Evaluation of a fluorescent immunochromatography test for fecal calprotectin

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Article:

Abstract

Background: Fecal calprotectin (FC) is widely used to discriminate between patients with inflammatory diseases such as inflammatory bowel disease (IBD) and functional diseases such as irritable bowel syndrome (IBS). ELISA is a time-consuming method for the measurement of FC, whereas a fluorescent immunochromatography test can obtain results in around 30 minutes and thus enables a rapid response to clinical decision.

Methods: Two methods, the Proglead® calprotectin (FC Proglead) and the BÜHLMANN fCAL® ELISA (FC BÜHLMANN), were used to quantitatively examine FC in 111 stool samples. The comparison and bias estimation of both assays were assessed using CLSI EP09c protocol.

Results: The two methods were highly correlated (rho = .96). Deming regression was employed to calculate the regression equation, with a slope of 1.01 and an intercept of −4.98 μg/g. The estimated median bias (FC Proglead – FC BÜHLMANN) was −4.19 μg/g with the 95% limits of agreement (−55.59 to 47.21 μg/g), and the estimated median percent bias was −8.71% with the 95% limits of agreement (−50.31% to 32.90%). There was 4.50% (5/111) of values outside the 95% limits of agreement. Percent biases at the FC cutoff values of 50 and 200 μg/g between both methods evaluated by Deming regression were 8.96% and 1.49%, respectively. The biases were all less than the acceptable standard (10%). And, 99.10% of FC results were in agreement between both methods (κ = .99, P < .001).

Conclusions: FC Proglead may be used as a suitable alternative to FC BÜHLMANN for the disease activity assessment for patients with IBD, considering its convenience and shorter turnaround time.

Keywords:
biomarkers, fecal calprotectin, inflammatory bowel disease, irritable bowel syndrome
1 | INTRODUCTION

Calprotectin, formed as a heterodimer of S100A8 and S100A9, constitutes about 60% of soluble cytosol proteins in human neutrophil granulocytes. Calprotectin is released by granulocyte activation and elevated level of fecal calprotectin (FC) is found in the GI tract inflammation that closely related to inflammatory bowel disease (IBD) activity. Increasing evidence indicates that FC can be used as a noninvasive marker for intestinal/colonic inflammation that helps clinicians distinguish organic inflammatory bowel disease (IBD) from functional irritable bowel syndrome (IBS). Markers of systemic inflammation, such as C-reactive protein (CRP) and white blood cells count, have low specificity and sensitivity for IBD, while the gold standard of ileo-colonoscopy is invasive and expensive. Thus, non-invasive method for monitoring disease activity is preferable. FC is more sensitive than serum CRP in reflecting disease activity in IBD and can be used to identify patients at risk of relapse and predict both endoscopic and histological mucosal healing.

Fecal calprotectin testing in laboratories may require stool samples transportation from outpatient departments to the laboratory. Furthermore, enzyme-linked immunosorbent assay (ELISA) is a time-consuming method for the measurement of FC, with a turnaround time of about 1 to 2 weeks, which may interfere with timely medical treatment and raise the risk of disease deterioration before clinical treatment, whereas a fluorescent immunochromatography test achieves a shorter turnaround time (around 30 minutes) and thus enables a rapid response to clinical decision. The study was aimed to evaluate the consistency of two methods in the determination of FC concentrations.

2 | MATERIALS AND METHODS

2.1 | Sample collection and storage

A total of 111 stool samples were collected from 111 different participants (only one sampling was done from each patient), who were treated in a tertiary hospital, Beijing Tsinghua Changgung Hospital (BTCH, Beijing, China), from September to October 2018. Out of the 111 participants, 99 were confirmed IBD patients and 12 were healthy controls. After collection, the samples were stored in the −20°C refrigerator until tested and frozen and thawed only once. Samples with insufficient size, samples contaminated, and samples without traceable information were excluded. FC concentrations of the 111 stool samples were parallelly detected by the Proglead® calprotectin method (FC Proglead) and the BÜHLMANN ICAL® ELISA method (FC BÜHLMANN).

2.2 | Stool sample extraction

Stool sample extraction could be achieved using two different methods. The first method was a stool weighing-based extraction protocol, and the second method was the use of the stool extraction device. To simplify stool extraction, collected stool samples were all extracted using a commercially available fecal extraction devices (CALEX® Cap Device) before testing for calprotectin by two methods. Briefly, stool sample preparation is diluted with extraction buffer and mixed well. Vortex the CALEX® Cap Device vigorously on a vortex mixer for 30 seconds and let the samples equilibrate for at least 5 minutes. Centrifuged the CALEX® Cap for 5 minutes at 3000 g. Take the supernatant into a fresh, labeled tube and continue with parallel testing by FC Proglead and FC BÜHLMANN methods for each stool sample.

2.3 | Calprotectin measurement

The FC Proglead method (Fujian Proglead Biotechnology Co Ltd) was introduced and used for the quantitative determination of calprotectin in fecal extracts. It was a double-antibody sandwich immunochromatography assay (not yet commercially available). Stool extraction was prepared following procedures described in 2.2 section. The supernatant (90 μL) was added into the sample port of the test card. Incubate at room temperature for 10 minutes. A mouse anti-human detection antibody (Ab) conjugated to fluorescent microsphere can bind to calprotectin in samples, and the antigen-antibody complexes flow to the detection area by the siphon action. The detection area of nitrocellulose membrane in the test card was coated with a mouse anti-human monoclonal capture antibody (mAb) highly specific to the calprotectin. A double-antibody sandwich complex of calprotectin antibody-calprotectin-fluorescent microsphere labeled calprotectin antibody was formed at the detection area. Then, the signal was generated by the fluorescent microsphere under excitation light, and the signal was detected using a matching fluoroimmunoassay instrument (PL-FL-01, Fujian Proglead Biotechnology Co Ltd). A S-shape calibration curve was established using seven calibrators provided in the reagent kit following instructions provided. Calprotectin results were calculated in comparison with the standard curve.

FC BÜHLMANN method was a commercially available ELISA method. Stool extraction prepared following procedures described in 2.2 section was parallelly tested by FC BÜHLMANN method for each stool sample following instructions provided. Briefly, the supernatant was incubated at room temperature onto a 96-well plate coated with a capture antibody. After incubation of 30 minutes, washing, a second incubation of 30 minutes with a detection antibody conjugated to horseradish peroxidase (HRP), and a further washing step, tetramethylbenzidine (blue color formation) followed by a stop solution (change to yellow color) were added. The absorption was determined at an optical density of 450 nm.

The same lot of reagent was used for both methods throughout the study. Two levels of quality control materials were used for quality control for both methods to ensure the reliability of the results.

2.4 | Statistical analysis

Clinical & Laboratory Standards Institute EP09C protocol was implemented for the method comparison and bias estimation of
TABLE 1: Spearman’s rank correlation and Deming regression equation of fecal calprotectin (FC) results by FC Proglead and FC BÜHLMANN methods

| Group               | n   | Spearman’s rank correlation \( \rho \) | \( P \) value | Deming regression equation | Slope (95% CI) | Intercept (95% CI) | \( r \) | \( P \) value |
|---------------------|-----|----------------------------------------|--------------|-----------------------------|----------------|---------------------|-----|-------------|
| Total group         | 111 | .96                                    | <.001        | \( y = -4.98 + 1.01x \)    | 1.01 (0.93 to 1.08) | ~4.98 (~10.87 to 0.91) | .98 | <.001       |
| Low-risk group (FC < 50 \( \mu g/g \)) | 58  | .75                                    | <.001        | \( y = -5.88 + 1.09x \)    | 1.09 (0.87 to 1.31) | ~5.88 (~12.50 to 0.74) | .78 | <.001 |
| Moderate-risk group (50 ≤ FC ≤ 200 \( \mu g/g \)) | 29  | .90                                    | <.001        | \( y = -32.81 + 1.25x \)   | 1.25 (0.99 to 1.50) | ~32.81 (~60.83 to -4.80) | .88 | <.001 |
| High-risk group (FC > 200 \( \mu g/g \)) | 24  | .94                                    | <.001        | \( y = -34.97 + 1.08x \)   | 1.08 (0.82 to 1.33) | ~34.97 (~116.10 to 46.17) | .92 | <.001 |

Note: Deming regression was employed to calculate the slope, intercept, and correlation coefficient. 95% CI, 95% confidence interval. \( \rho \), Spearman’s coefficient of rank correlation.

*The results of FC were not normally distributed, Spearman’s rank correlation was applied to analyze the method correlations for the different groups.

FIGURE 1 Deming regression analysis of fecal calprotectin (FC) concentrations between FC Proglead and FC BÜHLMANN methods. The dashed line displays the identity line, and the solid line displays the Deming regression line. \( N = 111 \), slope = 1.01 (95% CI, 0.93 to 1.08), intercept = -4.98 (95% CI, -10.87 to 0.91) \( \mu g/g \), \( r = .98 \), and \( P < .001 \)

FC concentrations by two methods. It indicated that FC results distribution were skewed leftward, medians were calculated, and biases were evaluated by Wilcoxon signed rank test. And, deming regression was employed to calculate the slope, intercept, and correlation coefficient. Percent biases at FC cutoff values (50 and 200 \( \mu g/g \)) between both methods were determined using three regression models, and percent biases were compared with the acceptable standard (10%, derived from the reagent instruction). All the 111 individuals were grouped into low-, moderate-, or high-risk groups by FC cutoff values of <50, 50 to 200, and >200 \( \mu g/g \), respectively.

A two-tailed \( P \) value of <.05 was judged to be statistically significant. EP evaluator software (version 12.0, Data Innovations LLC) and IBM SPSS Statistics software (version 24, IBM Corp) were employed for statistical analysis.

3 | RESULTS

Fecal calprotectin concentrations of 111 enrolled individuals were parallelly tested by FC Proglead and FC BÜHLMANN methods. The age of 43 women individuals was 57.6 ± 15.2 years and that of 68 men individuals was 56.5 ± 19.0 years. The median of FC concentrations determined by FC Proglead and FC BÜHLMANN method was 47.2 and 48.12 \( \mu g/g \), respectively.

3.1 | Correlation and regression analysis

All individuals were grouped into low-, moderate-, or high-risk groups according to FC concentrations of <50, 50 to 200, and >200 \( \mu g/g \), respectively. The results of FC were not normally distributed; Spearman’s rank correlation analysis was applied to analyze the method correlations. It showed a highly correlation for FC results determined by FC Proglead and FC BÜHLMANN methods for the total (\( \rho = .96 \)), low-risk (FC < 50 \( \mu g/g \) (\( \rho = .75 \)), moderate-risk (50 ≤ FC ≤ 200 \( \mu g/g \) (\( \rho = .90 \)), and high-risk (FC > 200 \( \mu g/g \) (\( \rho = .94 \)) groups, as shown in Table 1.

The regression equation was derived from the Deming regression, with a slope of 1.01 (95% CI, 0.93 to 1.08) and an intercept of -4.98 (95% CI, -10.87 to 0.91) \( \mu g/g \) for the total group;
LI ET AL.

3.2 | Method comparison and bias analysis

Biases between both methods presented a constant coefficient of variation. Referring to CLSI EP09C protocol, bias plots and percent bias plots between FC Proglead and FC BÜHLMANN methods were graphed. The estimated median bias (FC Proglead – FC BÜHLMANN) was $-4.19 \mu g/g$ (95% CI, $-10.68$ to $5.59 \mu g/g$) with the 95% limits of agreement (median ± 1.96 SD, $-55.59$ to $47.21 \mu g/g$) (Figure 2A). The estimated median percent bias [(FC Proglead – FC BÜHLMANN)/FC BÜHLMANN*100%] was $-8.71\%$ (95% CI, $-21.76\%$ to $-11.44\%$) with 95% limits of agreement (−50.31% to 32.90%). There was 4.50% (5/111) of values outside the 95% limits of agreement (median ± 1.96 SD) for percent bias evaluation plots. Right figure represents the distribution of percent bias frequency.

A, Left figure represents bias plot, the X-axis indicates FC concentrations by FC BÜHLMANN method and the Y-axis indicates the median method bias (FC Proglead – FC BÜHLMANN). The black thin dashed line displays the estimated median bias ($-4.19 \mu g/g$, 95% CI, $-10.68$ to $5.59 \mu g/g$). The red thin dashed lines displays the 95% limits of agreement ($-55.59$ to $47.21 \mu g/g$). There is 4.50% (5/111) of values outside the 95% limits of agreement (median ± 1.96 SD) for bias evaluation plots. Right figure represents the distribution of bias frequency. B, Left figure represents percent bias plot, the X-axis indicates FC concentrations by FC BÜHLMANN method and the Y-axis indicates the estimated median percent bias [(FC Proglead – FC BÜHLMANN)/FC BÜHLMANN*100%]. The black thin dashed line displays the estimated median percent bias ($-8.71\%$, 95% CI, $-21.76\%$ to $-11.44\%$). The red thin dashed lines displays the 95% limits of agreement (−50.31% to 32.90%). There is 4.50% (5/111) of values outside the 95% limits of agreement (median ± 1.96 SD) for percent bias evaluation plots. Right figure represents the distribution of bias frequency.
acceptable standard derived from the reagent instruction), as shown in Table 2.

### 3.3 Agreement evaluation

*Kappa* statistic was applied to evaluate the agreement between FC Proglead and FC BÜHLMANN methods. Percentages of low-, moderate-, and high-risk individuals were 52.25% (58/111), 26.13% (29/111), and 21.62% (24/111) for FC BÜHLMANN method, respectively, and that was 52.25% (58/111), 27.03% (30/111), and 20.72% (23/111) for FC Proglead method, respectively. A total of 99.10% (110/111) of the individuals were classified into the same group between both methods (*kappa* = .99, *P* < .001). In comparison with FC BÜHLMANN method, FC Proglead method regrouped 0.90% (1/111) of the individuals into a lower risk group, as shown in Table 3.

### 4 DISCUSSION

Patients with IBD often have increased calprotectin concentrations in blood or feces samples.9,16 FC is used to distinguish IBD from IBS. FC has been analyzed by ELISA methods, such as BÜHLMANN ICAL® ELISA (Bühlmann Laboratories AG).17 Although ELISA method offers accurate quantitative measurements, it is processed in a batch-like procedure (once or twice a week) increasing the turnaround time and requiring high expertise.10 So, we introduced the Proglead® fecal calprotectin testing kit (FC Proglead). It is easy to operate and can save hours, with a turnaround time of about 30 minutes.

A comparison between FC concentrations determined by different assays is required in order to determine their diagnostic consistence. Laboratories should be aware of the problem with varying calibrations and assay standardization.10 Jonas Halfvarson et al18 reported that the FC BÜHLMANN method produced higher concentrations of FC compared with the Phadia assay and especially with the Immundagnostik assay. Whitehead et al19 reported 3.8 times higher FC concentrations with BÜHLMANN Quantum Blue® than with Immundagnostik PhiCal® and Eurospital assays in anonymized surplus stool samples. The stool extraction step was required prior to analysis. Commonly, weighing-based extraction procedures are considered as a gold standard for calprotectin extraction when compared to commercial sample extraction devices. S J Whitehead et al19 reported that different extraction procedures contributed to the overall imprecision of the calprotectin assay and

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**TABLE 2** Bias evaluation of fecal calprotectin (FC) between FC Proglead and FC BÜHLMANN methods at FC cutoff values

| Regression models          | Regression equations | cutoff values (μg/g) | Predicted values (μg/g) | Biases (μg/g) | Percent biases (%) | Acceptable standard (%) |
|----------------------------|----------------------|----------------------|-------------------------|--------------|--------------------|-----------------------|
| Deming regression          | *y* = −4.98 + 1.01x  | 50                   | 45.52                   | 4.48         | 8.96               | ±10                   |
|                            |                      | 200                  | 197.02                  | 2.98         | 1.49               | ±10                   |
| Passing-Bablok regression  | *y* = −2.58 + 0.97x  | 50                   | 45.92                   | 4.08         | 8.16               | ±10                   |
|                            |                      | 200                  | 191.42                  | 8.58         | 4.29               | ±10                   |
| Ordinary Linear regression | *y* = −3.02 + 0.99x  | 50                   | 46.48                   | 3.52         | 7.04               | ±10                   |
|                            |                      | 200                  | 194.98                  | 5.02         | 2.51               | ±10                   |

Note: Percent biases at the FC cutoff values of 50 and 200 μg/g between both methods were estimated using three different regression models. It showed that percent biases were all less than 10% (the acceptable standard derived from the reagent instruction).

**TABLE 3** Grouping of the individuals by fecal calprotectin (FC) concentrations of FC Proglead and FC BÜHLMANN methods

| FC Proglead (μg/g) | FC BÜHLMANN (μg/g) |
|-------------------|-------------------|
|                   | Low-risk          | Moderate-risk      | High-risk          | Total |
|                   | (FC < 50 μg/g)    | (50 ≤ FC ≤ 200 μg/g) | (FC > 200 μg/g)   |       |
| Low-risk (FC < 50 03bcg/g) | 58 | 0 | 0 | 58 |
| Moderate-risk (50 ≤ FC ≤ 200 μg/g) | 0 | 29 | 1 | 30 |
| High-risk (FC > 200 μg/g) | 0 | 0 | 23 | 23 |
| Total             | 58 | 29 | 24 | 111 |

Note: The values were displayed as the numbers of individuals classified into the same group by fecal calprotectin (FC) concentrations of both methods. It indicated that 99.10% (110/111) of the individuals were classified into the same group (*kappa* = .99, *P* < .001). In comparison with FC BÜHLMANN method, FC Proglead method regrouped 0.90% (1/111) of the participants into a lower risk group.
comparison between methods. So, we used the same method to prepare the stool extraction prior to calprotectin measurement by both methods.

This study showed that FC concentrations measured by FC Proglead and FC BÜHLMANN method were highly correlated in analytical measurement range of 10-600 µg/g (rho = .96), with a slope of 1.01 and an intercept of -4.98 µg/g. Taking FC BÜHLMANN as the reference method, the estimated median bias and percent bias between two methods (FC Proglead – FC BÜHLMANN) were -4.19 µg/g and -8.71%, respectively. These biases were acceptable in clinical practice. Less than 5% of the points was outside the 95% limits of agreement. Different FC cutoff values to distinguish IBD from IBS have been reported. FC cutoff values were reported in the range of 150-250 µg/g. Percent bias at FC cutoff values of 50 and 200 µg/g between both methods was simultaneously estimated using three regression models. These differences were all less than the acceptable standard (10%). The agreement study demonstrated that 99.10% (110/111) of the individuals were classified into the same group between both methods (kappa = .99, P < .001).

Overall, this study proposes that FC results of FC Proglead are in good agreement with that of FC BÜHLMANN and FC Proglead may be used as a suitable alternative to FC BÜHLMANN for the gastrointestinal inflammation activity assessment for patients with IBD. FC Proglead method could significantly shorten the reporting turnaround time and enable timely response to patients, thus potentially improve treatment quality.

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ETHICAL APPROVAL

The study was approved by the Institutional Review Board of Beijing Tsinghua Changgung Hospital (approval number 18116), and the requirement for informed consent was waived.

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