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Coloscreen VPI Test Kit Evaluated for Detection of Fecal Occult Blood

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The usefulness of the Coloscreen VPI test kit in occult blood detection has been examined and compared with our current method. The results show the Coloscreen procedure to be a simple, sensitive method that is much less subject to vegetable peroxidase interference than are other available screening procedures.

Additional Keyphrases: colorectal cancer - pseudoperoxidase - dietary restriction - screening - variation, source of

The importance of screening for fecal occult blood for early detection of colorectal cancer is well recognized (1). The usefulness of this approach, however, has been hampered by the unreliability of many screening methods (2-4), because the incidence of false-positive results in subjects who are on an unrestricted diet may be very high (5). Most current methods are based on the pseudoperoxidase activity of the hematin portion of the hemoglobin molecule; the oxygen liberated from \( \text{H}_2\text{O}_2 \) is used to oxidize a dye to a chromogen. Because methods based on this reaction are affected by dietary blood, hemoglobin, and hematin, subjects to be screened should be advised to reduce their intake of red meat products.

Interference from vegetable peroxidase, enzymes present in many types of vegetables and fruit, is also a problem (6, 7). These enzymes demonstrate some resistance to heat denaturation and may not be inactivated by cooking. Dietary restriction of both hemoglobin- and vegetable peroxidase-containing substances is therefore essential for valid screening for occult blood. Dietary restriction is rarely completely effective, however, because the large number of food products involved generally provokes poor compliance. One approach to reducing interference at the assay stage has been to boil fecal samples thoroughly before analysis, thus inactivating the vegetable enzymes while leaving the pseudoperoxidase activity of hemoglobin largely intact. This procedure is inconvenient when large numbers of samples are assayed; so, also, is a more recently introduced method based on conversion of hematin to porphyrins (8).

Test sensitivity is also important. Normal blood loss in feces amounts to 2.5 mL per day (9), whereas >10 mL per day may be considered significant in the early detection of colorectal disease.

Our current laboratory method, the "Okokit II," is based on the oxidation of an \( \sigma \)-tolidine-like substance, in tablet form, to a green end product. "Coloscreen VPI" utilizes guaiac (impregnated into test cards) as the pseudoperoxidase substrate and also includes a vegetable peroxidase inhibitor to reduce dietary peroxidase activity. We describe here our evaluation of the Coloscreen VPI test procedure to determine its suitability as a screening method for fecal occult blood.

Materials and Methods

Specimens

Fecal samples (20–50 g) were obtained from routine collections for occult blood testing. These were from hospital inpatients and patients of general practitioners. Before analysis, samples were thoroughly mixed with a wooden spatula. Comparisons between Okokit II and Coloscreen VPI were made with duplicate samples from the same stool specimen. Patients were allowed an unrestricted diet. Fresh samples of blood, collected with EDTA present, were analyzed for hemoglobin (Hb) concentrations with a Coulter S-plus (Coulter Instruments, Hialeah, FL 33130), then used as Hb standards. Fecal iron concentrations were determined by the method of Peters et al. (11).

Reagents

The Okokit II was obtained from Hughes and Hughes Ltd., Romford, Essex, U.K., and the Coloscreen VPI from Cambridge Self Care Diagnostics Ltd., Ely, Cambridge, U.K. (manufactured by Helena Laboratories, Beaumont, TX 77704). Vegetable peroxidase was obtained from Sigma Chemicals, Poole, Dorset, U.K. "Analar"-grade water (BDH Chemicals, Poole, Dorset, U.K.) was used throughout. All other reagents were of at least Analar grade.

Test Procedures

Okokit II is based on the pseudoperoxidase activity of hemoglobin. The liberated \( \text{O}_2 \) is used to oxidize an \( \sigma \)-tolidine-like substance to a green-colored product (the identity of the chromogen is not revealed by the manufacturer). In the test procedure, a small sample of feces, ~0.1 g, is smeared onto filter paper provided in the kit, and one drop of reagent A (\( \text{H}_2\text{O}_2 \)) is added, followed by one drop of reagent B
(chromogen solution). The presence of a green color at 2 min indicates a positive result.

Coloscreen VPI is similarly based on pseudoperoxidase activity. This kit comprises a test card impregnated with guaiac, onto which fecal samples (0.1 g) are smeared. Three drops of VPI (vegetable peroxidase inhibitor) are added, followed by three drops of developer (H₂O₂). Positive results are indicated by a blue color at 30–120 s.

Sensitivity to Blood

To compare the sensitivity of each kit to lysed and intact erythrocytes, we diluted whole blood (Hb 150 g/L) with either water or saline (NaCl 154 mmol/L) to give a series of standard concentrations of hemoglobin ranging from 0.01 to 1 g/L. We tested 10 μL of each of these solutions by both methods.

Because pseudoperoxidase-based tests are less sensitive to hematin than to hemoglobin, we performed a further series of experiments, using blood in a fecal matrix. Whole blood containing EDTA as anticoagulant was diluted in water to give an Hb concentration of 100 g/L. Various volumes (0–40 μL) of this solution were then added to 0.5 mL of fecal suspension, prepared by homogenizing 5 g of feces in 10 mL of water. The concentration of Hb in the fecal matrix varied between 0 and 4 g/L. The fecal material used was only from fecal samples negative for occult blood by both methods. After addition of blood, the suspensions were allowed to stand for 30 min at room temperature before testing, to allow time for action by fecal bacteria.

Sensitivity to Vegetable Peroxidases

Sensitivity to horseradish peroxidase (EC 1.11.1.7) was compared by preparing serial aqueous dilutions of the enzyme (0.001–1.0 g/L) and testing them with both kits. These solutions were re-tested after the addition of fecal material, 1 g/mL, because this more closely approximated in vivo conditions.

Comparison of Test Results

Routine occult-blood samples (n = 220) were tested with both kits. Discrepant results were investigated by selecting 20 specimens without conscious bias and homogenizing 1–2 g samples of each in 5 mL of water. Glacial acetic acid, 5 mL, was added dropwise, with mixing, followed by 5 mL of diethyl ether. After mixing and centrifugation (2000 × g, 5 min, 20 °C), we examined the resulting supernate for hemoglobin content by scanning the sample with a recording spectrophotometer between 450 and 650 nm. During the procedure, hemoglobin is converted to acid hematin, which exhibits peaks at 510, 540, and 630 nm. Urobilin/sterobilin has a peak at 490 nm. Vegetable matter is not extracted, but chlorophyll may be (I2).

Results

Sensitivity to hemoglobin. For the aqueous standard series the detection limit for hemoglobin in the case of Okokit II was 0.1 g/L; for Coloscreen VPI, 0.05 g/L. Results for the saline standard series (intact erythrocytes) did not differ significantly from those for aqueous standards.

Sensitivity to blood in feces was found to be 0.5 mg of hemoglobin per gram of fecal material for Okokit II, 1 mg/g for Coloscreen VPI.

Sensitivity to vegetable peroxidase. Table 1 shows the various sensitivities of the kits to vegetable peroxidase in aqueous solution. Columns a and d show our results when we used the kits in their recommended form. Okokit II is sensitive to as little as 50 μg of peroxidase per milliliter, whereas Coloscreen is unaffected. If the vegetable peroxidase inhibitor supplied with the Coloscreen kit is used in conjunction with Okokit II (column b), however, this sensitivity is completely lost. Similarly, if the inhibitor is omitted from the Coloscreen test procedure, that method becomes sensitive to peroxidase (10 mg/L). When the tests were repeated with fecal material present, we noted a slight loss of sensitivity to horseradish peroxidase by Okokit II (0.2 g/L); Coloscreen VPI was unaffected.

Comparison of test results. Table 2 shows the results obtained for 220 fecal samples. The positive results do not include those considered marginal.

Because the results show a much lower percentage of positive results for Coloscreen VPI, we selected 100 samples without conscious bias and tested them with Okokit II, with the Coloscreen vegetable peroxidase inhibitor added. The results in this case were: Okokit II-positive 25, Okokit II-negative 75—a decrease in the positive rate from 36% to 25%. Of the 25 Okokit II-positive samples, 10 were Coloscreen-positive. Without the addition of VPI reagent, 36 of the 100 fecal samples studied were Okokit II-positive.

Spectrophotometry. Twenty specimens were treated with acetic acid and diethyl ether as described in Materials and Methods, and scanned for hemoglobin and its derivatives. A 1.5 g/L hemoglobin standard solution was treated similarly. Additionally, we re-tested the ether extracts by both methods. In all samples positive by Okokit II but negative by Coloscreen VPI (n = 10), spectrophotometry revealed well-defined absorbance peaks only at 490 nm, the wavelength expected for urobilin; one of these samples gave a trace peak at 540 nm. Similar results were obtained (n = 5) with samples negative by both methods. Samples positive by both methods (n = 5), however, produced well-defined peaks of 490 and 630 nm and small peaks at 510 and 450 nm. The hemoglobin solution produced peaks at 510, 540, and 630 nm, characteristic of an acid hematin solution. On re-testing the ether extracts with Okokit II and Coloscreen VPI only samples originally positive by both methods gave a positive result.

Heat treatment. A further 25 samples of feces were subjected to heat treatment (100 °C, 20 min) and re-tested with both kits. For the 15 samples with positive Okokit II and negative Coloscreen VPI results, heat treatment decreased the color produced, so much in nine cases as to give an

| Table 1. Sensitivity of Okokit II and Coloscreen VPI to Horseradish Peroxidase |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Peroxidase, g/L                 | 0.5             | 0.2             | 0.1             | 0.05            | 0.02            | 0.01            | 0.005           | 0              |
| Okokit II                       | ++              | ++              | +               | ±               | –               | –               | –               | –               |
| Okokit II + VPI                 | –               | –               | –               | –               | –               | –               | –               | –               |
| Coloscreen – VPI                | +++             | +++             | ++              | +               | ±               | –               | –               | –               |
| Coloscreen + VPI                | –               | –               | –               | –               | –               | –               | –               | –               |
| VPI, vegetable-origin peroxidase inhibitor. |

| Table 2. Okokit II and Coloscreen VPI Results for 220 Routine Fecal Occult-Blood Samples |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Okokit II positive | no. (and %) | Okokit II negative |
| Coloscreen positive     | 15 (7)         | 125             |
| Coloscreen negative     | 80 (36)        | 0               |

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Okokit II-negative result. Coloscreen-positive samples were unaffected. Possible interference from therapy with iron was considered. Five samples of stools with a characteristic "tarry" appearance were extracted with glacial acetic acid and analyzed for iron. All had an iron concentration >100 µmol/L, and all were Okokit positive, Coloscreen negative. Analysis of a series of standard concentrations of ferrous sulfate showed that both kits gave positive results at a ferrous sulfate concentration of 1 g/L.

Discussion

Because examination of fecal samples for occult blood loss is so common, screening procedures need to be simple, both for the patient and the laboratory. Many patients continue to take an unrestricted diet. We previously used the Okokit II method but believed that we were identifying a substantial number of false positives because as many as 40% of screened patients gave results indicative of significant fecal blood loss. Coloscreen VPI gave positive results in only ~5% of samples tested, which is in better agreement with the 2–3% found in surveys of well-controlled, dietary-restricted subjects (13).

We found that both kit procedures were easy to perform. Okokit II, however, was more difficult to interpret, because color development continued after the recommended incubation period. This may cause difficulties when sample numbers are large. The main problem with the Coloscreen VPI procedure was cross contamination between the three samples applied to each test card. This results from the comparatively large volume of liquid reagents applied to the test slide: three drops of inhibitor and two of color developer for each specimen. We found this excessive and decreased the amount applied to one drop of each reagent, with no effect on test results.

The sensitivity of Coloscreen VPI to aqueous hemoglobin and intact erythrocytes was identical, 0.05 g of hemoglobin per liter. Okokit II demonstrated similar sensitivities (Hb 0.1 g/L). The sensitivity of Okokit II to blood in a fecal matrix, however, was greater than that of Coloscreen (1 vs 2 mg of Hb per gram of feces). As stated previously, an ideal method should be able to detect a blood loss of as little as 10 mL/day. Assuming a mean fecal excretion rate of 200 g/day, the sensitivity of both Okokit II and Coloscreen VPI is ~3 mL of blood per day; both methods, therefore, have adequate sensitivity. Sensitivity to horseradish peroxidase differed markedly for the two kits, 0.2 g/L being sufficient to affect results by Okokit II, 0.02 g/L for Coloscreen. The addition of VPI however, decreases sensitivity to at least 0.5 g/L.

Clearly, a large proportion of positive tests with Okokit II can be attributed to vegetable peroxidase activity. The use of a peroxidase inhibitor in conjunction with the Okokit procedure and the heat-treatment experiments add further supporting evidence. In only one case did a Coloscreen-negative/Okokit-positive sample contain di-i spectrophotometric iron examination of this sample showed a small peak at 540 nm. This observation supports other data presented here that Okokit II is more sensitive than Coloscreen VPI.

Ferrous sulfate, albeit only in high concentrations, can produce false-positive results in both methods. The usual dose of iron in therapy of anemia is 100–300 mg/day. Both methods gave a slight positive result for 0.5 g of ferrous sulfate per liter. If the average excretion of feces per day is 200 g, then this may give an iron concentration of 100 mg/L in a 24-h fecal sample. Of course, in vitro experiments may not reflect sensitivity to iron in vivo.

Both methods have some minor procedural disadvantages, but they are simple to perform, have the required sensitivity, and are relatively inexpensive. Coloscreen VPI, however, offers the advantage of relatively low sensitivity to endogenous vegetable peroxidases. The elimination of such interference should considerably improve the reliability of non-diet-restricted occult-blood tests. The Coloscreen VPI test has been in routine use in our laboratory for three months, during which ~350 samples have been studied. Collaboration with one of our clinical hematologists has allowed us to assess the usefulness of the tests in patients with anemia. Before the Coloscreen VPI was introduced, a substantial number of positive occult blood tests were reported for anemic patients. Several of these patients, examined by barium series, were found not to have bowel lesions. In contrast, since the introduction of Coloscreen VPI, no false-positive results have been observed in this group of patients.

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