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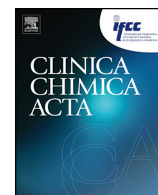

A novel sol particle immunoassay for fecal calprotectin in inflammatory bowel disease patients

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ABSTRACT

Background: We introduce a new assay method to measure the concentration of fecal calprotectin that can be applied in exclusive analyzers. The assay method uses gold colloidal reagents. In addition, we report performance evaluation results for the new method and the results of comparisons with enzyme-linked immunosorbent assay (ELISA) methods.

Methods: We evaluated the new method by linearity tests and within-run tests. In addition, we collected specimens from patients with a definitive diagnosis of inflammatory bowel disease ($n = 566$) and examined them using the new method. The results were compared with those from 2 commercially available ELISA kits.

Results: In the linearity tests, the correlation coefficients between the measured values and the theoretical values were 0.9980–0.9990. In the within-run tests, the CVs were 3.4–4.3%. The correlation coefficients for our method and the 2 ELISA kits showed high correlations of 0.945 and 0.942.

Conclusions: Our assay is capable of measuring calprotectin concentrations in feces, and has a similar performance to commercially available ELISA methods. Our method is an automated assay system, which is an easier, cheaper, and quicker measurement method than conventional ELISA kits. Therefore, our assay is suitable for daily clinical use.

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1. Introduction

Inflammatory bowel disease (IBD) is an unexplained chronic disease that causes inflammation or ulceration of the mucosa of the digestive tract. IBD includes Crohn's disease (CD) and ulcerative colitis (UC). In CD, there is a possibility that inflammation and ulceration may occur in any part of the gastrointestinal tract from the mouth to the anus. In UC, there are erosions and ulcers on the lining of the large intestine. The characteristic symptoms of the diseases include abdominal pain, diarrhea, and bloody stools.

Endoscopy is used in the diagnosis of IBD, and the active and remission phases are determined using the endoscopic images. Although endoscopic evaluation is necessary for confirmation of the mucosal status, colonoscopy is invasive and burdensome for patients [1]. In addition, there is a report that the symptoms of IBD can be exacerbated by the preparation required for endoscopy or by the endoscopy itself [2]. Therefore, noninvasive methods for evaluating and predicting the mucosal status are highly anticipated. Recently, some studies have focused on fecal calprotectin (Cp) in IBD and confirmed the utility of Cp in diagnoses, disease activity evaluations, medication effect evaluations, and relapse monitoring [3–7].

Cp is a 36-kDa calcium- and zinc-binding protein that represents 60% of the cytosolic protein in granulocytes [8–11]. It is a heterodimer composed of S100A8 (MRP8, calgranulin A) and S100A9 (MRP14, calgranulin B), and was first discovered and described in 1980 [12]. The amount of Cp in feces is proportional to the amount of neutrophil migration from the inflamed bowel wall to the mucosa. Cp is stable in feces for up to 7 days at room temperature [13].

Several different assays for measuring fecal Cp have been developed [14,15]. Recently, several enzyme-linked immunosorbent assay (ELISA) kits and point of care test (POCT) kits have become commercially available [16–18]. For the ELISA kit, patients collect a fecal sample directly and provide the feces to the laboratory. The samples are stored frozen until analysis. The preparation of samples is not easy and the measuring time also takes an hour or longer. With these kits, it is difficult to measure a large number of specimens at the same time and to measure each specimen separately. However, this method is highly specific and the quantitative performance is good. For the POCT kit, patients collect a fecal sample directly; however, a smaller amount of feces is necessary compared with the ELISA kit. After collection, the feces are then provided to the laboratory. The preparation of samples is not easy; however,

Abbreviations: Cp, calprotectin; IBD, inflammatory bowel disease; FIT, fecal immunochemical test; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; BSA, bovine serum albumin; CD, Crohn's disease; UC, ulcerative colitis; CDAI, Crohn's disease activity index; SPIA, sol particle immunoassay.

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Table 1
Patient characteristics.

	CD ($n = 255$)	UC ($n = 311$)
Median (interquartile range) age at entry	34 (16) years	41 (27) years
Males:females (n)	171:84	179:132
Disease activity		
Active	74 (29%)	94 (30%)
Remission	181 (71%)	217 (70%)
Location of CD (n)		
Small bowel	95 (37%)	
Large bowel	30 (12%)	
Small and large bowels	130 (51%)	
Extent of UC (n)		
Rectum		58 (19%)
Left-sided colitis		144 (46%)
Extensive colitis[a]		109 (35%)

CD, Crohn's disease; UC, ulcerative colitis.
[a] Involvement extended proximal to the splenic flexure.

the measuring time is approximately 10 to 30 min. With these kits, it is difficult to measure a large number of specimens at the same time. However, specimens can be measured separately. Majority of the kits provide semi-quantitative results; however, quantitative results through a dedicated reader can be obtained using a few of the kits.

Therefore, we developed another assay method for fecal Cp, which is quicker and easier and can be suitable for daily clinical use. Patients prepared fecal samples at home using a dedicated sample container; therefore, there was no need to prepare a sample in the laboratory. The assay is based on the sol particle immunoassay principle. The assay system has been used for measuring serum cystatin C [19] and a tumor marker in urine [20]. The assay can produce results in about 10 min.

2. Materials and methods

2.1. Materials

2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was from Dojindo Laboratories. Bovine serum albumin FAF (fatty acid-free) grade (BSA) was from Nacalai Tesque. A mouse anti-human Cp monoclonal antibody was from Mikuri Immunology Laboratory. Human neutrophil Cp was from Arotec Diagnostics. FIT NS-Prime Specimen Diluent and colloidal gold particles were obtained from Alfresa Pharma.

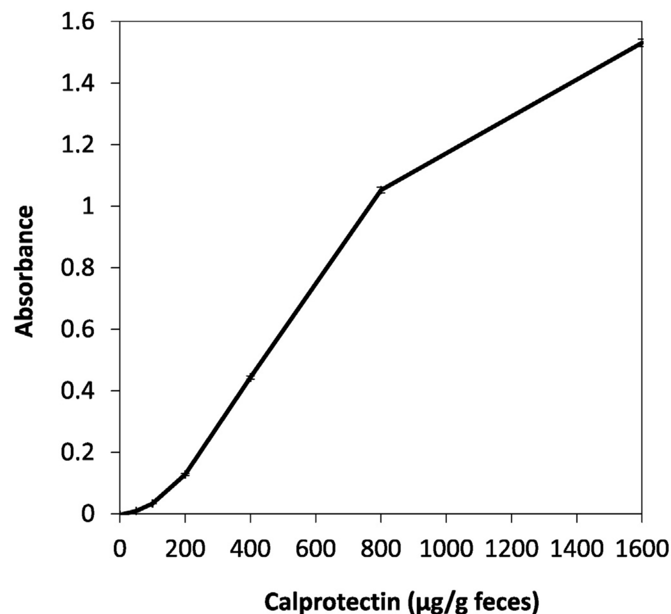

Fig. 1. Calibration curve for the determination of Cp obtained by Cp-SPIA. The absolute values of the absorbance changes for seven different known concentrations of Cp were measured. The mean values ± SD were plotted on the y-axis vs. the Cp concentrations on the x-axis. The calibration curve was in the shape of a sigmoid curve.

2.2. Assay reagents

Colloidal gold particles were prepared as described by De Roe et al. [21]. The colloidal gold particles were 50 nm in size. The particles were coated with the mouse anti-human Cp antibody by incubating the particle-containing solution (final concentration: 4 mg/l) for 2 h at 4 °C. BSA was then added to obtain a final concentration of 0.5 g/l and was incubated overnight at 4 °C. The solution of coated particles was centrifuged at 12,000 ×g for 40 min. The pellet was resuspended in HEPES containing 1 g/l BSA and 30 g/l mannitol, and adjusted with the same buffer solution to an $A_{540\ nm}$ value of 10. HEPES buffer (0.1 mol/l, pH 7.0) containing 10 g/l NaCl and 20 g/l polyethylene glycol 20,000 was used as the reaction buffer.

2.3. Calibrator

FIT NS-Prime Specimen Diluent was used for the dilution of samples and as the zero standard solution for our assay. The standard solution was prepared by diluting highly pure Cp from human neutrophils with the same dilution buffer (8000 μg/l). The standard solution was assigned a Cp concentration of 1600 μg Cp/g of feces, considering that the fecal concentration in our sample container is 5 mg/ml. By simply setting the standard solution and dilution buffer on the device, a standard curve was created through an automatic dilution (50, 100, 200, 400, 800, and 1600 μg Cp/g feces).

2.4. Patients

This study was approved by Yokkaichi Hazu Medical Center's Institution Ethics Review Board for human studies and patients signed an informed consent form. The assay was evaluated using samples from 255 CD patients and 311 UC patients treated at Yokkaichi Hazu Medical Center. For both CD and UC, the patients were classified by the lesion site, which was confirmed by endoscopic findings. All patients had an established diagnosis of IBD according to endoscopic and histologic assessments of their clinical symptoms. An outline of the clinical backgrounds of a CD patient and a UC patient are shown in Table 1. In CD, clinical activity was evaluated using the CD Activity Index (CDAI) [22]. Clinical active status was defined as CDAI ≥ 150, and clinical remission was defined as CDAI < 150. In UC, active phase was defined as bloody stools and endoscopically active lesions (absent vascular pattern, hemorrhages, erosions, and ulcerations), and remission phase was defined as disappearance of bloody stool and absent findings of endoscopic active phase [23].

2.5. Fecal sampling and a procedure

The patients prepared fecal samples at home using the sample container A (Alfresa Pharma Corp.) provided by the manufacturer. The patients rubbed the probe in several different areas of the stool, returned the probe to the tube, and tightly sealed the tube. Upon replacing the probe into the tube, excess stool was removed, meaning only 10 mg entered the container. Because the tube contained a buffer solution, the feces was suspended in the liquid at a concentration of 5 mg/ml. Cp is stable in sample container A for 7 days at room temperature. The reduction in Cp concentration after 3 days and after 7 days was 20% and 30%, respectively. In this study, patients submitted specimens at the time of their visit or within 3 days of the visit. The samples were stored frozen at −30 °C until measurement.

Measurements of Cp were carried out using our reagent and a commercially available FIT NS-Prime automatic analyzer (Alfresa Pharma Corp.). We used the following protocol, which is suitable for NS-Prime.

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