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Received 28 September 1995/Returned for modification 3 November 1995/Accepted 22 January 1996

Infectious diarrhea ranks second only to acute upper respiratory illnesses in terms of the number of individuals affected throughout the world (3). There is a wide array of reported etiologies, ranging from noninvasive, self-limited pathogens to more aggressive, inflammatory pathogens. The management of the former may be as simple as rehydration and electrolyte replacement, while the treatment of the latter may require specific antimicrobial therapy or involve a prolonged hospital course. Culturing the stool in question and determining the responsible pathogen can simplify the management of diarrhea. However, results are often delayed, and studies have shown that the yield from these tests is quite low. Of 2,468 patients whose stools were tested at the Massachusetts General Hospital, Koplan et al. reported that only 2.4% of cultures were positive (9). Of 2,020 patients tested at the University of Virginia Hospital, Guerrant et al. noted that only 1.5% of the cultures were positive (7), making the use of stool culture as a screening tool prohibitively expensive. For example, the costs per positive result in these two above-mentioned studies were $952 and $1,200, respectively. A rapid, simple method of identifying the majority of specimens that are more likely to yield an invasive pathogen on routine culture for Salmonella, Shigella, and Campylobacter spp. would greatly reduce costly, unnecessary cultures and greatly improve the quality of the stool culture.

Microscopic examination of stool samples prepared by methylene blue staining provides a clue in the diagnosis of acute diarrheal illness. The presence of fecal leukocytes in the smear suggests that the diarrhea has an inflammatory etiology, i.e., Shigella species, Salmonella species, Campylobacter jejuni, or Clostridium difficile (1, 2, 8, 10). This finding also suggests that a more serious illness is present and that further diagnostic workup, namely, stool culture and antibiotic therapy, may be indicated (7).

Studies performed at the University of Virginia have suggested that using fecal leukocyte detection with freshly obtained specimens to select those for culture can indeed increase the yield of positive results by as much as fivefold, with a simultaneous reduction in the cost per positive culture of from $1,000 to $150 (7).

Despite these promising results, there are several significant limitations to the use of fecal leukocyte detection as part of an algorithm for diagnosing infectious diarrhea. First, the microscopic smear must be performed on a fresh specimen, preferably collected in a cup rather than on a swab or a diaper. Swab specimens have been shown to be only 44% sensitive, while cup specimens are 95% sensitive in the detection of fecal leukocytes (10). Even if a fresh, cup specimen is collected, the result of the microscopy is largely dependent on the skill, experience, and bias of the microscopist.

To address these concerns, we have developed the lactoferrin latex agglutination (LFLA) assay and licensed this to Techlab, Blacksburg, Va. It is a simple in vitro test for a leukocyte marker that is highly sensitive to the presence of polymorphonuclear neutrophils. This test has been shown to consistently detect as little as 0.31 ng of purified lactoferrin per μl and as few as 120 to 280 polymorphonuclear leukocytes per μl (4). These are levels that are considerably below the level expected with inflammatory diarrhea. Furthermore, the test will continue to detect lactoferrin despite the morphologic loss of leukocytes that occurs with storage and refrigeration or placement on swabs (4). The test requires minimal training, and there is minimal subjectivity in the agglutination reading.

This assay has been tested against different types of experimentally induced diarrhea to determine whether it may have a potential use in distinguishing between inflammatory and non-inflammatory diarrheas (4, 12). Whereas 23 healthy controls and 10 patients with noninflammatory diarrhea caused by Vibrio cholerae consistently had LFLA titers of $<1:50, all 9 patients with inflammatory diarrhea caused by Shigella spp. and 11 of 12 patients with inflammatory diarrhea caused by C. difficile had titers of $≥1:50. All 7 patients with enteropathogenic Escherichia coli infections had titers of $≥1:100, while the titers of 17 patients with enterotoxigenic E. coli infections were

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consistent with a mild inflammatory process, ranging from 1:50 to 1:400 (12).

The purpose of this study was to evaluate the clinical usefulness of the LFLA assay in the detection of different types of naturally acquired diarrheal illnesses, including rotaviral and invasive bacterial diarrheas. With this added information, the role of the LFLA assay in the diagnostic algorithm for infectious diarrhea can be better defined and the indications for stool culture can be narrowed. In addition, this study compares the actual cost per positive culture in the experience of a busy community hospital and the cost per positive culture that would have been accrued if the LFLA assay had been used to guide the diagnostic workup.

MATERIALS AND METHODS

Sample collection. Upon the request of attending physicians, based on a clinical suspicion of bacterial or viral gastroenteritis, fecal samples were submitted to the laboratory. The patients from whom these samples were taken were located on the emergency ward, in the outpatient clinics, or on the inpatient, pediatric, or general medicine wards of Fairfax Hospital. All stools, collected between October 1994 and September 1995, were from patients who, if hospitalized, had been in the hospital for less than 72 h. Ten infants were breast-fed, and their stools were collected but were considered separately. Cultures were performed at Fairfax Hospital by using standard methods for abnormal stools only, i.e., stools that were loose or watery, bloody, or filled with mucus. The samples were tested for rotavirus with the Meritec latex agglutination assay (Meridian Diagnostics, Inc., Cincinnati, Ohio). The samples were then frozen, transported to the University of Virginia, and kept at −70°C until the lactoferrin assays were performed.

We retrospectively tested for lactoferrin all samples which were positive for Salmonella, Shigella, or Campylobacter spp. or for rotavirus (n = 38) and the eight samples which had sufficient material and had been negative by both culture and rotavirus testing. Altogether, the 46 patients with diarrhea included 28 pediatric (ages, 2 months to 18 years) and 18 adult (ages, 20 to 84 years) patients. Control samples were collected between February and September 1995 from the same inpatient and outpatient population as that used for the study samples. The control patients included 8 children (ages, 1 to 15 years) and 1 adult (age, 20 years). They were without diarrhea for a period of at least 2 weeks, were not on antibiotics, and were not breast-fed.

Determination of LFLA titers. The LFLA assay was performed according to the manufacturer’s instructions (Leukotest, Techlab). In brief, 1 drop (50 μl) of diluted sample and 1 drop (50 μl) of antibody-coated latex bead suspension were mixed on a disposable agglutination card. After 3 min of mixing with a clinical rotator, the agglutination cards were read with the unaided eye. A scale of + to ++++ was used as follows: +, definite, easily visible agglutination with a milky background; ++, agglutination with a white ring beginning to form at the perimeter of the liquid; ++++, greater agglutination with a more pronounced ring and a clearing background; ++++, highly pronounced agglutination with a highly pronounced ring and a clear background. Negative controls were performed simultaneously with a suspension of latex beads not coated with the antilactoferrin antibody plus the diluted stool sample. Each sample was tested initially at a 1:50 dilution which was prepared by mixing 50 μl of liquid stool with 2.5 ml of diluent (buffered protein solution containing 1% sodium azide). If the sample was semiformed, the dilution was calculated by weight. Serial twofold dilutions were performed, and the highest dilution at which definite agglutination was seen was recorded as the lactoferrin titer.

RESULTS

Figure 1 shows the LFLA titers obtained from the tested specimens and controls. The levels of inflammation were divided into three categories: none (titer of <1:50), mild (titers from 1:50 to 1:200), and high (titer of 1:400 or greater). Like numerous control specimens from healthy adult volunteer studies (4, 12), control specimens from patients without diarrhea had titers of less than 1:50, or no detectable inflammation. Those with rotaviral diarrhea had titers of ≤1:200, with 9 of 10 (90%) titers being less than 1:50. The presence of mild inflammation in one of these patients could reflect an unusual, mild
degree of inflammation with the rotavirus infection or an additional unknown pathogen that was missed by routine cultures. Of the 28 specimens that had positive cultures for Salmonella, Shigella, or Campylobacter spp., 26 (93%), including all 8 with Shigella or Campylobacter infection, had LFLA titers of $\geq 1:100$. Most (17 of 28, or 61%) had titers of $\geq 1:400$, suggesting high levels of inflammation. The two specimens that were culture positive for Salmonella spp. but negative for lactoferrin were from a 1-year-old patient who had no diarrhea and no fecal leukocytes but had a stool culture as part of a fever evaluation and from another 1-year-old, afibrile patient with apnea and watery diarrhea that was atypical for Salmonella enteritis. As colitis was not documented by endoscopy or histology, the significance of the Salmonella spp. isolated in these two patients remains unclear. Among the patients with negative cultures for Salmonella, Shigella, and Campylobacter spp. and with a negative assay for rotavirus, the specimens from two patients had titers of 1:100. One of these individuals was suspected of having disaccharidase deficiency, and biopsy of the small bowel showed eosinophilic infiltration, suggesting the presence of a noninfectious inflammatory process. The other patient may have had either another inflammatory process, such as inflammatory bowel disease, or an invasive pathogen that was missed on culture. Potentially invasive pathogens for which routine cultures are not done, such as Yersinia spp., certain Vibrio species, or enteroinvasive E. coli, may have been present, or the specimens in question may represent the relative insensitivity of culture for Salmonella, Shigella, and Campylobacter spp.

Figure 1 also summarizes the lactoferrin data for the 28 samples that were culture positive (Salmonella, Shigella, and Campylobacter spp.) for comparison with those of the 18 specimens from patients with rotaviral diarrhea and those with unknown etiologies despite tests for rotavirus and cultures. The sensitivity of fecal lactoferrin at $\geq 1:50$ is 93% for culture-positive diarrhea, while only 17% of culture-negative diarrhea specimens were positive for lactoferrin at $\geq 1:50$. At $\geq 1:400$, while the sensitivity falls to 61%, the specificity (albeit with small numbers) increases to 100% for a culturable invasive pathogen.

Slides were examined for 8 patients who were negative for rotavirus and negative by culture, for 10 patients who were positive for rotavirus and negative by culture, and for 2 patients who were positive for Salmonella spp. by culture. In the first group, 4 of 8 specimens were negative for both lactoferrin and fecal leukocytes; 2 of 8 specimens were negative for lactoferrin but showed one or two and three to five cellular forms approximating the size of neutrophils when observed at $\times 100$ magnification; and 2 of 8 specimens had lactoferrin titers of 1:100 but no definite leukocytes. In the rotavirus-positive group, 9 of 10 specimens were negative for lactoferrin, with fecal leukocyte readings (per $\times 100$ field) as follows: 4 specimens had no leukocytes, 4 specimens had one to two cellular forms approximating the size of neutrophils, and 1 specimen had five cellular forms. The last specimen in the second group had a lactoferrin titer of 1:200 and 5 to 10 cellular forms approximating the size of neutrophils per $\times 100$ field. In the Salmonella-positive group, both samples had elevated lactoferrin titers of 1:200, and they showed 6 to 10 and 11 to 15 definite polymorphonuclear leukocytes per $\times 100$ field. When 10 of these slides were coded and reviewed by two blinded investigators, only the 2 with Salmonella spp. (and lactoferrin titers of 1:200) had definite fecal leukocytes.

**DISCUSSION**

The results of this study confirm previous investigations proposing that the LFLA assay may have clinical utility in distinguishing between noninflammatory and inflammatory diarrheas (4, 12, 14, 16). In contrast to previous studies of experimental infections (4, 12, 14, 16), the present study was conducted with samples from patients with sporadic, community-acquired illnesses seen at a suburban, community hospital. Pathogens generally accepted as being invasive in their pathogenesis, including Salmonella, Shigella, and Campylobacter spp., did not have titers greater than or equal to 1:100 in 26 of 28 (93%) cases of those patients with rotaviral diarrhea. In 9 of 10 specimens (90%) had titers of $< 1:50$. These results are confirmed by our experience at the University of Virginia Hospital with the same pathogens from November 1994 to June 1995. All three Shigella samples had titers of $\geq 1:400$, while 2 of 3 Campylobacter samples had titers of 1:400 and the third had a titer of 1:200. Five of six rotavirus samples were negative for lactoferrin. The sixth rotavirus sample was positive at a titer of 1:200. Thus, it has once again been shown that the LFLA assay detects the vast majority of invasive diarrheas while excluding most of the noninvasive illnesses.

While microscopy is somewhat useful in predicting inflammatory diarrheas (1, 2, 8, 10), our study demonstrates that, in practice, it has many drawbacks relative to testing for fecal lactoferrin. Not only does microscopy require prompt examination of specimens obtained in cups (which is often difficult), but it requires careful examination by a skilled Microscopist. Even ignoring the problems with specimen decomposition that may compromise the reliability of stool smears (4), subjective interpretation of our sample slides, even by experienced microscopists, was fraught with difficulty. Definite polymorphonuclear leukocytes were few and sparse when present. The potential for either underestimating the number of leukocytes by using strict selection criteria or overcalling them by including oval or round cellular forms approximating the size of neutrophils cannot be overstated. The lactoferrin assay eliminates much of this subjectivity; only those agglutination reactions with definite, easily visible precipitation are considered positive. The level of lactoferrin (reflecting the number of leukocytes) that needs to be present to cause such a precipitation should certainly be exceeded by any significantly inflammatory diarrhea. For this reason alone (elimination of subjectivity), the fecal lactoferrin test may be superior to microscopic examination for fecal leukocytes. Furthermore, several studies have reported the lactoferrin assay to be considerably more sensitive than microscopy in detecting inflammatory diarrhea caused by invasive pathogens (12, 14, 16).

Regarding the lactoferrin-positive samples that were culture negative, these could have been due to an idiopathic inflammatory colitis or to an invasive pathogen, such as Yersinia spp. or invasive E. coli, that was not cultured. Indeed, the culture for Shigella spp. is relatively insensitive, as documented in one report in which 7 of 21 (33%) culture-negative, lactoferrin-positive specimens were found to contain Shigella spp. by PCR analysis (14).

Despite its superior sensitivity in the prompt detection of inflammatory diarrhea caused by recognized invasive pathogens, several limitations of the lactoferrin assay must be remembered. First, the LFLA assay should be avoided for infants who are being breast-fed, because it may give false-negative results in this situation. In the experience of Quiroga et al., 8 of 8 healthy children fed breast milk had positive titers for fecal lactoferrin, with five of the titers being $> 1:540$ (13). Of 17 healthy children drinking other types of milk, 17 were found to

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be negative for lactoferrin in the same study (13). The 10 breast-fed infants from our study (7 of these 10 had unknown culture results, and 4 were rotavirus positive) all had positive titers, ranging from 1:50 to 1:3,200. Six (60%) of these patients showed high levels of lactoferrin ($\geq 1:400$). In an additional three healthy, breast-fed infants from studies at the University of Virginia, titers were found to be elevated to $1:800$, whereas three healthy, non-breast-fed infants tested negative.

FIG. 2. Algorithm for the use of fecal lactoferrin in screening for inflammatory diarrhea (adapted from reference 5). WBC, leukocytes; ELISA, enzyme-linked immunosorbent assay. AR, attack rate; LT and ST, heat-labile and heat-stable toxins, respectively; AF, acid-fast stain; ORS, oral rehydration solution; EIEC, enteroinvasive E. coli; LGV, lymphogranuloma venereum.
positive for a titer of 1:50. Thus, the likelihood of false-positive results in breast-fed children must be recognized. Although infectious diarrheal illnesses are distinctly less common in breast-fed children (6), the LFLA test is not interpretable and should therefore not be used for specimens from children who are being breast-fed.

The other major caveat regarding interpretation of elevated fecal lactoferrin is that any inflammatory process will result in increased lactoferrin; i.e., elevation of fecal lactoferrin is not specific for any single invasive pathogen. Indeed, it may be useful in following the activity of idiopathic inflammatory bowel disease (15). However, in the patient without known inflammatory bowel disease who is suspected of having a bacterial diarrheal illness, those organisms for which routine cultures are obtained and for which antimicrobial therapy may be indicated are those that cause most inflammatory diarrheas (e.g., Salmonella, Shigella, and Campylobacter spp., and in the setting of recent antibiotic use, cytotoxicogenic C. difficile). Therefore, the LFLA assay, along with a careful medical history, may be extremely useful in screening for specimens that should be cultured for potentially treatable pathogens.

Thus, a reasonable proposal for the LFLA assay would be to use it as a screening tool for deciding when to perform a stool culture; i.e., cultures could be done only if the LFLA screen is positive at 1:50 (or if the physician specifically requests it because of exposure or severe, febrile illness), as suggested in the algorithm in Fig. 2 (5). For example, given that approximately 1,800 stool cultures are done per year at Fairfax Hospital (at $30 per culture), with an approximate yield of cultures positive for Salmonella spp., Shigella spp., or C. jejuni of 2.9%, if 83% of the 97% that are culture negative could be excluded by being LFLA negative at 1:50, this could save over $800 per positive result.

Additional testing of specimens positive at $1:50 for a titer of $1:400 would further enhance one’s confidence that a “highly” inflammatory process is present (albeit with the loss of some sensitivity for some invasive diarrheas). In our study, a titer of $1:400 was specific for the presence of invasive pathogens. At the University of Virginia Hospital, we have seen a stool culture positive for Salmonella spp. in an asymptomatic, lactoferrin-negative parent of a child with diarrhea, suggesting a carrier state. Thus, the clinical presentation (i.e., presence of fever, bloody diarrhea, contact history, etc.) should also help guide the decision concerning when to obtain a stool culture or to consider empiric antibiotic therapy.

In conclusion, the lactoferrin assay is a useful, quick, easy laboratory test that can be added to one’s armamentarium in the optimal, cost-effective diagnosis and management of infectious diarrhea. While it is not helpful for breast-fed infants, a negative result can mitigate the need for stool culture, while a positive result will greatly enhance the likelihood of a positive culture. The provision that the physician should be able to override a negative lactoferrin test and request culture anyway if epidemiologic (such as exposure to known infection) or clinical presentation (such as high fever) requires it should be added. In addition, a recent report notes that the lactoferrin Leukotest provided the best predictor for C. difficile diarrhea at Johns Hopkins Hospital (11). When one considers the prevalence of diarrhea in hospitals and communities around the world, the savings possible by limiting the number of unnecessary cultures is quite substantial, and the improved yield of invasive pathogens in specimens positive for fecal lactoferrin may greatly enhance a cost-effective approach to the diagnosis of microbial diarrheas.

ACKNOWLEDGMENTS

We thank Leah Barrett for assistance throughout this study. This work was supported in part by Techlab, Inc., Blacksburg, Va., and by a grant from Virginia’s Center for Innovative Technology.

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