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# Measurement of Fecal Lactoferrin as a Marker of Fecal Leukocytes

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**While diarrheal illnesses are extremely common in communities and hospitals throughout the world, an etiologic diagnosis may be expensive and cost-ineffective. Although the presence of fecal leukocytes are helpful in the diagnosis and specific therapy of inflammatory diarrheas, this requires prompt microscopic examination of fecal specimens (preferably obtained in a cup rather than a swab or diaper) by a trained observer. We developed a simple, sensitive test for the detection of leukocytes in fecal specimens using antilactoferrin antibody. Whereas radial immunodiffusion detected 0.02 μg of lactoferrin (LF) per μl or ≥2,000 leukocytes per μl, latex agglutination (LA) readily detected ≥0.001 μg of LF per μl or ≥200 leukocytes per μl added to stool specimens. Despite the destruction or loss of morphologic leukocytes on storage for 1 to 7 days at 4°C or placement of specimens on swabs, measurable LF remained stable. Initial studies of stool specimens from six patients with *Salmonella* or *Clostridium difficile* enteritis were positive and those from three controls were negative for LF by LA. Of 17 children in Brazil with inflammatory diarrhea (≥1 leukocyte per high-power field), 16 (94%) had LF titers of >1:50 by LA, whereas only 3 of 12 fecal specimens with <1 leukocyte per high-power field on methylene blue examination and none of 7 normal control specimens had an LF titer of >1:50 by LA. Of 16 fecal specimens from patients with *C. difficile* diarrhea (cytotoxin titers, ≥1:1,000), 95% ($n$ = 15) had detectable LF by LA (in titers of 1:100 to 1:800). Finally, of 48 fecal specimens from healthy adult U.S. volunteers before and after experimental shigellosis and of 29 fecal specimens from children with documented shigellosis and hospitalized controls in northeastern Brazil, fecal LF titers ranged from 1:200 to ≥1:5,000 in 96% (25 of 26) samples from patients with shigellosis (and reported positive for fecal leukocytes), while 51 controls consistently had fecal LF titers of ≤1:200. We conclude that fecal LF is a useful marker for fecal leukocytes, even when they are morphologically lost on swab specimens or when they are destroyed on transport or storage or by cytotoxic fecal specimens.**

Diarrheal illnesses are extremely common throughout the world, causing 2 to 16 or more illnesses per person per year in developed and developing countries (7, 15) and often posing diagnostic and therapeutic questions for physicians. The causes of diarrhea include a wide variety of etiologic agents, many of which have been recognized only in the past two decades (7). However, these agents do not need to be exhaustively sought in every instance of this common problem (6, 23), and the cost of indiscriminate use of etiologic studies for diagnosis is prohibitive. The cost for each positive routine stool culture result has exceeded $900 to $1,000 (8, 11).

An important diagnostic clue in considering whether diarrhea is a noninflammatory or an inflammatory process is the examination for fecal leukocytes (4, 9, 12). If fecal leukocytes are present, they suggest an inflammatory process caused by *Salmonella* species, *Shigella* species, *Campylobacter jejuni*, or *Clostridium difficile*. Although the majority of cases are noninflammatory (rotaviruses, Norwalk-like viruses, enterotoxigenic *Escherichia coli*) and often respond to simple oral rehydration therapy, it is important to distinguish the invasive, inflammatory diarrheal illnesses that may be more severe and that should be the focus of more extensive diagnostic studies and cultures and/or antimicrobial therapy (8). However, the methylene blue examination for fecal leukocytes requires that the physician or a skilled microscopist promptly examine under a microscope mucus from a fecal specimen in a cup. The specimen is stained so that leukocytes are clearly distinguishable in the fecal debris. This requires the immediate availability of a skilled person with a microscope to stain and examine fresh fecal specimens in the clinic or emergency areas where the patient is seen. It may also be difficult to obtain fecal specimens in a cup (these specimens are superior to swab or diaper specimens for examination of fecal leukocytes; swab or diaper specimens are only 44% sensitive, whereas cup specimens are 95% sensitive for fecal leukocyte detection by methylene blue examination of specimens from patients with culture-documented shigellosis [12]). Therefore, a method that remains sensitive when swabs are used and that remains sensitive when specimens are transported or stored (overnight) would be helpful and might allow a wider application of a selective diagnostic and treatment algorithm.

We developed a simple in vitro test for a leukocyte marker that is highly sensitive to the numbers of fecal leukocytes typically found in inflammatory diarrheal specimens and that can be quickly and easily done either in the clinic or later (after transportation or storage) in the laboratory. Only a minimum amount of training is required to learn how to perform the test.

We initially explored the leukocyte marker leukocyte esterase (an enzyme used in detecting leukocytes in the urine) (16) and found that, in contrast to its usefulness in testing leukocytes in urine, normal fecal specimens gave a positive result, so we abandoned further studies with leukocyte esterase. Instead we found that lactoferrin, an iron-binding glycoprotein found concentrated in secondary gran-

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ules in leukocytes (10, 19), was not readily detected in normal stool specimens unless neutrophils were added. The neutrophils were then readily detected in fecal specimens when lactoferrin was used as a marker. While we initially demonstrated the feasibility of detecting leukocytes in fecal specimens using a radial immunodiffusion assay for lactoferrin, the greater sensitivity and speed of latex agglutination led to a focus on that method, as noted below.

## MATERIALS AND METHODS

**Preparation of latex beads.** Latex beads (Bacto-Latex 0.81 beads; Difco Laboratories, Detroit, Mich.) were coated with rabbit anti-human lactoferrin (product L-3262; Sigma Chemical Company, St. Louis, Mo.) as follows: 2.5 ml of beads was centrifuged at 1,800 × *g* for 30 min, washed with 5 ml of glycine buffer (7.3 g of glycine and 10 g of NaCl in 1 liter of distilled water adjusted to pH 8.2 to 8.3), and then resuspended in 5 ml of glycine buffer to provide an approximately 1% suspension of beads. To this latex bead suspension was added 0.35 ml of undiluted rabbit antilactoferrin antibody, to provide a 7% antibody dilution in the bead suspension. The mixture was incubated at 38°C for 1 h, after which the antibody-coated beads were spun and resuspended in 5 ml of buffer to which 0.005 g of azide (0.1%) and 0.05 g of bovine serum albumin (1%) were added. The coated bead suspension was then stored at 4°C until use. Twenty microliters of this antibody-coated latex bead suspension was mixed on a microscope slide with 20 μl of sample, and agglutination was graded after 2 min with an unaided eye as follows: 0, no agglutination; ±, barely detectable, trace agglutination with a milky background; 1+, definite, fine agglutination with a milky background; 2+, definite, fine agglutination with a clearing background; 3+, larger agglutination with a clear background.

For a negative control, latex beads were prepared as described above, but rabbit anti-human lactoferrin was not added. The assay has been licensed and is being developed as a commercial product by Techlab Inc., Blacksburg, Va. (a patent is pending).

**Isolation of neutrophils.** Neutrophils were obtained from normal heparinized (10 ml) venous blood by a one-step Ficoll-Hypaque separation procedure (Neutrophil Isolation Medium; Los Alamos Diagnostics, Los Alamos, N.M.) (3). The polymorphonuclear leukocytes (PMNs) were washed three times with Hanks balanced salt solution (HBSS). Residual erythrocytes were lysed by hypotonic lysis with 3 ml of iced 0.22% sodium chloride solution for 45 s and then with 0.88 ml of 3% sodium chloride solution, this was followed by the addition of 5 ml of HBSS and centrifugation. A 1:2 suspension of PMNs in diluted stool was prepared by using normal stool of mixed with 1 ml of HBSS to make a cloudy suspension and adding 0.5 ml of previously counted PMNs to 0.5 ml of the stool suspension. A 1:2 suspension of PMNs in HBSS was prepared as a control for comparison with fecal suspensions.

To standardize our antibody-coated latex bead preparation, we determined dose-response curves using a solution of lactoferrin (L-0520; Sigma) diluted in HBSS.

**Testing of PMNs in fecal specimens after refrigeration or placement on a swab.** To test for PMNs in specimens that were refrigerated or placed on swabs, aliquots of previously counted PMN-stool suspensions were refrigerated or placed on swabs for the indicated times. For swab specimens, 130 μl (we found that this volume saturates the swab) of a previously counted PMN-stool suspension was used to saturate a rayon-tipped swab (Culturette II collection and transport system; Marion Scientific, Kansas City, Mo.). The swab was then placed in 390 μl of HBSS and shaken, and the excess fluid was squeezed out to make a calculated 1:4 dilution that we could examine for leukocytes directly and by the lactoferrin assay.

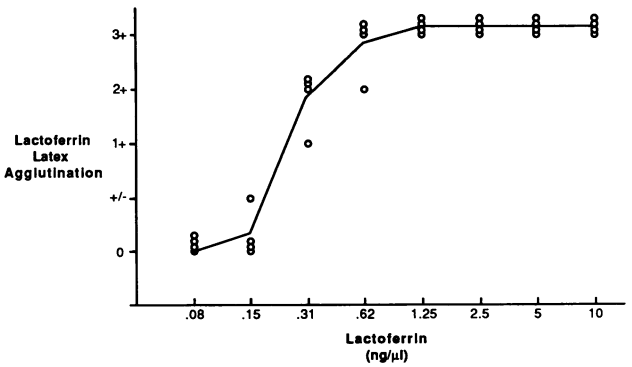


FIG. 1. Sensitivity of the latex agglutination assay for lactoferrin. Data represent the mean (line) and individual results from four different experiments (○) by using two different preparations of lactoferrin and three different preparations of antibody-coated beads.

PMNs were quantified morphologically at time zero and at subsequent intervals by using a ruled Neubauer-type hemacytometer chamber. To aid in visualizing the nuclei, methylene blue stain was incorporated into the counting solutions (20 μl of solution, 20 μl of methylene blue, 160 μl of HBSS).

## RESULTS

**Sensitivity of lactoferrin latex agglutination assay for lactoferrin.** As shown in Fig. 1, the lactoferrin latex agglutination assay done with three different antibody-coated bead preparations was sensitive to less than 1 ng of purified lactoferrin per μl, with readily apparent agglutination of the latex beads. The lowest concentration of lactoferrin that consistently gave 1+ or greater agglutination was 0.31 ng/μl, a concentration that, from previously published data (10), would be expected to be present in 60 PMNs per μl or 60 PMNs per $mm^3$, a number much lower than that in normal peripheral blood and substantially lower than that expected in an inflammatory fecal specimen.

**Sensitivity of lactoferrin latex agglutination assay for PMNs in HBSS or stool suspensions.** By using human PMNs that were separated by Ficoll-Hypaque and suspended in HBSS or normal stool suspensions, the sensitivity of the lactoferrin latex agglutination assay that gave detectable trace agglutination was 60 to 140 PMNs per μl, and in stool specimens, 120 to 280 PMNs per μl gave definite 1+ to 2+ agglutination (Fig. 2), with slightly greater sensitivity seen when the PMNs were suspended in stool specimens than when they were suspended in HBSS. The stool specimens, like the detergent Triton X-100 (0.1%), therefore appeared to release lactoferrin. There was no further increase in the sensitivity for PMNs in fecal suspensions when 0.1 or 1% Triton X-100 was used. This number of PMNs was in the range expected from the assay sensitivity for lactoferrin and, again, was substantially below that which would be expected from microscopic examination of inflammatory fecal specimens.

To compare the feasibility of detecting leukocytes by morphologic counts or by latex agglutination for lactoferrin

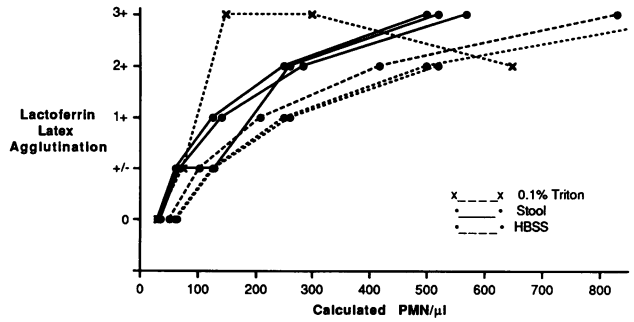


FIG. 2. Sensitivity of the lactoferrin latex agglutination assay for PMNs in HBSS or stool suspensions. Each line represents a separate experiment. Stool suspensions (like Triton X-100) appear to release lactoferrin and, thus, increase the sensitivity of the lactoferrin latex agglutination assay. The sensitivity of the lactoferrin latex agglutination assay that gave definite fine agglutination (1+) was a lactoferrin content of 120 to 240 PMNs per µl.

after storage in a refrigerator or on swabs, the number of PMNs in the suspensions were counted and then the PMNs were placed in 1% Triton X-100 or in a cloudy suspension of normal stool or both. Then, we reexamined the suspension for PMN counts by microscopy and lactoferrin titer by the latex agglutination assay immediately and after storage in a refrigerator or on swabs for 1 to 6 days (Fig. 3 and 4). In contrast to the lability of PMN numbers and morphologies seen after refrigeration, lactoferrin titers were remarkably stable even when the stool or Triton X-100 suspensions were refrigerated for several days.

There was a striking loss of PMN numbers after PMNs were placed on swab specimens (Fig. 4). In contrast, as quantified by determining the lactoferrin titer of leukocytes in fecal suspensions placed on swab specimens remained stable. Lactoferrin titers were relatively stable after PMNs were placed in a fecal suspension and then placed on rayon-tipped swabs (in comparison with the lactoferrin titers that were determined before placement of the suspension on swabs). In contrast, the number of morphologically evident leukocytes was extremely variable, with a mean loss of 3 to 4 log units after placement on a swab for 1 to 6 days.

**Clinical studies.** Initial pilot studies of stool specimens from six patients with *Salmonella* ($n$ = 2) or *C. difficile* ($n$ =

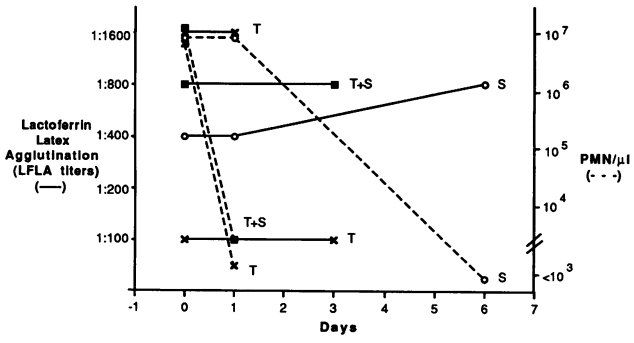


FIG. 3. Stability of lactoferrin latex agglutination assay (LFLA) titers after refrigeration of specimens for 1 to 6 days. Each line represents a separate experiment. In contrast to the lability of PMN numbers and morphologies seen in paired experiments, lactoferrin titers were remarkably stable, even when solutions were refrigerated for several days. T, 1% Triton X-100; S, stool specimen.

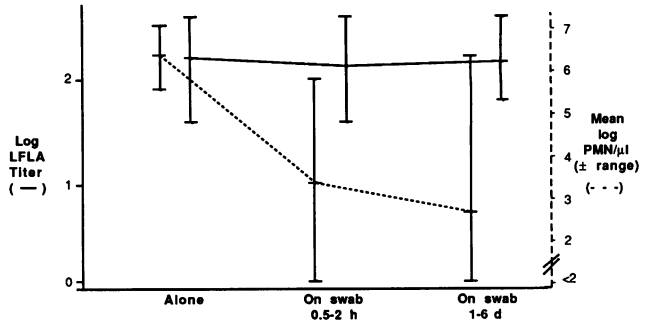


FIG. 4. Stability of lactoferrin latex agglutination assay (LFLA) titers despite the instability of PMN counts with swab specimens in 1 to 6 days. "Alone" indicates PMN counts and lactoferrin titers determined in the fecal-PMN suspension just before their placement on the swabs. Lactoferrin was consistently detected in swab specimens with minimal or no loss, in contrast to the striking variation with a mean 3- to 4-log-unit loss of morphologic PMN counts after placement on swab specimens. Results are the means and range of three and four different experiments for PMN and lactoferrin measurements, respectively.

4) enteritis were positive and three control stool specimens were negative for lactoferrin by latex agglutination, thus demonstrating the feasibility of using this test to examine patient fecal specimens. In preliminary studies to test whether the lactoferrin latex agglutination method correlated with evidence of fecal leukocytes on microscopy of methylene blue-stained specimens in a field setting, fecal specimens in cups from 29 children with acute diarrhea presenting to a Rehydration Center in Fortaleza, Ceará, Brazil, where stool cultures are not routinely available, were promptly examined. Of 17 children whose specimens had one or more leukocytes per high-power field on microscopic examination, we determined by latex agglutination that 16 (94%) had fecal lactoferrin at a titer of greater than or equal to 1:50. In only 3 of 12 children (25%) whose specimens had less than one leukocyte per high-power field was lactoferrin present at a titer of greater than or equal to 1:50. In addition, none of the seven normal control specimens from healthy individuals without diarrhea in Charlottesville, Va., was positive for lactoferrin at a titer of greater than or equal to 1:50. When we systematically examined by cell culture assay 16 fecal specimens from patients with nosocomial diarrhea at the University of Virginia Hospital that had *C. difficile* cytotoxin titers greater than or equal to 1:1,000, we found by latex agglutination that 95% (15 specimens) had lactoferrin titers that ranged from 1:100 to 1:800, even though PMNs were not seen in 6 of 9 of these specimens that were promptly examined after methylene blue staining. Of 14 fecal specimens with cytotoxin titers of 1:10 to 1:100, 9 (64%) had moderate lactoferrin titers (1:100 to 1:400), while only 1 of 16 specimens (6%) with no detectable cytotoxin had a lactoferrin titer of >1:50 (1:100).

Finally, we tested fecal specimens from patients with culture-documented shigellosis from the University of Maryland Center for Vaccine Development and Hospital das Clinicas in Fortaleza, Ceará, Brazil (Fig. 5). In contrast to 0 of 34 fecal specimens from healthy control adults in the United States that had a titer of >1:50 or 0 of 17 hospitalized children without diarrhea in Fortaleza with fecal lactoferrin titers of >1:200, in 25 of 26 (96%) fecal specimens from adult

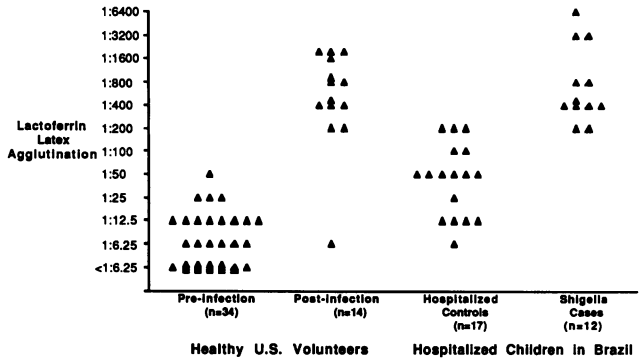


FIG. 5. Fecal lactoferrin titers in controls and patients with both experimental and naturally occurring acute shigellosis. (A) Lactoferrin latex agglutination assay titers in the stools of 34 different healthy adult volunteers before experimental challenge in vaccine studies and in 14 individuals who developed acute inflammatory diarrhea after experimental *Shigella flexneri* infection. (B) Lactoferrin latex agglutination assay titers in the stools of 12 children with acute, culture-documented shigellosis and 17 hospitalized control children without diarrhea in Fortaleza, Ceará, Brazil.

and pediatric patients with documented shigellosis with fecal leukocytes on examination by methylene blue staining, fecal lactoferrin titers were ≥1:200 (ranging to ≥1:5,000).

## DISCUSSION

Results of the studies described here demonstrate the practical feasibility of using latex agglutination for determination of fecal lactoferrin as a simple, semiquantitative marker for PMNs in fecal specimens. Lactoferrin latex agglutination provided a highly sensitive means to quantify PMNs in fecal specimens. It detected lactoferrin at <1 ng/μl and <200 PMNs per μl, a number far less that the expected number of PMNs in inflammatory fecal specimens. Although fecal leukocytes have long been recognized as being of diagnostic value, there is little information on the expected number of PMNs in inflammatory fecal specimens. Reported numbers of leukocytes have ranged from multiple cells on five or more high-power fields (18) or two or more leukocytes per high-power field seen in 86 to 91% of patients with *Shigella*, *C. jejuni*, and *Salmonella* infections (versus 0 to 2% for patients with viral, *Giardia*, or toxigenic bacterial diarrhea) (1) to >50 leukocytes per high-power field reported in fecal specimens from 39 to 85% of patients with shigellosis (20, 21). Speelman et al. (20) noted that the mean number of leukocytes per cubic millimeter determined by hemacytometer counts was 28,700 ± 4,300 for 33 patients with shigellosis (correlated with a mean of 81.6 ± 6 leukocytes per high-power field). Therefore, one leukocyte per high-power field would equate to approximately 350 leukocytes per $mm^3$. Therefore, our 1:50 dilution would represent a threshold of approximately 8 to 30 leukocytes per high-power field (based on the sensitivity of our test of 60 to 200 leukocytes per μl). Lactoferrin is found in specific (secondary) granules in PMNs and is not found in lymphocytes or monocytes (5, 13, 14). Although lactoferrin is present in several bodily secretions, the amounts range from 4.7 to 26 ng/μl in saliva (22) to 3.8 to 218 μg/mg of protein in vaginal mucus (depending on the time from the patient's last menses [2]) and are substantially lower than the amounts of lactoferrin expected from the number of leukocytes typically seen in patients with recognized inflammatory enteritis. At our 1:50 screening dilution, we may detect as little as 15 ng of lactoferrin per μl, or about 3,000 PMNs per μl. In our pilot field studies in Brazil, this correlated with ≥1 PMN per high-power field. Although Stoll et al. (21) noted that 99% of 304 stool specimens from patients infected with *Shigella* species, 96% of 262 stool specimens from patients infected with *C. jejuni*, and 98% of 137 stool specimens from patients with *Entamoeba histolytica* showed >1 leukocyte per high-power field, 20 to 29% of patients with rotaviral, enterotoxigenic *E. coli*, and cholera (all presumably noninflammatory diarrhea) had 11 to 20 leukocytes per high-power field, suggesting that the threshold separating patients with primary inflammatory diarrhea from those with noninflammatory diarrhea may be higher in areas where multiple bacterial and parasitic infections are common. Harris et al. (9) noted >25 PMNs per high-power field in 68% of patients with *Shigella* or invasive *E. coli* colitis.

Lactoferrin was stable in fecal specimens, even after transportation, storage, swab, or toxin destroyed the leukocyte morphology. Preliminary experience showed that in patients with inflammatory diarrheas caused by *Salmonella* species and *C. difficile* (cytotoxin positive, antibiotic-associated diarrhea), lactoferrin was readily detectable by this method. Field studies showed that for 94% of patients (16 of 17 patients) with fecal specimens with one to five or more fecal PMNs per high-power field on methylene blue staining, lactoferrin latex agglutination titers were >1:50. According to the sensitivity of our assay, this corresponds to 3,000 to 12,000 PMNs per μl, a concentration range that would be expected to correlate with one or more PMNs per high-power field in a blood smear. Furthermore, we found a correlation of *C. difficile* toxin titers with lactoferrin positivity, despite an apparent lack of morphologically evident PMNs in many of the toxin-containing stools. Finally, in contrast to none of 51 controls (34 healthy U.S. adult volunteers and 17 children hospitalized with diagnoses other than diarrhea in Fortaleza) who had fecal lactoferrin titers of >1:200, 96% (25 of 26) of specimens from patients with acute shigellosis (under experimental or field conditions) and fecal leukocytes reported on initial examination by methylene blue staining had fecal lactoferrin titers of ≥1:200. The one specimen reported on initial examination to have leukocytes but low lactoferrin concentrations was unconfirmed, because examination of all the frozen specimens failed to reveal leukocytes, despite their presence in large numbers in the initial examination of most specimens. A possible explanation for this includes a false-positive reading of the wet mount for fecal leukocytes (false-positive wet mount readings are well documented when fresh fecal specimens are also fixed for subsequent staining and examination by oil-immersion microscopy by additional trained microscopists and for methylene blue staining and lactoferrin studies). Alternatively, frozen fecal specimens may not always preserve lactoferrin or an inhibitor may rarely be present. The controls show that, whereas healthy adults who reside in the United States consistently have fecal lactoferrin titers of ≤1:50, children without diarrhea may have fecal lactoferrin titers of as high as 1:200, perhaps reflecting a subclinical inflammatory response to a common enteric parasite such as *Ascaris* species, *Trichuris* species or hookworm (17) or to subclinical malabsorption of milk, salivary, or serum protein. In this setting, the fecal lactoferrin test may help to define mild subclinical inflammatory enteritis, and the threshold for highly significant inflammatory enteritis may thus be interpreted more cautiously, perhaps with a border-

line zone of titers ranging from 1:50 to 1:200 suggesting mild inflammation or protein malabsorption. While breastfed infants are much less likely to develop serious diarrhea, we tested stool specimens from four healthy breastfed infants in Charlottesville, Va., in whom lactoferrin titers ranged from <1:50 to 1:100. Additional studies with larger numbers of culture-documented inflammatory and noninflammatory etiologies of diarrhea are needed. However, the lactoferrin latex agglutination test correlates with evidence of PMNs in fecal specimens determined by methylene blue staining, and lactoferrin remains intact even when the leukocyte morphology is destroyed on transfer or storage or by cytotoxic fecal specimens. Furthermore, it is of considerable practical importance that the lactoferrin assay can detect PMNs in specimens obtained on swabs that adsorb most morphologically detectable PMNs.

In conclusion, fecal lactoferrin can readily be detected by a simple latex agglutination assay and provides a useful marker for inflammatory diarrhea, even when fecal leukocytes are destroyed by transportation, storage, swab, or toxins. The ultimate utility of this assay in clinical or field settings and its potential application to specimens other than stool specimens require prospective clinical studies in different field, hospital, clinical, and laboratory settings.

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