Evaluation of a desk top instrument for the automated development and immunochemical quantification of fecal occult blood

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Summary

Background:
The guaiac fecal occult blood test (FOBT) for colorectal cancer (CRC) screening is user dependent and not specific for human hemoglobin (Hb). The automated-developed, quantitative, immunochemical human Hb FOBT (I-FOBT) is specific, allows for quality control and selection of a suitable Hb level, with optimal sensitivity and specificity, for colonoscopy.

Material/Methods:
We evaluated a desktop instrument, OC-MICRO™ (Eiken, Japan), which automatically develops and quantifies 50 fecal tests/hr for Hb; for ease of use, test reproducibility and stability and intra-patient daily I-FOBT variation; clinical evaluation included sensitivity and specificity for neoplasia in patients undergoing colonoscopy.

Results:
Five hundred patients prepared 3 fecal tests which were quantified for Hb, I-FOBT samples were: (1) repeatedly re-examined; (2) stored at 4°C or 20°C or 28°C and re-examined; (3) I-FOBT levels correlated with colonoscopic findings. Five I-FOBTs re-examined 6 times had no significant changes; 30 tests stored ≥21 days had a decay/day of: 0.3%±0.4 at 4°C (NS), 2.2%±1.7 at 20°C (NS) and 3.7%±1.8 at 28°C (P<0.05). Receiver operating characteristic curve analysis showed that at the 100 ng Hb/mL I-FOBT level 76.5% of CRCs and advanced adenomas were detected with a specificity of 95.3%.

Conclusions:
The instrument provided reproducible results and refrigerated I-FOBT samples were stable 21 days. An I-FOBT level can be chosen to provide optimal sensitivity and specificity for significant neoplasia.

key words: colorectal neoplasia • fecal occult blood • immunochemical test • screening

Abbreviations:
AAP – advanced adenomatous polyph; CRC – colorectal cancer; G-FOBT – guaiac fecal occult blood test; Hb – hemoglobin; HGD – high-grade dysplasia; I-FOBT – immunochemical fecal occult blood test; LGD – low-grade dysplasia

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**BACKGROUND**

Population screening for asymptomatic colorectal cancer (CRC) or pre-cancerous adenomatous polyps is usually based on identifying the presence of fecal occult blood as bleeding almost inevitably occurs from clinically significant neoplasia [1,2]. The standard fecal occult blood test uses the chemical guaiac (G-FOBT), which is sensitive to the hemoglobin (Hb) peroxidase activity [1]. The guaiac test card is extensively used worldwide as it is inexpensive and its use in randomized, controlled, population screening trials had demonstrated a small but significant reduction in CRC mortality [2].

However, the standard G-FOBT has low sensitivity for clinically significant colorectal neoplasia and has low specificity due to its non-specificity for human Hb [2]. The test is also user dependent, which leads to the possibility of inaccurate preparation, storage, development and evaluation by inadequately trained personnel [1–4]. A further limitation of the G-FOBT is its sensitivity to dietary peroxidases, found in meat, fruits and vegetables, which can give a false positivity for neoplasia [1,2]. The G-FOBT has been extensively used, mainly in western countries, but not found useful in Asian countries because of their high-peroxidase diet [5].

For these reasons, screening tests have been developed based on the immunochemical detection of human fecal Hb. This test is specific, and so eliminates the need for diet restrictions. The office-developed immunochemical fecal occult blood tests (I-FOBT) improved test specificity and became the standard CRC screening test used in Japan [6–11]. However, for these office-developed tests, the I-FOBT manufacturers have predetermined the degree of sensitivity for Hb at a level similar to a sensitive G-FOBT [8].

A further improvement has been the introduction of instruments for the automated immunochemical development and quantification of fecal Hb. The automated-development allows for standardization and quality control and the Hb quantification allows selection of a suitable I-FOBT level for follow-up colonoscopy [5–7,10–12]. Such instruments have been developed by an Australian-American company (Insure™, Enterix), and also in Japan (MagStream™, Fujirebio and OC-MICRO™ Eiken) and only now are the latter instruments becoming available elsewhere [13,14].

A critical issue in the use of the FOBTs is the stability of the fecal Hb sample. Degradation of the Hb due to bacterial activity is temperature dependent. For this reason, it is usual to air-dry the prepared fecal sample-card used for both the G-FOBTs and office-developed I-FOBTs. They are then recommended to be kept in cool surroundings until development which should be no longer than 14 days from sample preparation [1,2]. A fluid-storage environment for the fecal sample and uncontrolled temperature will potentiate Hb degradation, and is not usually recommended and used for occult blood screening tests. However, this is the collection system used for some of the automated instruments for Hb quantification such as OC-MICRO™.

Little has been published in the English language on the I-FOBT test performed on the OC-MICRO™ instrument [15–19]. So, the use of such methodology for our population-screening program required our pilot evaluation of the test and instrument and their performance characteristics. Our clinical evaluation has been reported in detail [19]. Here we report on the technical features of the test as prepared by the screenee and of the instrument, and recommendations we made for its clinical use.

**MATERIAL AND METHODS**

**Fecal sampling**

The fecal sampling device is a small probe that has a serrated tip. The probe is removed from the container tube and pushed several times into different areas of the stools and then reinserted into the tube, past a scraper and through a membrane and thereby closing and sealing the sample tube. This action removes most of the excess feces and leaves a semi-standard amount of the stool in the probe tip serratations (Figure 1-left A,B,C) [17]. The tip is then in a closed amount of Hb-stabilizing buffer to produce a suspension. Each sample tube has its own bar code and before preparing the sample the patient writes his/her name and date of preparation on the tube. The examinees were requested to prepare 3 consecutive daily samples, which were double closed in zip-lock bags and refrigerated until returned to the developing laboratory where they were also kept at 4°C until development.

**Instrument and testing**

The instrument evaluated for developing and quantification of the I-FOBT is OC-MICRO™ Eiken, Japan. This is a desktop instrument weighing 26 kg. It is self-contained with reagents, buffer, washing and fluid-disposal bottles, requiring a standard power supply (Figure 1-right). Technical information and evaluations performed in Japan is available in English translations of Japanese language publications [15–18].

Ten patients prepared fecal sample tubes that are loaded into the sample rack and in parallel there is another rack with the corresponding disposable reaction cells (Figure 1). The instrument is preheated to its operating temperature of 37°C, which is reached after 30 min. It then automatically punctures the membrane sealing the base of the fecal sample tubes with a needle-pipette which removes and then inserts 25 μL of the fecal-buffer solution into the corresponding reaction tube and with a second pipette mixes it with 60 μL of the provided latex-Hb reagent and 300 μL of diluent buffer fluid (HEPES). Between sampling both pipettes are automatically washed inside and outside with distilled water.

Following the development cycle, there is automatic flushing of the system (sample needle, tubing etc.) with a 5% sodium hypochlorite solution, which is collected into the disposal bottle. The entire cycle takes about 10 min. and about 50 samples/hr can be processed. The instrument automatically prints the results (ngHb/mL of test fluid), giving also the bar code numbers, development date, as well as error messages. Data for 999 samples can be stored in the instrument’s memory for re-call and there is also the possibility to connect the instrument to a computer for data management.
A qualified medical technician, who twice received instruction from the instrument company, performed the testing.

I-FOBT analysis and quality control

The immunochemical method is an anti-human HbA IgG antibody (prepared in rabbit)–antigen reaction. The reagent is provided and had been prepared by absorbing the anti-human Hb antibodies onto polystyrene latex particles, suspended in an ammonium buffer. These reactions take place in an automatically heated, 37°C temperature-controlled system.

The agglutination turbidity is read as an optical change at 600 nm and compared to a standard calibration curve that is prepared every 2 weeks and kept in the instrument’s memory. Standardizing calibration is prepared for each day’s analyses using the provided known high and low-value control test fluids. The range of measurements is 50–2000 ng Hb/mL, approximately equivalent to 40–400 μg Hb/g feces. The 100 ng Hb/mL level for determining positivity had been recommended, based on clinical follow-up of a screened population [12,13]. This was now reevaluated in our clinical study [19].

Evaluations performed

These included evaluations of test reproducibility and fecal sample stability to different temperatures and durations of storage. Samples were taken from the tubing and disposal bottle for bacteriological examination. Clinical evaluation was by requesting patients, without visible rectal bleeding and scheduled for colonoscopy, to prepare 3 consecutive I-FOBTs following explanation of the tests and written instructions on preparing the I-FOBT [19].

The Ethics Committee of the Rabin Medical Center approved the clinical study in 2004. All participants gave an informed, signed consent for the I-FOBT and colonoscopy examination.

Analysis and statistical methods

Statistical analysis was performed using the SAS system for Windows, version 8.01. Results are given as means ±SD. Stability of the test measurements and effects of duration of storage and temperature were examined by analysis of variance with repeated measurements. Weekly changes in the stored I-FOBTs were determined by comparing the initial values measured with the lowest values measured during weeks 1, 2 and 3.

For clinical correlation with the I-FOBT levels, the most severe pathology finding was recorded. “Significant” neoplasia included CRC or “advanced” adenomatous polyps (AAP). This latter category includes adenomas ≥10mm, or having more...
than 20% of villous histology or any amount of high grade dysplasia independent of polyp size. A receiver operator characteristics curve (ROC) was then generated and the I-FOBT measurements correlated with the presence of neoplasia.

RESULTS

Instrument stability, bacteriology examination and test reproducibility

During its use for more than 2 years, other than for routine periodic maintenance, it has remained free of technical problems. The instrument’s tubing was found free of fecal bacteria, but the collection bottle was contaminated. The bottle is now emptied and cleaned daily with antiseptic fluid. The five prepared I-FOBTs that were quantified and then were each repeatedly examined 5 more times during 1 day, showed no significant variations in measurements, F(5,20)=0.24, P=0.66.

Fecal test stability

Thirty fecal tests, highly positive for Hb and 12 tests with low but elevated levels of fecal Hb, and stored at 4°C, 20°C or 28–30°C were repeatedly re-examined 2–3 times a week for 3 weeks. The calculated decay/day was 0.3%±0.4 at 4°C (NS), 2.2%±1.7 at 20°C (NS) and 3.7%±1.8 at 28°C (P<0.05). Their weekly changes in I-FOBT measurements, as compared to their initial values, are shown in Figure 2. At 20°C and at 28°C the maximum decay occurred by the end of the first week. At 4°C, I-FOBT samples maintained their elevated fecal Hb levels for ≥21 days. But 2 samples, with the lowest initial values, had repeated measurements <100 ng Hb/mL by 3 weeks from time of preparation by the patient.

Colonoscopy results

The complete data from the initial 500 examinees was analyzed and is reported in detail elsewhere [19]. Clinically significant neoplasia was found in 34 patients, these included 6 with CRC and 28 with AAP. Non-advanced adenomas were found in a further 85 examinees.

Fecal occult blood results

For the 500 examinees, having 3 daily I-FOBT measurements, there was nonsignificant inter-daily variation in fecal Hb levels [19].

The ROC demonstrated good separation of I-FOBT levels in CRC and AAP-bearing patients from those with only non-advanced adenomas. These were obtained with high specificity of CRC and at a 95% specificity for 71% for AAP (Figure 3). This was obtained at the 100 ng Hb/mL level by utilizing the highest results from all 3 days sampling. Similar analyses at lower and higher I-FOBT Hb levels are given in Table 1.

DISCUSSION

The technical evaluation of ease of use, degree of bacterial contamination, ease of test performance and reproducibility were found favorable by our technician. Evaluation included examination of the prepared fecal occult blood for stability at high ambient temperatures. This latter point was carefully evaluated and showed that the prepared I-FOBT could be 2–3 weeks in refrigeration without significant degradation of the test antigen. However, for low-elevated levels, the measurement could drop below the 100 ng Hb/mL threshold by 3 weeks even when refrigerated. So, when using this type of liquid I-FOBT sample, it is important to emphasize, to the patient and medical service, the correct storage temperature and the limitations to duration of storage; a 2-week time period is adequate for batch processing of accumulated test samples. An evaluation previously performed in Japan had shown sample stability, at room temperature, for 5 days [15]. We did not feel that this was suitable for our population as it did not take into consideration that at present we request 3 daily samples, which would then be sent for processing, and could be exposed for a longer duration of time to the uncontrolled room and transport temperature.

The instrument-developed, quantitative fecal occult blood testing, allows the physician to choose the optimal fecal Hb threshold level that leads to a follow-up colonoscopy. To demonstrate this, we performed this study in high-risk for
CRC patients and symptomatic patients undergoing colonoscopy. From this study, we confirmed that the optimal sensitivity/specificity ratio was obtained at the I-FOBT threshold of 100 ng Hb/mL. This threshold allowed detection of all the cancers and the majority of advanced adenomas, giving for both together the optimal sensitivity of 76.5% with an acceptable specificity of 95.3%, at this round of screening in a group of patients at high-risk for CRC [2,19]. The assumption being that future rounds of annual I-FOBT screening would detect additional, but at present unidentified, significant neoplasia. As shown by the ROC analysis, the test does not identify the presence of non-advanced adenomas that have a low risk for CRC at present. The clinical conclusions drawn from this initial experience with OC-MICRO™ needs to be extended to a larger population. This will allow further evaluation of the optimal I-FOBT level for recommending colonoscopy follow-up and a calculation of cost-efficiency as compared to the present G-FOBT screening test being used.

At present, we recommend collecting 3 annual fecal tests as, in this high-risk population, we have confirmed the additive clinical value of repeated occult blood testing. This recommendation is in contrast to the annual 2-day I-FOBT collection in the average-risk population, as routinely used in Japan, US and Australia and 1-day biennial testing as performed in Italy [11,12,20–22]. In the Japanese experience, the number of samples collected and threshold chosen were different from the experience in Italy, namely 2 annual samples and 150 ng/mL fecal Hb threshold in Japan vs. 1 biennial sample and 100 ng/mL threshold in Italy [20–22].

The goal is to extend this automated-developed, quantitative, I-FOBT to colorectal neoplasia screening of the asymptomatic average-risk population. This has been done with the InSure™ (Enterix, USA and Australia) test in pilot studies in Australia and to the public in the US [10,11]. Another automated test is MagStream™ (Fujirebio, Japan) developed in Japan and also being evaluated in Australia, France and Hong Kong [14,23]. It has been evaluated in a large colonoscopy screening study performed in Japan, but they used only a 1-time sample and did not provide details on the sample storage conditions before processing [24]. Outside of Japan, the automated version of OC-MICRO™ is being used in a large population, biennial, single sample study in Northern Italy [15]. It is also being used in a population study in Uruguay (Dr. E. Fenocchi, personal communication) and will be used in a large Spanish asymptomatic population screening trial (Dr. E. Carballo, personal communication). It is now being marketed in the USA as OC-AutoMicro 80.

To facilitate average-risk compliance, there are two issues that need to be addressed. The provision of a toilet-disposable paper stool-collecting device facilitates preparing the test [25]. This is available in Japan and we are now evaluating such a device. As we have seen, the storage of the prepared I-FOBT in a refrigerator, until development, is important for maintaining the stability of the prepared test. This is critical in countries with high ambient temperatures, even when homes are air-conditioned. The opaque double zip-lock bags we provided answered some of the screenees’ hesitancy about keeping the prepared test transiently in their home refrigerator. This packaging has been improved. We have also advised adding a reusable cold pack to the kit for storing in the freezer and adding to the prepared samples when returning the prepared FOBTs to the developing laboratory.

**CONCLUSIONS**

We have found that this desktop, automated developed and quantified Hb version of the I-FOBT, to provide a sensitive test for detecting significant colorectal neoplasia with an acceptable specificity and consequent high negative predictive value. Its suitability for average-risk population screening will now be evaluated.

**Acknowledgments**

To the medical and secretarial staff of the Endoscopy Units and the patients for their cooperation. We thank Dr. Ester Shabtai and Doron Comaneshter for statistical analyses, Ms. Sally Zimmerman for secretarial assistance, the Katzman Family Foundation for supporting publication costs and Mr. Takuo Ichiyanagi for providing English language translation. The InSure™ test was developed in Japan and also being evaluated in Australia, France and Hong Kong. Table 1. Sensitivity, Specificity and Predictive Values for Significant Colorectal Neoplasia, (N=34) at Differing Fecal Hb Levels (N=500)***.**

<table>
<thead>
<tr>
<th>Fecal Hb ng/mL</th>
<th>Sensitivity N (%)</th>
<th>Specificity %</th>
<th>Predictive Value %</th>
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<td>50</td>
<td>27 (79.4)</td>
<td>89.7</td>
<td>36.0</td>
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<tr>
<td>150</td>
<td>24 (70.6)</td>
<td>95.9</td>
<td>55.8</td>
</tr>
<tr>
<td>200</td>
<td>22 (64.7)</td>
<td>96.3</td>
<td>56.4</td>
</tr>
</tbody>
</table>

* 6 colorectal cancers and 28 advanced adenomatous polyps;
** Utilizing the highest of the 3 I-FOBT measurements in each patient;
*** Derived from reference 19.
REFERENCES:


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