c111. There was a strong agreement between the two instruments with a Passing-Bablok fit of -3.963 (95% CI: -14.41 - 11.51) + 0.898 x Mindray BS-200E (95% CI: 0.812 - 0.956), Figure 2.

DISCUSSION

Fecal calprotectin has become a widely used IBD marker that usually is analyzed by ELISA in a microtiter format. The microtiter format favors batch analysis of samples which leads to longer turnaround time compared to random access testing. ELISA testing is also more labor intensive than the majority of assays in chemistry laboratories. The labor cost increases the total costs of analysis. In a recent review comparing the fecal immunochemical test (FIT) with the fecal calprotectin ELISA, a price of USD 180 per sample for a fecal calprotectin ELISA result was compared to USD 20 per sample for the FIT result performed on an automated analyzer [16]. A lower examination cost allows more frequent assessments to be conducted, resulting in closer follow-up of patients and earlier detection of relapses that should lead to a decrease in the number of severe relapses and improved quality of life for the patients.

The BÜHLMANN fCAL® turbo has an assay time of approximately 10 minutes, and, if the instrument has the capability of automatic reruns with a higher dilution, a rerun can be performed within 10 additional minutes expanding the measuring range up to a fecal calprotectin concentration of 20,000 µg/g.

We evaluated the reagents on two different instrument platforms, cobas® c111 and Mindray BS-200E. These instruments are bench-top chemistry analyzers. They

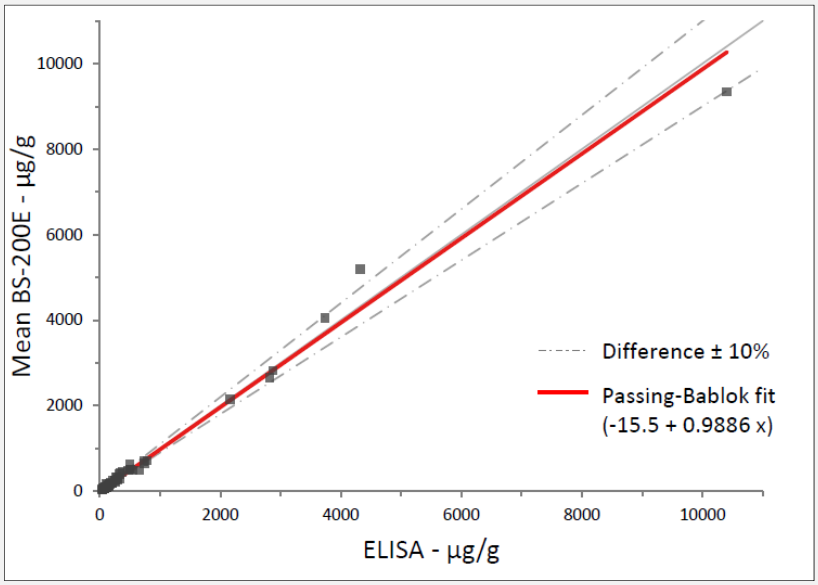

Figure 1. Method comparison between the BÜHLMANN fCAL® ELISA and the BÜHLMANN fCAL® turbo performed on the Mindray BS-200E (n = 56).

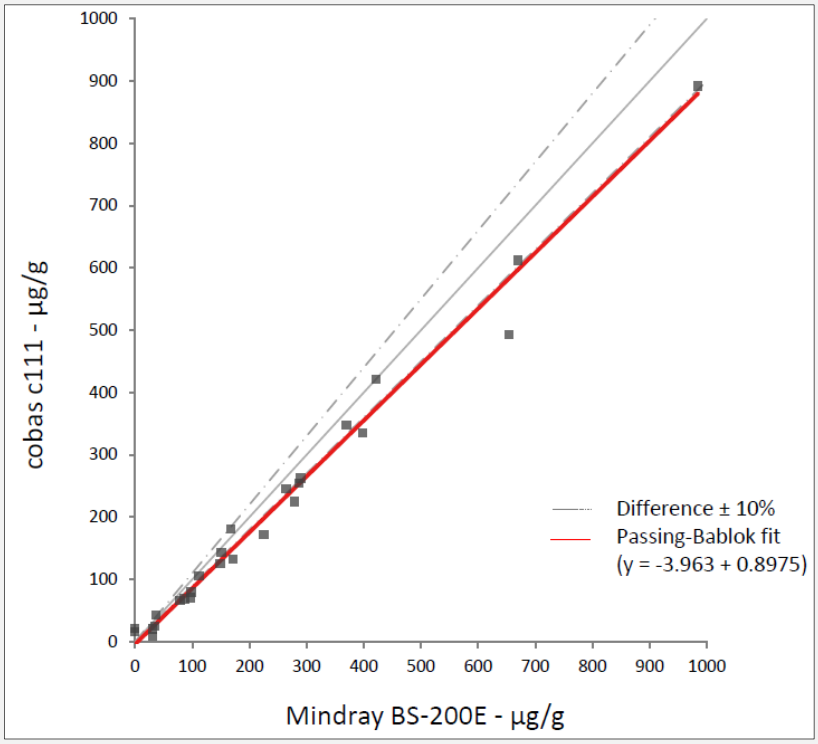

Figure 2. Passing-Bablok agreement between the BÜHLMANN fCAL® turbo performed on the Mindray BS-200E and cobas® c111 (n = 28).

have less capacity than larger instruments but are less expensive to purchase. Even if the assay capacity is lower than for larger chemistry analyzers the capacity is sufficient even for laboratories that have large fecal calprotectin volumes such as 20,000 assays per year. The instruments evaluated in this study could thus be suitable as dedicated instruments for fecal samples for laboratories that do not want to run fecal samples on the same instrument as used for their serum or plasma samples even as many validated protocols for such instruments are available from the manufacturer. The fecal samples are pre-diluted 1:500 with extraction buffer and centrifuged prior to loading the samples onto the instrument. Most laboratories today are running urine samples on their serum platforms without any problems and several of these samples have high bacterial counts due to urinary infections compared to the fecal calprotectin extracts used. In the present study, we did not observe any issues running fecal samples. The study was performed with instruments that were only used for fecal samples during the study. We can thus not exclude the possibility that fecal samples could cause interferences with serum samples and assays.

CONCLUSION

The BÜHLMANN fCAL® turbo is well suited for rapid analysis of fecal calprotectin on Mindray BS-200E and cobas® c111 analyzers providing short turnaround times using bench-top analyzers dedicated for fecal samples. The reagents had good stability and the method had good linearity and good precision on the evaluated instruments. The test results show a high correlation to the BÜHLMANN fCAL® ELISA.

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Declaration of Interest:
Tom Nilsen and Kathrin Sunde are employed by Gentian who developed the turbidimetric immunoassay evaluated in this paper. The authors alone are responsible for the content and writing of the paper.

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