A New Method of Quantitative Fecal Fat Microscopy and Its Correlation With Chemically Measured Fecal Fat Output

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Abstract

Fecal fat microscopy using the Sudan stain has suffered from a relative lack of specificity, and results are "qualitative." Therefore, we developed a quantitative fecal fat microscopic method with hopes of improving diagnostic accuracy. One hundred eighty patients with chronic diarrhea collected stools for 1 to 3 days, and fecal fat output was measured by a standard chemical method, and microscopy was performed by the old qualitative and new quantitative methods. There was a highly statistically significant linear correlation between quantitative fecal fat microscopy and chemically measured fecal fat output. The quantitative microscopic method had a sensitivity of 94% and a specificity of 95%; the traditional method had a sensitivity and specificity of 76% and 99%, respectively. Fecal fat Sudan microscopy performed by a dedicated approach to counting and size measurement of fat globules can yield a quantitative result that correlates well with chemically measured fecal fat output and has a high diagnostic accuracy.

Patients with chronic diarrhea should be evaluated for nutrient malabsorption because if present, diagnostic pursuits and disease possibilities are very different from those when malabsorption is absent. For more than 50 years, the "gold standard" for detection of malabsorptive states has been a 72-hour quantitative stool collection measured for its fatty acid content by the titration method of van de Kamer et al.[1] With this test, fecal excretion of greater than 7 g/d of fat is considered abnormal,[1-4] although in the setting of diarrhea, as much as 14 g/d may be excreted by healthy persons.[4] Although quantitating fecal weight and fat can provide vital information for the approach to patients with chronic diarrhea, performing this test is difficult logistically for centers unaccustomed to the procedure and usually abhorred by patients and laboratory technicians. Moreover, for patients with chronic diarrhea, the test is complicated further by bowel movement frequencies that may reach 20 or more per day and/or fecal volumes as high as 2 or 3 L per day, both of which decrease the likelihood that patients will collect all of the fecal effluent they pass.[5,6] This is problematic because daily fecal fat output is a calculated value derived from the measured fat concentration and average daily fecal weight; consequently, its accuracy depends on the completeness of the collection, and missed fecal specimens lead to underestimation of the true amount of fecal fat excretion.

For these reasons, physicians evaluating chronic diarrhea often avoid tests for steatorrhea altogether, blindly pursuing possible causes of malabsorption, or resort to the more simple screening method of fecal fat microscopy. Like van de Kamer's original method of fecal fat quantitation, the method of fecal fat microscopy by Drummey et al[7] using Sudan III staining has stood the test of time and has

been used essentially unchanged since 1961. Fecal fat microscopy usually is viewed as a "qualitative fat" test but with results reported in a graded fashion as follows: 1+, normal; 2+, slight increase; and 3+, definite increase.[7] This method has been shown to be fairly accurate in its ability to detect severe steatorrhea when measured in a quantitatively collected stool sample, but as originally described, the method suffered from a relative lack of specificity in defining normal and in separating mild and possibly inconsequential steatorrhea from more severe steatorrhea. A separate and perhaps lesser problem with the original report by Drummey et al[7] is that quantitative fecal fat output was expressed as a percentage of intake rather than in grams per day, the traditional unit of measurement.

The microscopic method of Drummey et al[7] relied on the number and size of fat globules to distinguish the 3 ranges of fecal fat. The implication from this, as well as from the hydrophobic physicochemical properties of lipid in a water-based solution, is that as more fat is present in stool, fat globules become more numerous and increase in size. From this, we hypothesized that with a slightly more dedicated approach to counting and size measurement of fat globules, the accuracy of fecal fat microscopy could be improved, and the results could become quantitative. Therefore, we devised a new method of assessing fecal fat by Sudan microscopy to determine whether measurement of fecal fat output by a quantitative microscopic method would directly correlate with fecal fat output assessed by chemical analysis of stool collected for 1, 2, or 3 consecutive days. We also studied a separate and previously uninvestigated question: whether Sudan microscopy of a single random stool specimen is adequate to detect or rule out steatorrhea.

Methods

Patients and Fecal Specimens

One hundred fifty-six consecutive patients with chronic diarrhea (fecal weight >200 g/d) submitting quantitative stool collections during an 18-month period (September 1996 to March 1998), and 24 patients with moderate to severe steatorrhea having collected stools in the past (which were kept frozen) constituted the study population. Thus, 180 patients' fecal samples were studied. The patients were instructed to eat a high fat diet and to collect all stools passed during the defined period in refrigerated containers provided to them. Twenty-five, 113, and 42 patients collected stools for 24, 48, or 72 hours, respectively. On return, the specimens were weighed, homogenized in a blender, and evaluated for their fat content by microscopy and by chemical analysis.

Methods of Fecal Fat Microscopy

Quantitative Fecal Fat Microscopy

Glass slides for fecal microscopy were prepared according to the split fat method of Drummey et al.[7] A small amount of stool from the homogenized quantitative collection (an amount occupying about a 5-mm diameter) was placed on a glass slide, and 2 drops of 36% acetic acid were added, followed by 2 drops of 1% Sudan III stain. The slides were held by hand over a hot plate until bubbles appeared, then quickly removed, and reheated 2 additional times. They were interpreted immediately with a microscope containing a calibrated ocular micrometer at high power ($\times 400$). Areas of the slide appropriate for analysis were those showing fat droplets but little if any opaque fecal material. The number of fat globules were counted separately in the diameter ranges of 0 to 5 μm, 6 to 10 μm, 11 to 20 μm, 21 to 40 μm, 41 to 80 μm, and greater than 80 μm. Five high-power fields were counted. The average number of fat globules counted within each size range from the 5 high-power fields was multiplied by the size-range midpoint (ie, 2.5, 8, 15.5, 30.5, and 60.5 μm) to obtain a statistically weighted size-number product for each of the size ranges measured. (The size range midpoints represent an assumed average size of the fat droplets within each size range.[8]) If globules were greater than 80 μm in diameter (which was rare), the actual measured average size of these globules was multiplied by the average number in 5 high-power fields to calculate this size-number product. All size-number products were then summed to obtain a single numeric result that we call fecal fat droplet total size-number product.

Qualitative Fecal Fat Microscopy

For comparison, fecal microscopy from all patients also was interpreted by using the original qualitative method of Drummey et al.[7] Again using the average values obtained from assessment of 5 high-powered fields, samples were rated as follows: normal, up to 100 fecal fat droplets between 1 and 4 μm in size; slightly increased, more than 100 droplets between 1 and 8 μm; and definitely increased, more than 100 droplets between 6 and 75 μm.

Quantitative Fecal Fat Microscopy of Spot Stools

To study the relationship of quantitative fecal fat microscopy of randomly collected spot stools to that assessed in continuously collected fecal homogenates, 38 patients collected a random stool before beginning the high-fat diet and collected what they thought would be their last stool of a 48- or 72-hour fecal collection in a separate small container provided to them. (No 24-hour collections were performed in this subgroup of patients.) On return of

the stools, the spot specimens were stirred vigorously with a wooden stick (tongue depressor), and a small specimen from each was placed on separate glass slides for quantitative fecal microscopic analysis as described. The remainder of the stool from the second spot specimen (which was part of the 48- or 72-hour collection) was then mixed with the rest of the quantitatively collected stool, and the entire specimen was weighed and homogenized. From this homogenate, a third microscopic slide was prepared and analyzed like the others. Thus, fecal microscopy from a spot stool specimen obtained while the patient was on his or her usual diet could be compared with that from a spot stool obtained following 2 to 3 days of a high fat test diet (the last specimen from the collection period), and each of these could be compared with the fecal microscopy from a homogenized stool specimen collected over 48 or 72 hours while on a high-fat diet.

Quantitation of Fecal Fat Output by Chemical Analysis

Fecal fat concentration was determined chemically by the titration method of van de Kamer et al.[1] Five grams of the homogenized stool was emulsified with 40 mL of 95% ethyl alcohol containing 0.4% amyl alcohol, and 10 mL of 33% potassium hydroxide, and transferred into a 250-mL Erlenmeyer flask. Contents were boiled on a hot plate for 20 minutes after the flask was connected to a reflux condenser. After cooling, 17 mL of 25% hydrochloric acid was added. Fifty milliliters of petroleum ether then was added, and the flask was topped and shaken manually for 1 minute. After separation of the fat and water layers, 25-mL was removed from the ether-fat layer with a graduated pipette and placed into a 125-mL flask. The flask contents were evaporated to dryness on a hot plate. Ten milliliters of neutral alcohol was added, and the solution was titrated with 0.1N sodium hydroxide until a thymol blue indicator (added to the neutral alcohol) displayed its color. Grams of fatty acid per 100 g of stool were calculated from the milliliters of sodium hydroxide necessary for titration and the average molecular weight of dietary fatty acid.[1] This expression of fecal fat concentration was converted to a daily output value by multiplying by the mean daily fecal weight.

Results

Quantitative Fecal Fat Microscopy vs Chemically Determined Fecal Fat Output

Figure 1 shows the relationship of quantitative fecal fat microscopy and chemically measured fecal fat output. There was a highly statistically significant linear correlation
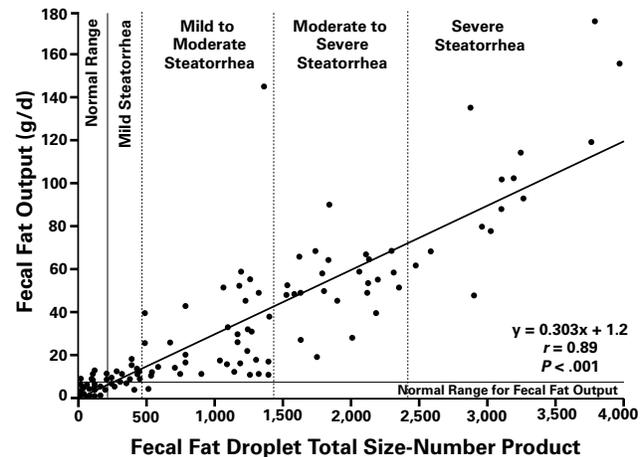

Figure 1 Linear correlation of fecal fat output and microscopic fecal fat droplet total size-number product for 180 patients with chronic diarrhea.

between the 2 values. According to the individual data points, the upper limit of normal for fecal fat total droplet size-number product was optimal at 200. Only 6 of 81 patients with total size-number products less than 200 had elevated fecal fat output values, and these were only mildly elevated (8-13 g/d). Similarly, only 4 of 99 patients with a size-number product greater than 200 had fecal fat output values less than 7 g/d; all 4 of these patients had fecal weights less than 275 g per day. The microscopic results for these 4 patients were only mildly elevated (ie, values were <500, to be contrasted with the highest values in patients with steatorrhea approaching 4,000 [Figure 1]). There was no appreciable difference in the data obtained from patients collecting their stools for 24, 48, or 72 hours. The individual data presented in Figure 1 also defined ranges of microscopic size-number products that, in turn, defined ranges of varying severity of steatorrhea as denoted in the Figure. Examples of the microscopic appearance of fecal samples from patients with varying degrees of steatorrhea are shown in **Image 1**.

Qualitative Fecal Fat Microscopy vs Chemically Determined Fecal Fat Output

Figure 2A shows the distribution of results from our 180 patients using the qualitative microscopy method of Drummey et al.[7] Twenty-three of 101 patients meeting the "normal" microscopic criteria had steatorrhea (false-negative results), with values as high as 16 g/d. In contrast, only 1 of the 79 patients in the 2 groups with "increased" microscopic values had normal fecal fat values (false-positive result).
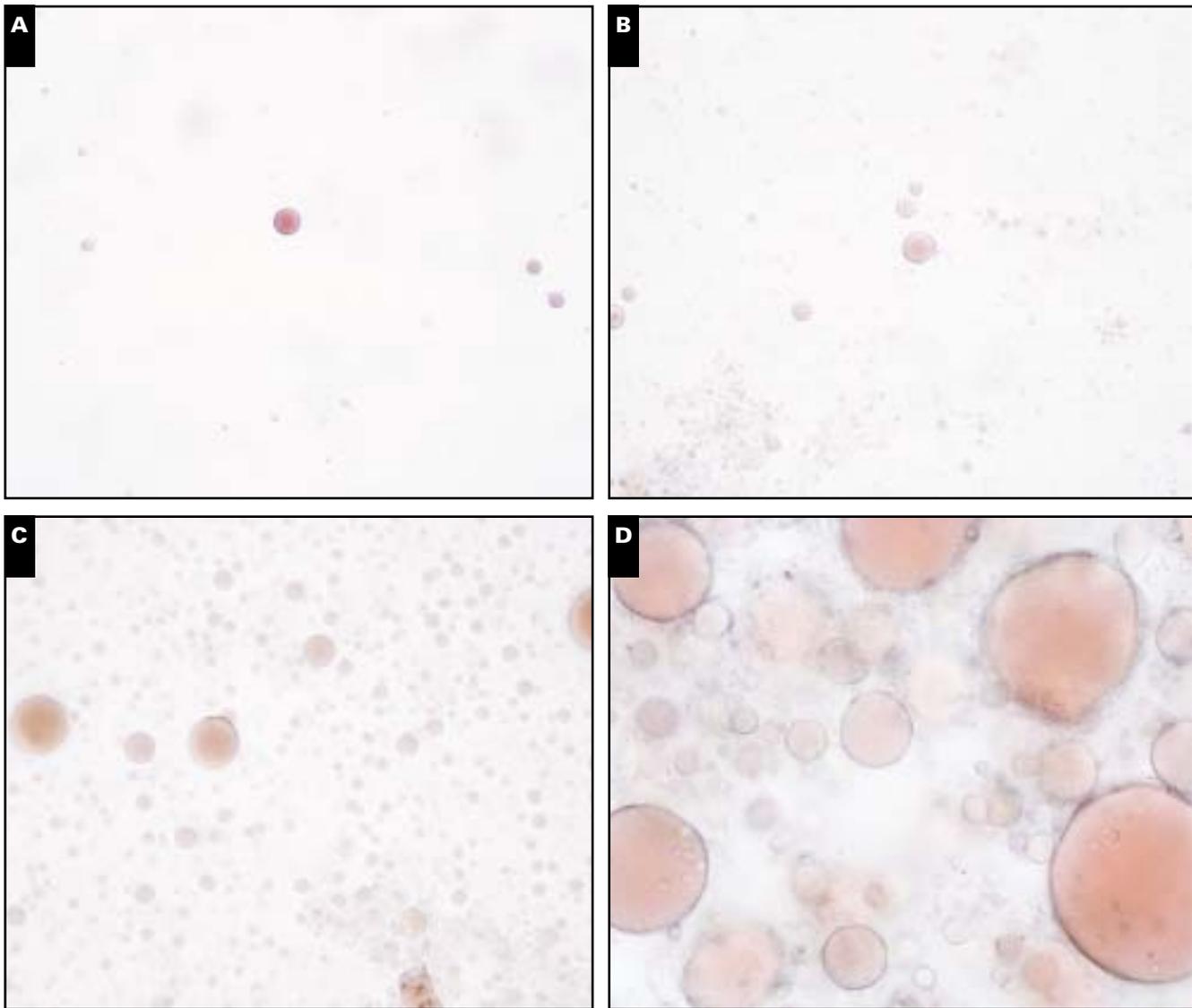
❙Image 1❙ Microscopic appearance of fecal samples containing varying amounts of fat stained with Sudan III after acidification and heating. A, No steatorrhea; few microscopic fat droplets ranging in size from 2-20 μm; fecal fat output was 3 g/d; fecal fat droplet total size-number product was 36. B, Mild steatorrhea; fat droplets of similar size range as Image 1A but in greater number; fecal fat output was 13 g/d; fecal fat droplet total size-number product was 480. C, Moderate steatorrhea; greater number and size of fat droplets (up to 30 μm) compared with Image 1B; fecal fat output was 25 g/d; fecal fat droplet total size-number product was 804. D, Severe steatorrhea; numerous and even larger fat droplets (up to 90 μm) in multiple planes of the slide; fecal fat output was 64 g/d; fecal fat droplet total size-number product was 2,682.
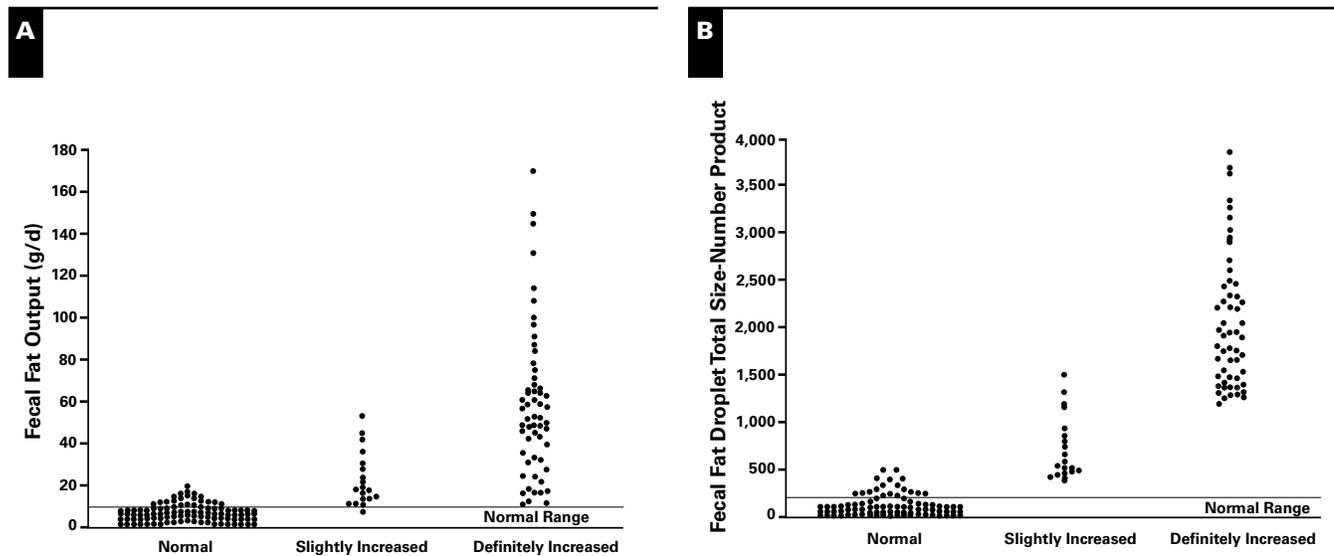
Comparison of Quantitative and Qualitative Fecal Fat Microscopic Methods

❙**Figure 2B**❙ compares our fecal fat droplet total size-number product results with the results obtained using the qualitative method. All patients meeting the slightly or definitely increased criteria of Drummey et al[7] had abnormal results by our quantitative method. Of the 101 samples in the "Drummey-normal" group, 20 had elevated quantitative microscopic values by our criteria, 17 of which had elevated fecal fat output values. In other words, 17 of 23 patients with steatorrhea but with falsely negative

microscopic values by the older qualitative method (ie, the 23 false-negative results mentioned in the preceding paragraph) would have been diagnosed correctly by our quantitative fecal fat microscopic method.

Parameters of Diagnostic Accuracy for the Old and New Methods of Fecal Fat Microscopy

For calculations of sensitivity and specificity, results of quantitative stool collection were considered the gold standard, with a fecal fat output value greater than 7 g/d representing a positive test result. Based on this, our quantitative

Figure 2 A, Distribution of fecal fat output for 180 patients with chronic diarrhea separated into 3 groups according to the results of fecal fat microscopy performed using the qualitative method of Drummey et al[7] (normal, n = 101; slightly increased, n = 22; definitely increased, n = 57.) B, Distribution of fecal fat droplet total size-number product by our quantitative fecal fat microscopic method separated according to the results of fecal fat microscopy performed using the qualitative method of Drummey et al.[7]

Table 1

Quantitative Fecal Fat Microscopy in Spot Stools on a Free or High-Fat Diet Compared With That From Homogenized 48- or 72-Hour Stool Collections from 38 Patients With Chronic Diarrhea

| | Quantitative Fecal Fat Microscopy | | | | | |
| | Spot Stool on Free Diet | | Spot Stool on High-Fat Diet | | 48- or 72-h Stool on High-Fat Diet | |
Range of Measured Fecal Fat Output	Correctly Diagnosed	Incorrectly Diagnosed	Correctly Diagnosed	Incorrectly Diagnosed	Correctly Diagnosed	Incorrectly Diagnosed
No steatorrhea (<7 g/d) (n = 20)	20	0	20	0	20	0
Mild steatorrhea (8-14 g/d) (n = 9)	5	4*	5	4*	6	3*
Moderate steatorrhea (24-48 g/d) (n = 6)	6	0	6	0	6	0
Severe steatorrhea (62-97 g/d) (n = 3)	3	0	3	0	3	0
Total (n = 38)	34	4	34	4	35	3

* Three patients with mild steatorrhea and high fecal weights (740-1,240 g/d) had negative microscopic readings in all 3 samples. The fourth incorrect diagnosis in each of the spot stool groups also was a false-negative result but for different patients.

fecal fat microscopic method had a specificity of 95% (95% confidence interval [CI], 87%-97%) and a sensitivity of 94% (95% CI, 90%-98%). The qualitative method of Drummey et al[7] in our hands had a specificity of 99% (95% CI, 93%-100%) but a sensitivity of only 77% (95% CI, 68%-85%). According to a z test for proportions, the sensitivity of our new quantitative microscopy method was statistically significantly greater than that of the older qualitative method (z value, 3.265; $P < .0012$), whereas the specificities were not significantly different (z value, 0.938; $P = .35$).

Comparison of Quantitative Fecal Fat Microscopy of Spot Fecal Specimens With Quantitative Microscopy of 48- or 72-Hour Fecal Collections

Table 1 shows the number of correct or incorrect diagnoses by quantitative fecal fat microscopy when spot stools from patients eating a normal or high-fat diet or their 2- or 3-day stool collections were analyzed microscopically. All 3 samples gave similar results. There was only 1 specimen in each of the 2 spot specimen groups (from different patients) that failed to reveal mild steatorrhea when analysis of the complete collection did so. Three patients with high fecal weights but with only mild steatorrhea (meaning that

fecal fat concentration was very low) had negative microscopic results in all 3 specimens. No diseases typified by malabsorption were found in theses patients, implying that their mild steatorrhea was likely due to diarrhea per se.[4] Thus, quantitative microscopy of 1 spot fecal specimen from 38 patients displayed 98% concordance with microscopy of their 2- or 3-day fecal collections; analysis of 2 spot specimens was 100% concordant.

Discussion

We hypothesized that the traditional "qualitative" fecal fat microscopy test could be modified to become quantitative. By using a dedicated assessment of the microscopic fat droplet size and number in a way that did not add substantial difficulty or completion time to the old method, we were able to successfully detect or rule out steatorrhea with 95% accuracy. Furthermore, the severity of steatorrhea could be estimated within mild, moderate, or severe ranges. Thus, with only 1 spot stool specimen, the absence or presence of steatorrhea and its relative severity can be assessed with confidence. Consequently, baseline and posttreatment measurements of fecal fat excretion can be compared in patients with treatable forms of nutrient malabsorption, such as celiac sprue and exocrine pancreatic insufficiency, while avoiding the unpopular practice of having patients collect stools for 48 to 72 hours.

The original qualitative fecal fat microscopic method as reported by Drummey et al[7] was very sensitive at 97% but at the consequence of lower specificity, which was 86%. This means that 14% of persons without steatorrhea had a false-positive test result. In contrast, in our hands, the method of Drummey et al[7] applied to our 180 patients was highly specific at 99% but with a sensitivity of only 77%. Although the explanation for the difference of our results with those of Drummey et al[7] cannot be determined, several possibilities exist. These include interobserver technical differences, the different units of expression for fecal fat output used then and now (percentage of fat intake malabsorbed vs grams per day, respectively), and the possibility that the patient population of Drummey et al[7] differed from ours. Regardless of the true explanation, a specificity of 86% or a sensitivity of 77% is less than ideal for a diagnostic test. Although a low sensitivity (the results we obtained) would be particularly undesirable for this clinical setting because potentially treatable causes of malabsorption might be missed, a specificity of 86% also would be problematic because 14% of patients would undergo an esophagogastroduodenoscopy with small intestinal biopsy, small intestinal radiography, and/or a test of pancreatic function or anatomy (which by some would include an esophagogastroduodenoscopy with small intestinal biopsy, small intestinal radiography, and/or a test of pancreatic function or anatomy (which by some would include an endoscopic retrograde cholangiopancreatography) unnecessarily. Our new method circumvents these problems since it had a sensitivity of 94% and a specificity of 95%, with similar positive and negative predictive values.

It is pertinent to mention that all 6 of the patients with "falsely" negative fat microscopy results by our method but who had mildly elevated fecal fat output (8-13 g/d) did not have diseases normally thought of as malabsorptive syndromes. Steatorrhea in this range has been shown to occur even in healthy persons when they have diarrhea induced with laxatives.[4] A related fact is that the 4 patients in the present study with falsely positive quantitative microscopy results had very low collected stool weights for patients with chronic diarrhea (225-275 g/d), suggesting the possibility that fecal collection was incomplete or, alternatively, that with smaller fecal outputs, a 24- to 72-hour collection period may not be long enough to accurately assess average daily fecal fat output. Regardless of whether the quantitative microscopy results for these 4 patients were falsely positive or their measured fecal fat outputs were falsely negative, the fact is that the microscopic total size-number products were only mildly above the upper limit of normal of 200, with values less than 500. Therefore, if 500 was considered the lower limit of disease, the range above which would have a 100% specificity for steatorrhea regardless of fecal weight, then values between 200 and 500 could be considered indeterminate for steatorrhea (even though 25 of 29 patients with size-number products in this range had mild steatorrhea). Patients found to have microscopic fecal fat total size-number products in this "indeterminate range" subsequently could undergo a quantitative stool collection to confirm the presence of steatorrhea before undergoing endoscopic or radiographic tests for malabsorption.

There have been several appraisals of fecal fat microscopy in the medical literature. One of the earliest studies of which we are aware found microscopic estimates of fecal fat to have fairly reliable diagnostic accuracy in infants and children when compared with chemical analysis by old gravimetric methods.[9] However, Weijers and van de Kamer wrote in the 1950s that fecal fat microscopy left "very much to be desired" when compared with fecal fat concentration values measured by their titration method.[10] More recent authors, on the other hand, have found qualitative Sudan microscopy to be a worthwhile test.[11,12] However, these studies have involved relatively few patients and required special diagnostic materials not widely available[11] or studied only artificial fecal media.[12] In one of these studies,[11] an attempt was made to correlate the concentration of fecal fat with microscopic results, but this was done by progressively diluting a single stool specimen rather than correlating measured fecal fat concentration values in stools from different patients.

Interest has reemerged in the so-called steatocrit, which seems to be a relatively effective screening test for steatorrhea but requires centrifugation and chemical manipulation of stool.[13-16]

Since previous reports of the diagnostic accuracy of fecal fat microscopy used multiday fecal samples for analysis,[7,9,11] we thought it was necessary to confirm that results obtained from analysis of spot samples were accurate if the method is to be used optimally by avoiding the difficulty of quantitative stool collection. To evaluate this, we asked 21% of our patients to collect 2 spot specimens, 1 before and 1 at the end of their 2- or 3-day stool collection period, the latter of which was conducted while patients ate a high-fat diet. The results of the microscopic analysis of both sets of spot specimens were nearly identical and as accurate as that performed in the 2- to 3-day collection homogenate; mild steatorrhea was missed in only 1 specimen in each spot specimen group. However, since these falsely negative spot tests were not from the same patient (ie, the 1 time that the first spot specimen was negative, the second was positive, and vice versa), and all other analyses were 100% concordant, we can conclude that placing a patient on a high-fat diet before collecting a spot specimen for fecal fat microscopy is unnecessary, and a negative microscopic result in 2 randomly collected stool specimens can rule out clinically significant steatorrhea with full confidence.

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