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# Fecal lactoferrin as a parameter in determining invasive causes of acute diarrhea

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## Abstract

### Introduction

Diarrhea may be acute, which subsides by 7–14 days and is related to infections (bacterial or viral) or reaction to medications, or chronic, which lasts more than 2 weeks and is mainly caused by inflammatory bowel disease, functional bowel disorder, food allergies, or parasitic infestations. The infective etiologies may be noninvasive or invasive pathogens that have the capacity to invade intestinal mucosa and stimulate local inflammatory response [by activated neutrophils that secrete lactoferrin (LF)] and sometimes cause ulceration, hemorrhage, and systemic inflammatory response. Stool culture is the standard method for the diagnosis of invasive diarrhea; however, it is laborious and time consuming. LF is a major constituent of the secondary granules of neutrophils. It activates innate immunity and has microbicidal and anticancer effects. It is stable in feces for several days at room temperature and resistant to proteolysis. The presence of LF in the intestinal lumen is proportional to the flux of neutrophils, and its assessment can provide a reliable biomarker for inflammation and intestinal invasion.

### Aim

The aim was to evaluate the clinical usefulness of fecal lactoferrin (FL) assay in differentiating invasive from noninvasive types of acute diarrhea.

### Patients and methods

This cross-sectional study was performed on 90 children with acute diarrhea ( $\leq 1$  week). Their ages ranged 4–168 months, with a mean of  $48.18 \pm 39.89$  months. The patients were divided into two groups: group 1 included 31 patients with invasive diarrhea, and group 2 included 59 patients with noninvasive diarrhea.

### Results

Serum C-reactive protein was significantly higher in group 1 than group 2 (28.46 and 9.7 mg/l, respectively;  $t = 2.7$ ,  $P < 0.005^*$ ). Moreover, the neutrophilic count of white blood cells was significantly higher in group 1 than group 2 ( $9.2 \times 10^3$  and  $3.8 \times 10^3$ /dl, respectively;  $t = 1.79$ ,  $P < 0.05^*$ ). FL was significantly higher in group 1 than group 2 (16.8 and 6.4 ng/ml, respectively;  $t = 1.99$ ,  $P < 0.05^*$ ). There was a significant positive correlation between FL and neutrophilic count of white blood cells ( $r = 0.71$  and  $P < 0.005^*$ ) in group 1 and ( $r = 0.58$  and  $P < 0.005^*$ ) in group 2], and a strong significant positive correlation between FL and serum C-reactive protein [in group 1,  $r = 0.8$  and  $P$  less than 0.0001\* and in group 2,  $r = 0.62$  and  $P$  less than 0.0005\*).

### Conclusion

FL testing is a good useful noninvasive test in differentiating invasive from noninvasive acute diarrhea.

**Keywords:** Diarrhea, fecal lactoferrin, neutrophilic count

## INTRODUCTION

Diarrhea is a leading cause of malnutrition in children under 5 years of age [1]. It is defined as the passage of three or more loose or watery stools per day, more frequent passage than

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normal for the individual or a single loose stool, containing blood, pus, or mucus (invasive diarrhea) [2].

Diarrhea may be acute or chronic: acute diarrheal episodes subside by 7 up to 14 days, whereas chronic diarrhea lasts for more than 2 weeks [3].

Acute diarrhea usually is related to infection (bacterial or viral) or reaction to medications[4] whereas chronic diarrhea is caused by inflammatory bowel disease, functional bowel disorder such as irritable bowel syndrome, food intolerances or allergies, and parasites such as giardia [5].

The infective etiologies range from noninvasive, self-limited pathogens to more aggressive inflammatory pathogens. The management of the former may be as simple as rehydration and electrolyte replacement, whereas the treatment of the latter may require specific antimicrobial therapy or prolonged hospital course [6].

The main causative agents of infective pediatric diarrhea are bacteria such as *Shigella*, *Salmonella*, *Campylobacter*, *Escherichia coli*, or *Vibrio*, and parasites, such as *Giardia lamblia*, *Entamoeba histolytica*, or *Cryptosporidium*. Other less frequent organisms are *Listeria*, *Yersinia*, or *Clostridium difficile*. These pathogens have the capacity to invade the mucosa of the distal small intestine and colon, stimulate local and systemic inflammatory responses, and may sometimes cause hemorrhage and ulceration of the mucosa [7].

Unlike most diarrheal illnesses which are self-limited, invasive diarrhea necessitates extensive diagnostic studies and specific antimicrobial therapy to shorten the clinical course, to decrease the excretion of multiresistant organisms, and to prevent complications [8,9]. Stool culture is the standard method for diagnosis; however, it is tedious, laborious, and time consuming [10].

An intense intestinal infection involves intense infiltration of neutrophils, macrophages, mast cells, lymphocytes, and other inflammatory cells in the epithelial lining of colonic mucosa. These cells secrete various enzymes and metabolites, including myeloperoxidase and lactoferrin (LF), produced by activated neutrophils [11].

The presence of fecal leukocytes suggests an inflammatory process caused by invasive pathogens that also can invade mucosal cells causing hemorrhage, resulting in bloody diarrhea. If the organisms are noninvasive, they do not produce either leukocytes or hemorrhage [12].

LF (a major whey protein) is an 80 kDa iron-binding glycoprotein produced by many exocrine glands[13] It is a major component of the secondary granules of polymorphonuclear neutrophils that are released during the inflammatory process and is secreted by most mucosal membranes. During intestinal inflammation, polymorphonuclear neutrophils infiltrate the mucosa, resulting in an increase of LF concentration in feces [14]. LF displays diverse biological activities, ranging from the

activation of innate immunity, microbicidal effects, and anticancer cells [15].

LF is stable in feces for several days at room temperature, and even longer if the stool is refrigerated, and it is resistant to proteolysis [11]. The presence of LF in the intestinal lumen is proportional to the flux of neutrophils, and its assessment can provide a reliable biomarker for inflammation. It can be tested using commercial enzyme-linked immunosorbent assays [16].

## Aim

The purpose of this study was to evaluate the clinical usefulness of the LF assay in differentiating different types of acute diarrhea (invasive and noninvasive), so that early treatment could be started, unnecessary treatment could be avoided, and the indications for stool culture could be restricted.

## PATIENTS AND METHODS

This cross-sectional study was performed on 90 children with acute diarrhea (three or more loose or watery stools per day or a single loose stool containing blood, pus or mucus for a period of  $\leq 1$  week) from outpatient clinic, inpatient wards, or emergency pediatric ward of Banha Teaching Hospital during the period from February to December 2017. Their ages ranged from 4 to 168 months, with a mean of  $48.18 \pm 39.89$  months.

### Ethical considerations

The study purpose and procedures were explained to the parents, and written consents were obtained before the study.

Approval of the Local Ethical Committee in the Pediatrics Department and General Organization for Teaching Hospitals and Institutes was obtained before the study.

The authors declared no potential conflict of interest with respect to the research and publication of this article.

All data of the patients and results of the study are confidential, and the patient has the right to keep it.

The authors received no financial support for the research and publications of the article.

### Inclusion criteria

Pediatric patients with acute diarrhea ( $\leq 1$  week) irrespective of age or sex were included.

### Exclusion criteria

Patients with factors affecting gastrointestinal tract immunity or functions, such as malnutrition, vitamin deficiency, immunodeficiency, active inflammatory bowel disease, surgical causes of diarrhea, newborn or breast-fed babies (due to high breast milk LF), and secondary diarrhea owing to any systemic disease as respiratory tract infection and otitis media were excluded.

The patients were subjected to the following:

Complete history taking, including age, sex, residence, type of feeding, type and frequency of diarrhea, associated tenesmus,

history of fever, vomiting, abdominal pain, daily intake, appetite, manifestations of upper respiratory tract infection, history of repeated diarrhea, or operations.

Complete clinical examination included signs of dehydration and its degree if present, temperature recording, signs of malnutrition, associated systemic infections, abdominal tenderness, and signs of vitamin or micronutrient deficiency.

The patients were divided into two groups:

- (1) Group 1 included 31 patients with invasive diarrhea, diagnosed by the following:
  - (a) Bloody, mucoid diarrhea and/or clinical history of fever associated with abdominal pain, anorexia, vomiting, and/or signs of toxemia.
  - (b) Laboratory finding including high C-reactive protein (CRP) ( $\geq 12$  mg/l) [17] and leukocytic count ( $\geq 14 \times 10^3/\text{mm}^3$ ) [18].
  - (c) Stool containing blood cells and/or occult blood, leukocytes, and/or parasites such as giardia lamblia and amebae.
- (2) Group 2 included 59 patients with noninvasive diarrhea, diagnosed by the following:
  - (a) Watery diarrhea and/or vomiting, clinical history of mild, moderate or no fever or signs of toxemia, and mild to moderate dehydration.
  - (b) Laboratory finding including negative CRP ( $< 12$  mg/l), leukocytic count  $< 14 \times 10^3/\text{mm}^3$ , stool containing no blood cells or occult blood, no parasites, and leukocytes less than 5/HPF.

### Laboratory studies

Blood examination included the following:

- (1) White blood cells/differential counts.
- (2) CRP.

Stool examination included the following:

- (1) Direct microscopic examination within 2 h after collection for fecal mucus, leukocytes (the test was considered positive if  $\geq 5$  leucocytes were detected in 20 fields or  $> 10$  leucocytes in one field) [19], blood cells, and parasitic infestations (for trophozoites, cysts, helminthic ova, or larvae).
- (2) Stool culture using standard methods for pathologic bacteria excluding *Yersinia enterocolitica* and *C. difficile* owing to lack of suitable culture techniques or diagnostic kits in our hospital.
- (3) Fecal lactoferrin (FL) was determined by enzyme-linked immunosorbent assay.

### Methods

Stool examination including microscopic testing was done by two laboratory technicians and checked within 2 h after collection. Fecal occult blood was examined by using immunochemical occult blood test (OC-Sensor Diana; Eiken Chemical Co. Ltd. Tokyo, Japan). A result that was greater than or equal to 100 ng/ml was considered positive.

### Lactoferrin detection

LF assay employs a quantitative sandwich enzyme-linked immunoassay technique. Stool samples were prepared and analyzed for LF according to the manufacturer's instructions (Hycult Biotech, Netherlands). The samples were extracted using the 0.15 M NaCl extraction buffer. Overall, 5 ml of extraction buffer was added to 100 mg sample and then filtered to remove coarse particles ( $> 0.6$  mm). The filtrate was shaken and then centrifuged, and the supernatant was used for analysis. Samples were stored at  $-70^\circ\text{C}$  in polypropylene tubes to be used later. Measurable concentration range was 0.4–100 ng/ml [20].

### Statistical analysis

The collected data were statistically analyzed using the SPSS program for Windows (version 24; SPSS Inc., Chicago, Illinois, USA), and continuous variables were presented as means  $\pm$  SD. The relationship between FL levels in both groups and different laboratory parameters was determined using the Spearman correlation analysis and the linear regression method.

*P* less than 0.05 was considered statistically significant.

## RESULTS

Table 1 shows clinical data and laboratory findings of the studied groups.

Table 2 shows the enteric pathogen frequency. In group 1, bacterial pathogen frequency was 25% (19% single and 6% mixed) and parasitic infection as follows: Entamoeba 22.5%, Giardia 9.5%, other parasites 12%, and mixed infestations 25.8%. In group 2, bacterial pathogen frequency was 0% and parasitic infections as follows: Entamoeba 15%, Giardia 1.5%, other parasites 11%, and mixed infestations 13.5%.

Table 3 shows that FL was 16.93 ng/ml in group 1 and 6.47 ng/ml in group 2.

Fig. 1 shows that FL was higher in group 1 than in group 2.

Fig. 2 shows that there was a significant positive correlation between FL and neutrophilic count of white blood cells (WBCs) in group 1 ( $r = 0.71$  and  $P < 0.005^*$ ).

Fig. 3 shows that there was a strong significant positive correlation between FL and serum CRP in group 1 ( $r = 0.8$  and  $P < 0.0001^*$ ).

Fig. 4 shows that there was a significant positive correlation between FL and neutrophilic count of WBCs in the group 2 ( $r = 0.58$  and  $P < 0.005^*$ ).

Fig. 5 shows that there was a significant positive correlation between FL and serum CRP in group 2 ( $r = 0.62$  and  $P < 0.0005^*$ ).

Table 4 shows the following:

- (1) Serum CRP was 28.46 mg/l in the group 1 and 9.7 mg/l in the group 2. It is significantly higher in group 1 than group 2 ( $t = 2.7$ ,  $P < 0.005^*$ ).

(2) Neutrophilic count of WBCs was  $9.2 \times 10^3/\text{dl}$  in group 1 and  $3.8 \times 10^3/\text{dl}$  in group 2. It is significantly higher in group 1 than group 2 ( $t = 1.79$ ,  $P < 0.05^*$ ).

(3) FL was 16.8 ng/ml in group 1 and 6.4 ng/ml in group 2. It is significantly higher in the group 1 than group 2 ( $t = 1.99$ ,  $P < 0.05^*$ ).

**Table 1: Clinical data and laboratory finding of studied groups**

	Group 1 (n=31)	Group 2 (n=59)
Age (months)		
Range	9-168	4-98
Mean±SD	55.47±42.08	36.39±30.1
Sex (male/female)	19/12	30/29
Fever		
Duration	2-5	2-3
Level	38.3±0.93	37.9±0.9
Diarrhea		
Duration	2-4	1-3
Times/day	3-5	8-10
Type bloody and/or mucoid	++	-
Watery	-	++
Laboratory finding [n (%)]		
Fecal examinations	31 (100)	13 (7.67)
Positive leukocytes	29 (93.5)	0
Positive blood and mucous	7 (22.5)	0
Positive occult blood	14.3-21.3	5.2-13.8
Hematological examinations		
Leukocytic count ( $\times 10^3/\mu\text{l}$ ) (mean±SD)	15.93±1.67	7.9±2.11
Neutrophil count ( $\times 10^3/\mu\text{l}$ ) (mean±SD)	9.2±2.3	3.88±2.9
CRP (mg/l)	28.8±7.9	9.76±2.86

CRP, C-reactive protein.

**Table 2: Enteric pathogen frequency of studied groups**

Enteric pathogen	Frequency [n (%)]	
	Group 1 (n=31)	Group 2 (n=59)
Bacterial		
Single pathogen	6 (19)	0
Mixed pathogen	2 (6)	0
Parasitic		
Entamoeba (cyst or trophozoite)	7 (22.5)	9 (15)
Giardia (cyst or trophozoite)	3 (9.5)	1 (1.5)
Other parasites	4 (12)	7 (11)
Mixed	8 (25.8)	8 (13.5)

**Table 3: Fecal lactoferrin levels in group 1 and group 2**

	Group 1 (n=31)	Group 2 (n=59)
Fecal lactoferrin (ng/ml)	16.93±4.54	6.47±2

**Table 4: Summary of laboratory inflammatory markers of both groups**

	Group 1 (n=31)	Group 2 (n=59)	t	P
Serum CRP (mg/l)	28.46±7.8	9.7±2.8	2.7	<0.005*
Neutrophilic count of WBCs ( $\times 10^3/\text{dl}$ )	9.2±2.3	3.8±1.9	1.79	<0.05*
Fecal lactoferrin (ng/ml)	16.8±3.4	6.4±2	1.99	<0.05*

CRP, C-reactive protein; WBC, white blood cell.

## DISCUSSION

Although acute infectious diarrhea is a common clinical disease in children, few reliable and noninvasive diagnostic tools have been used [15].

The presence of fecal leukocytes is believed to suggest an inflammatory etiology and a more serious illness in patients with acute diarrhea, and further diagnostic workup may be indicated. However, the results of microscopy are largely dependent on the technician and the freshness of the specimen [21].

LF, an iron-binding glycoprotein, is a major constituent of the secondary granules of neutrophilic leukocytes and can be a useful marker for fecal leukocytes, as it can be detected even after the morphologic loss of leukocytes [14].

We measured FL in two groups of pediatric patients with acute diarrhea: group 1 included 31 patients with invasive diarrhea diagnosed by clinical history of bloody and mucoid diarrhea and fever associated with signs of toxemia with laboratory finding of CRP greater than or equal to 12 mg/l, white blood cell count greater than or equal to  $14 \times 10^3/\text{mm}^3$ , and stool containing blood cells and/or occult blood, leukocytes, and/or parasites. Group 2 included 59 patients with noninvasive diarrhea diagnosed by clinical history of mild, moderate, or no fever associated with watery diarrhea and/or vomiting, no signs of toxemia, a laboratory finding of CRP less than 12 mg/l, white blood cell count less than  $14 \times 10^3/\text{mm}^3$ , stool with no blood cells or occult blood, and leukocytes less than 5/HPF.

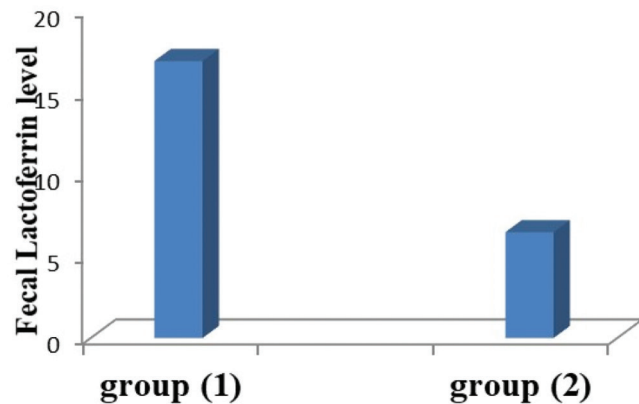
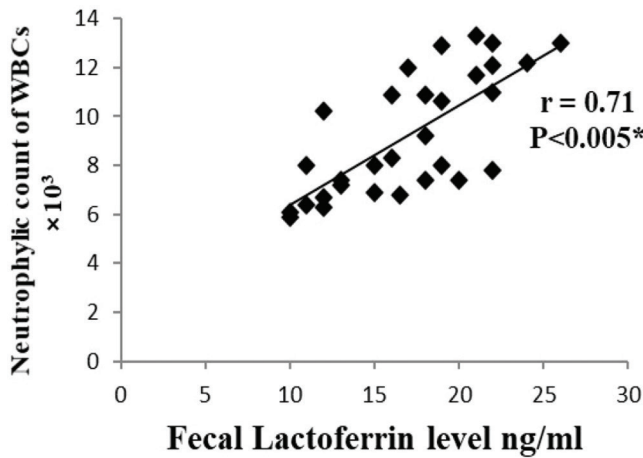
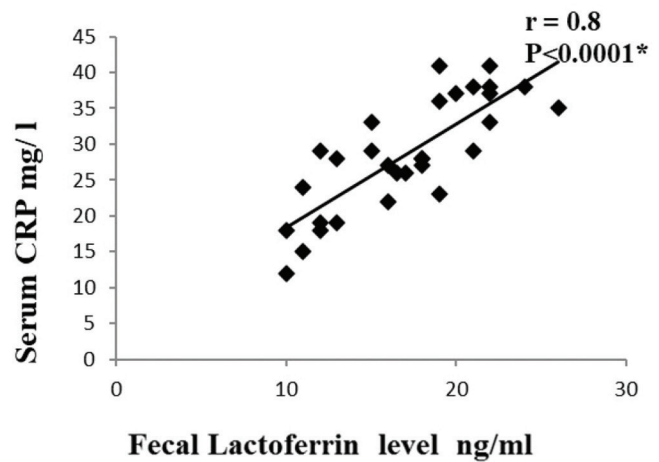


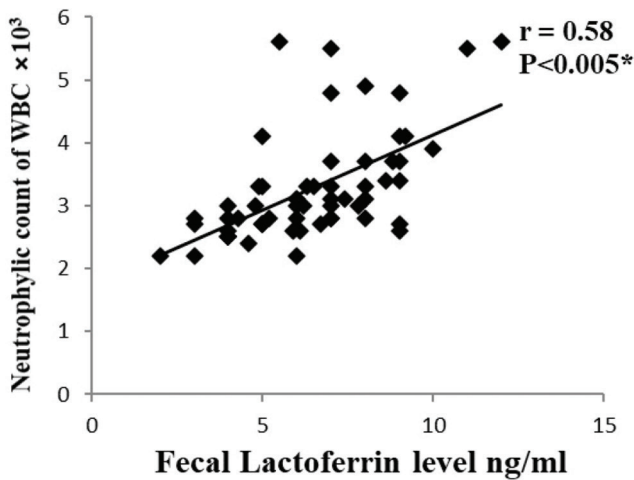
Figure 1: Fecal lactoferrin in group 1 and group 2.



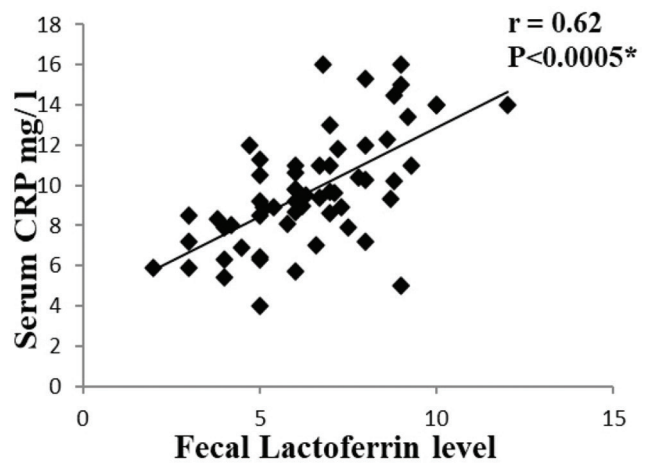
**Figure 2:** Correlation between fecal lactoferrin and neutrophilic count of white blood cells in group 1.



**Figure 3:** Correlation between fecal lactoferrin and serum C-reactive protein in group 1.



**Figure 4:** Correlation between fecal lactoferrin and neutrophilic count of white blood cells in group 2.



**Figure 5:** Correlation between fecal lactoferrin and serum C-reactive protein in group 2.

On comparing the LF level in both groups, we found that the LF level was higher in the group 1 ( $16.93 \pm 4.54$  ng/ml) than in group 2 ( $6.47 \pm 2$  ng/ml), and this difference was statistically significant ( $t = 1.99, P < 0.05$ ). LF was detected months after the disintegration of stool leukocytes, and it was positive because it is stable for long periods, so it can detect the presence of leukocytes in non-fresh fecal samples. These findings were in concordance with the study of Voravuthikunchai *et al.* [22], who stated the practical feasibility of using LF/hemoglobin as simple markers of invasive organisms of inflammatory diarrhea and demonstrated that LF and Hb titers are consistently present in the inflammatory process.

Moreover, Hayakawa *et al.* [13], who tested the sensitivity and specificity of different markers of intestinal inflammation associated with invasive pathogens (e.g. fecal leukocytes, occult blood in the stool, and FL) found that FL had the best diagnostic accuracy.

Chen *et al.* [15], found that the FL level is higher in bacterial gastrointestinal infections and lower in patients with viral

infections. In the same way, Lee *et al.* [21], found that FL can be a more useful clinical marker in patients with acute diarrhea than fecal leukocyte. Positive FL was significantly associated with the presence of fecal bacterial pathogen detection by multiplex PCR.

Similarly, Park *et al.* [23], reported that although LF was less sensitive in determining the bacterial etiology of acute pediatric diarrhea than CRP, it was better than the other fecal neutrophil-derived inflammatory biomarkers, including calprotectin, MPO, and PMN-e. Most importantly, the combination of CRP and LF showed a higher diagnostic capability for bacterial etiology in acute pediatric diarrhea compared with their use alone.

On testing the correlation of LF level with some systemic markers of invasion of GIT such as CRP or neutrophilic portion of WBCs, we found that there was a significant positive correlation between FL and CRP in both groups, but it was also higher in group 1 ( $r = 0.8, P < 0.0001$ ) than in group 2 ( $r = 0.62, P < 0.0005$ ), as CRP rises during the acute-phase response

upon stimulation by IL-6 and other cytokines originating at the site of inflammation, and reflects a summation of the systemic immune response. These findings were similar to the studies done by Kim *et al.* [24] and Lee *et al.* [21], who stated that CRP has moderate sensitivity and specificity to detect bacterial infection in children with fever and can discriminate inflammatory from non-inflammatory diarrhea in young adults. Similar findings were reported by Park *et al.* [23], in which they reported that CRP, FL, fecal leukocytes, and fecal occult blood were correlated with bacterial etiology in acute pediatric diarrhea.

Moreover, there was a significant positive correlation between FL and neutrophilic count of WBCs in both groups, but it was higher in group 1 ( $r = 0.71$ ,  $P < 0.005$ ) than in group 2 ( $r = 0.58$ ,  $P < 0.005$ ). These findings are comparable with the results of a previous report by Chen *et al.* [15], who showed an increase of FL during bacterial infection and association with greater disease severity.

## CONCLUSION

FL testing is a good useful noninvasive test in differentiating invasive from noninvasive acute diarrhea. A negative result rules out an invasive pathogen in the diarrheal attack.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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