Evaluation of a New Technique for iFOBT Utilising a New Sample Collection Device with Increased Buffer Stability

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Abstract. *Aims: The aims of the present study were: (i) Evaluate specificity and sensitivity of Hb Smart enzyme-linked immunosorbent assay (ELISA) (ScheBo Biotech) compared to colonoscopy results and (ii) assess stability of a new sample collection device containing a newly formulated buffer to extract haemoglobin using buffer and stool samples spiked with defined concentrations of haemoglobin. Materials and Methods: Stool samples were quantified with the ELISA method. The stability of haemoglobin in the extraction buffer and in native stool samples, respectively, was determined daily by ELISA during storage for 5 days at 4°C and at room temperature after addition of haemoglobin. Results: Haemoglobin ELISA had a sensitivity of 78.4% for detection of CRC with a specificity of 98%. Haemoglobin extracted in corresponding extraction buffer demonstrated stability throughout storage for 5 days at 4°C and at room temperature. Conclusion: Hb Smart represents a very promising tool for large-scale screening of CRC with regard to sample handling, stability and analysis of haemoglobin in faeces.*

Colorectal cancer (CRC) has a major impact on public health and public health costs. It is the most common cancer with worldwide 1.23 million cases diagnosed annually, and more than 600,000 deaths from this disease (1-3). In its early stages, CRC has a good prognosis. Several screening methods have been shown to be effective in reducing mortality from CRC (4-7). Colonoscopy is currently regarded as gold standard for CRC screening. However, its acceptance by the general population is very poor, costs are high and availability is low, despite inclusion in screening programs in the German health system for over 10 years. Therefore, there is great interest in evaluating additional or alternative screening tools. Guaiac-based or immunochemical faecal occult blood testing (iFOBT),

genetic stool tests by DNA and the metabolic enzyme M2-PK are some existing stool tests for CRC screening.

The M2-PK has been identified as a key metabolic enzyme in CRCs and polyps. The dimeric isoform of M2-PK exists completely independently from the presence of faecal occult blood and is specific for a change in glucose metabolism, which occurs in polyps and tumours (direct testing) (11, 12).

iFOBT for the detection of human blood relies on the presence of a source of bleeding in the bowel (indirect method). iFOBT, which exclusively uses antibodies specifically targeted at human haemoglobin, has been clinically proven to detect more bleeding-associated CRCs and polyps than traditional guaiac-based FOBTs. Its superior specificity for lower gastrointestinal bleeding compared to guaiac-based FOBT reduces the number of false-positive test results, making it an improved screening tool for the presence of human haemoglobin from bleeding CRCs or polyps in faeces (3, 8-10). Immunological FOBT is increasingly being chosen for cost-effective large-scale CRC screening (13, 14). iFOBT might replace guaiac-based FOBT completely in clinical laboratory practice (15, 16).

The result of iFOBT is not affected by food. However, haemoglobin in faeces can be degraded within a few days, bringing its concentration below the cut-off limit if samples require storage for several days. Therefore, an efficient method for stabilization and quantification of haemoglobin from faeces is necessary (17).

Our study aimed to quantify haemoglobin concentrations in stool samples of individuals who underwent complete colonoscopy, using Hb Smart enzyme-linked immunosorbent assay (ELISA) of ScheBo Biotech. Furthermore, we analysed the stability of haemoglobin in a new extraction buffer by the use of defined concentrations of haemoglobin.

Materials and Methods

Reagents. Human haemoglobin was purchased from Sigma-Aldrich (Dreisenhofen, Germany). The extraction system filled with ready-to-use extraction buffer and the Hb Smart iFOBT ELISA tests were provided by ScheBo Biotech AG (Giessen, Germany).

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Faecal sampling and preparation method. A total of 156 stool samples from male and female patients undergoing colonoscopy aged between 40-80 years (median=62) were collected in plastic containers and kept frozen at −20°C. Samples were thawed before performing the haemoglobin ELISA.

The Master Quick-Prep sample preparation system was used to extract haemoglobin from the stool samples. It is a new sample collection device containing a newly formulated buffer to extract stool samples. The tube contains ready-to-use extraction buffer, a dosing tip and a cone. The dosing tip is inserted into the thawed stool sample until all notches are filled with faeces. It is then inserted into the tube through the cone to yield an exact mass of stool, with a coefficient of variation of 2.7%. The analyte is extracted from the stool sample into the buffer for 10 minutes at room temperature.

Haemoglobin quantification by iFOBT. Haemoglobin quantification was performed with the commercially available ELISA kit (ScheBo Biotech AG), according to the procedure described in the manufacturer's instruction manual. The test uses monoclonal antibodies directed against human haemoglobin which do not cross-react with haemoglobin of animal origin. Haemoglobin concentration is determined within the range of 15-60 μg haemoglobin/g stool *via* a standard curve. The manufacturer's stated cut-off value for a positive test result is ≥25 μg haemoglobin/g stool. After stool extraction with Master Quick-Prep buffer, extract supernatant was pipetted into the wells of the ELISA plate. The plates were incubated for 1 hour, followed by washing and pipetting of anti-Hb-bio POD-streptavidin complex into each well for 30 minutes' incubation. After washing the plate, a colour reaction was generated by the addition of tetramethylbenzidine. After 15 min, the reaction was stopped by adding stop solution. The optical density at 450 nm, with 620 nm as a reference wavelength, was then measured. The concentration of samples was evaluated by optical density on a log-log scale using a standard curve.

Stability test. The ready-to-use extraction buffer was spiked with haemoglobin in concentrations from 2-60 μg haemoglobin/g and each sample was vigorously shaken. The samples were then stored at 4°C and at room temperature at 20-25°C. The concentration of haemoglobin in each sample was then quantified every day for 5 days *via* ELISA.

Furthermore, faecal samples from healthy controls undergoing colonoscopy were tested to confirm absence of haemoglobin. The stool samples were extracted in extraction buffer and spiked with haemoglobin concentrations from 5-60 μg haemoglobin/g. The samples were stored at 4°C and at room temperature (20-25°C). They were also tested every day for 5 days using the Hb Smart ELISA.

In addition, stool samples from patients with CRC confirmed by colonoscopy, and healthy individuals as controls, were thawed and extracted in buffer. They were then stored at 4°C and room temperature (20-25°C) for 5 days and haemoglobin concentrations were quantified with the iFOBT ELISA to assess the stability of haemoglobin once extracted into the buffer.

Results

Two distinct sets of experiments were performed: the first evaluated the sensitivity and specificity of Hb Smart ELISA compared to colonoscopy results. The second experiment tested the stability of known haemoglobin concentration
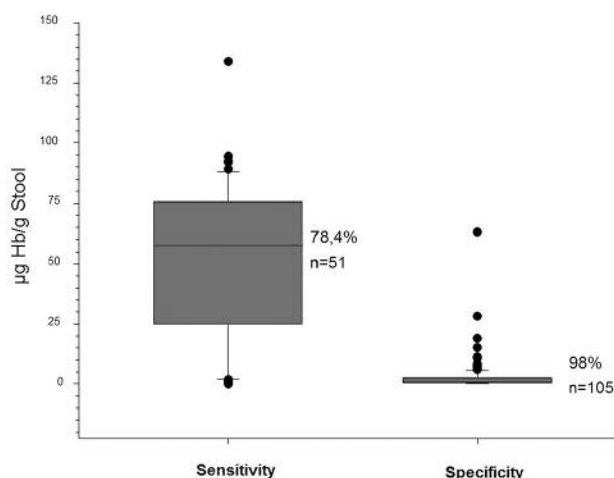

Figure 1. *Sensitivity and specificity in patients who underwent total colonoscopy, n=156. A total of 51 stool samples were analyzed from patients with proven colorectal cancer using the manufacturer's cut-off of ≥25 μg haemoglobin/g stool (by Hb Smart ELISA). Overall, 78.4% of samples had a haemoglobin concentration ≥25 μg/g stool. No haemoglobin was detected in 11 (21.6%) stool samples. Sensitivity was therefore 78.4%. A total of 105 stool samples from individuals without CRC were analysed to determine specificity of Hb Smart ELISA. Overall, 2% of the stool samples assayed had a haemoglobin concentration higher than 25 μg/g stool. The specificity was therefore 98%.*

using the iFOBT ELISA dosing device extraction buffer at 4°C and room temperature (20-25°C). In addition, the stability of stool samples spiked with different concentrations of haemoglobin which were extracted in the extraction buffer and analyzed with the Hb ELISA was tested.

Sensitivity and specificity. We analysed 156 stool samples from males and females aged between 40-80 years (median=62 years). All stool samples were from individuals who subsequently underwent colonoscopy (2, 18). The stool-filled containers were stored at −20°C upon arrival at the laboratory and were thawed for about 24 h at 4°C before assaying. The thawed stool samples were extracted with Master Quick-Prep to ensure consistent stool weight and buffer volume for every sample. Haemoglobin quantification by ELISA was performed automatically on a DSX auto-analyser (Dynex Technologies, Denkendorf, Germany).

In total, 51 of the analyzed stool samples were from patients in whom CRC was found at colonoscopy. After colonoscopy, the haemoglobin concentration was quantified in the respective stool samples. The manufacturer's specified cut-off value was applied to categorize the results. Values ≥25 μg/g were classified as elevated (positive). A total of 78.4% of analyzed samples had a haemoglobin concentration ≥25 μg/g stool, whereas 11 (21.6%) stool samples did not have raised haemoglobin concentrations (Figure 1).
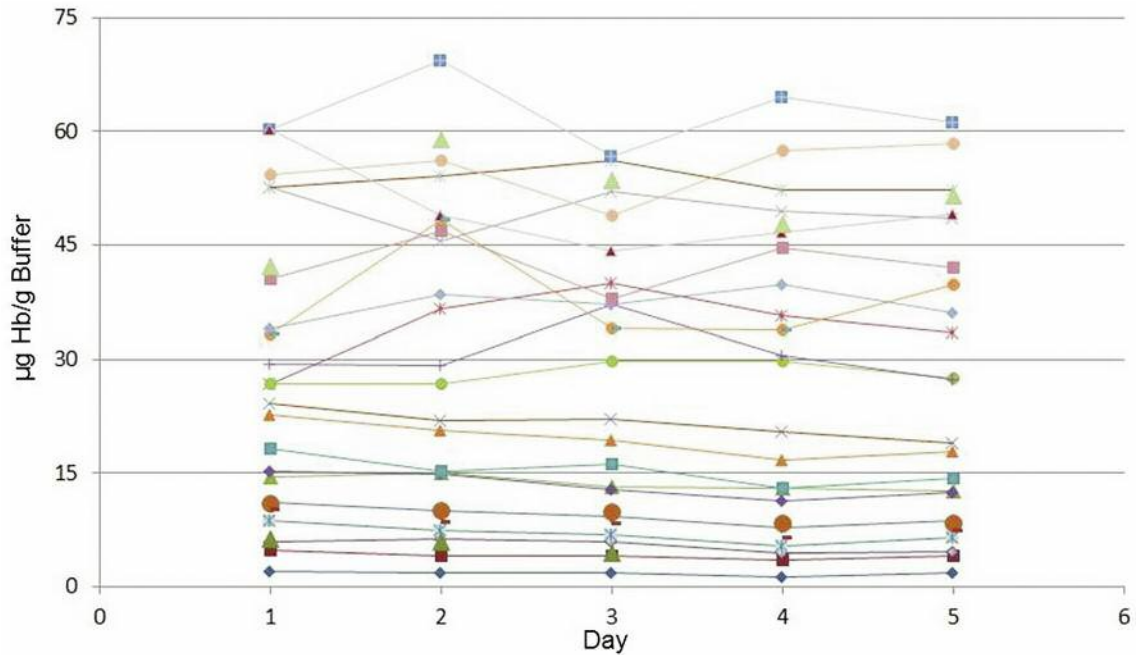
Figure 2. *Stability study: 5 days at room temperature - extraction buffer spiked with haemoglobin. Master Quick prep buffer of the Hb Smart kit was spiked with human haemoglobin in a range from 5-60 μg haemoglobin/g. The spiked samples were stored at room temperature and quantified every day for 5 days by Hb Smart ELISA. All of the individual spiked samples showed only small variations during storage at room temperature for 5 days. The haemoglobin concentration did not decrease below the cut-off for any of the haemoglobin-positive sample extracts nor did any negative extracts become positive after 5 days' storage at room temperature.*
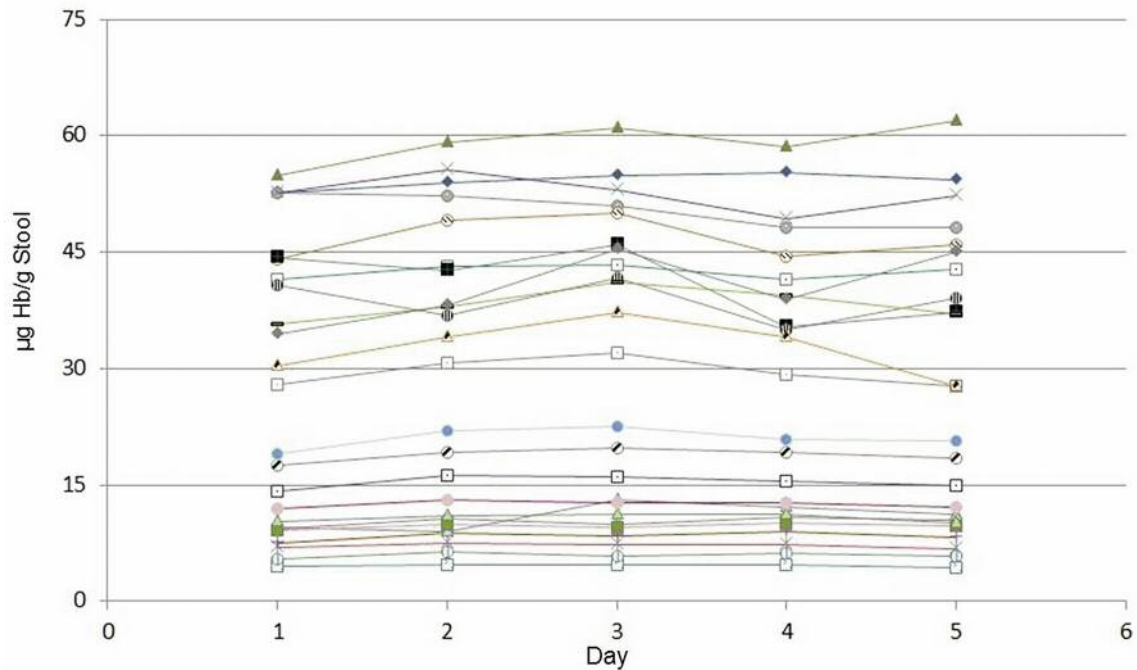

Figure 3. *Stability study: 5 days at room temperature – stool sample spiked with haemoglobin. Extracted faecal samples from healthy individuals were spiked with defined concentrations of haemoglobin in a range from 5-60 μg/g stool. The spiked samples were stored at room temperature and quantified every day for 5 days by Hb Smart ELISA. The variation of the haemoglobin concentration between day 1 and day 5 at 4°C and at room temperature was not significant; the median variation was 5%. None of the samples changed from a positive to a negative result during storage, nor switched from below to above the cut-off concentration.*

In order to calculate specificity for the healthy control group, we analysed 105 stool samples from asymptomatic participants without CRC or other medical disorders at screening colonoscopy. Overall, 98% of samples had a haemoglobin concentration <25 μg/g stool, whereas 2% of the 105 stool samples had a haemoglobin concentration ≥25 μg/g stool (Figure 1).

Therefore we found Hb Smart ELISA has a sensitivity of 78.4% for CRC with a specificity of 98%.

Stability of haemoglobin. The extraction buffer for iFOBT analysis must fulfil a number of important criteria to be acceptable for routine laboratory use. Since haemoglobin is easily degraded at room temperature, there is a substantial risk that an elevated faecal haemoglobin concentration may fall below the cut-off limit within a few days unless a stabilizing agent is added. Therefore, an extraction buffer containing a haemoglobin stabilizer is needed in order to extract reliable results. We assessed a new extraction buffer in sample collection tubes to determine the resultant stability of haemoglobin in faecal samples during storage at different temperatures. In addition, we aimed to test the stability of defined concentrations of haemoglobin in the extraction buffer and of extracted faecal samples in regard to haemoglobin concentration, time and temperature. Therefore, the extraction buffer used for Hb Smart ELISA was spiked with human haemoglobin in a range from 2-60 μg/g. The spiked extraction buffer was stored at 4°C (data not shown) and at room temperature (Figure 2) and analysed by Hb Smart ELISA on five consecutive days.

The analysis of the daily measurements of the spiked extraction buffer stored for 5 days at 4°C revealed a non-significant variation in haemoglobin concentration of 11%. Interestingly, buffer stored at room temperature revealed the same average variation between the concentrations of spiked haemoglobin buffer measured on day 1 compared to day 5. The spiked extraction buffer showed only small variations around the cut-off of 25 μg haemoglobin/g when stored at either room temperature or 4°C. The haemoglobin concentration did not decrease below the cut-off for any of the haemoglobin-positive sample extracts nor did any negative extracts become positive after 5 days' storage at 4°C or at room temperature (Figure 2).

In a second step, extracted faecal samples from healthy individuals were spiked with a defined concentration of haemoglobin. After confirming the initial absence of haemoglobin in the sample by ELISA, haemoglobin was added to the stool extracts in defined concentrations in a range from 5-60 μg/g. The stool extracts were stored at 4°C and at room temperature and the haemoglobin concentration was analysed daily for five consecutive days (Figure 3). The variation of the haemoglobin concentration between day 1 and day 5 at 4°C and at room temperature was not significant;
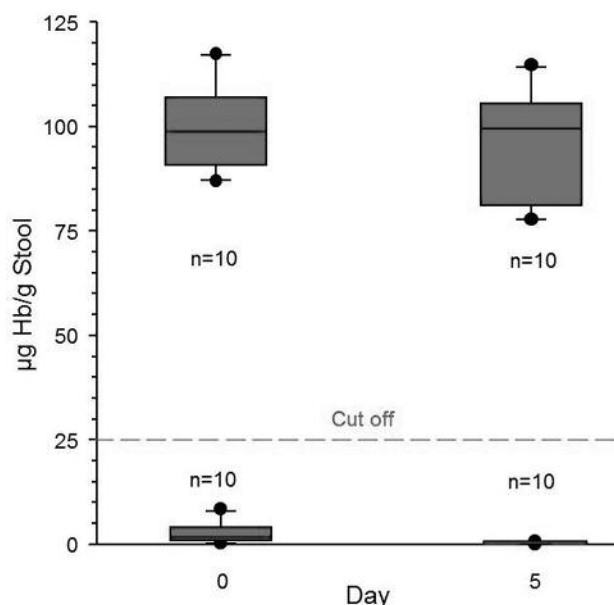

Figure 4. *Haemoglobin concentration in native stool samples from patients with colorectal cancer (n=10) and healthy controls (n=10) who underwent colonoscopy, after 5 days' storage at room temperature. The stool samples were extracted in buffer, analysed by Hb Smart ELISA and stored at room temperature. After 5 days, the stool extracts were assayed by iFOBT. No significant variations were found in haemoglobin concentrations. None of the samples exhibited a change in haemoglobin concentration that resulted in a false-negative or false-positive result after 5 days storage at room temperature.*

the median variation was 5%. None of the samples changed from a positive to a negative result during storage, nor switched from below to above the cut-off concentration.

As a third step, stool samples from patients with CRC (n=10) and healthy controls (n=10) were freshly extracted and haemoglobin concentrations were quantified. The stool extracts of both groups were then stored at 4°C and room temperature. After 5 days, the extracts were analysed again *via* ELISA. No significant variations in haemoglobin concentrations were found, neither in the extracted stool samples from those with CRC nor in the healthy control group. None of the samples exhibited a change in haemoglobin concentration that resulted in a false-negative or false-positive result (Figure 4).

In summary, haemoglobin extracted into Master Quick Prep buffer demonstrated consistent haemoglobin concentration during 5 days' storage at either 4°C or at room temperature.

Discussion

Colorectal cancer (CRC) is an important public health problem. Colonoscopy is currently generally regarded as the best screening tool for CRC. The guaiac-based FOBT

has been historically often recommended as a screening test for CRC, with a high specificity but a low sensitivity. However, the test needs special dietary restriction and 3-day collection of faeces. Quantitative and semi-quantitative iFOBT are becoming increasingly accepted as CRC screening tools (5, 16, 17, 19-23). They can be effective for the prevention of advanced CRC and reduce the risk of developing fatal CRC by 23.0% to 60.0% (24). The costs arising from CRC might be substantially reduced when iFOBT is used in place of a guaiac-based test for population screening (1). iFOBT screening is more cost-effective and influences colonoscopy referral and detection rates. It is highly likely that more people will participate in CRC screening programs using iFOBT as a primary screening method than those who are willing to undergo initial investigation by colonoscopy (17, 22).

This study demonstrates the merit of quantifying haemoglobin in faeces with Hb Smart ELISA, which is an accurate and reliable screening tool for CRC with a sensitivity of 78.4% and a specificity of 98%. The cut-off value of ≥25 μg haemoglobin/g stool is compatible with the published literature (7, 14, 25, 26).

It may take 3 to 5 days for sample collection and transport by mail or personally to the medical scientist prior to laboratory quantification of haemoglobin. However, haemoglobin in faeces is very unstable and, without a stabilizing agent, haemoglobin concentrations can decrease to below the cut-off value within a few days. Therefore, a buffer which stabilizes haemoglobin in faeces is necessary (27).

Here we tested a new buffer in conjunction with the Hb Smart iFOBT from ScheBo Biotech AG for sample stability under conditions designed to simulate routine laboratory practice and including pre-analytical sample collection and transport. The extraction buffer and collected stool samples were spiked with defined different haemoglobin concentrations. In addition, native faeces from patients with CRC at colonoscopy were analysed for haemoglobin stability during 5 days' storage at 4°C and at room temperature. In summary, measurement of haemoglobin extracted in Master Quick-Prep buffer revealed that concentrations remained consistent over 5 days' storage at either at 4°C or at room temperature.

Such CRC testing relies upon the detection of human blood originating from a source of bleeding in the bowel. Therefore, for comprehensive CRC screening, detection of non-bleeding cancers and polyps, for example by M2-PK ELISA or DNA testing, is also required.

Considering current guidelines and published literature the Hb Smart ELISA and its corresponding extraction buffer is a very promising tool for large-scale screening of CRC with regard to sample handling, stability and analysis of haemoglobin in faeces.

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