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Bacterial and Parasitic Profile of Acute Infectious Pediatric Diarrhoea and the Role of Faecal Screening Tests in Prediction of The Invasive Type of Diarrhoea

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Abstract: Diarrhoea is one of the leading causes of morbidity and mortality in children worldwide. Faecal screening methods as detection of faecal leucocytes, faecal lactoferrin and faecal occult blood, have diagnostic and therapeutic implications in the provisional diagnosis of invasive diarrhea before culture results made available. Aim of the work was to outline the bacterial and parasitic profile of acute pediatric diarrhoea and to evaluate faecal screening tests in preliminary diagnosis of invasive diarrhoea. Three hundred children under five years of age, suffering from acute diarrhoea (<4 days) and attending the out-patient clinic of El Shatby Children's University Hospital in Alexandria, over a period of 1 year, were recruited in the study. Stool samples were collected from the children and were subjected to bacteriological examination, parasitological examination and 3 faecal screening tests to distinguish invasive (inflammatory) from non invasive (non inflammatory) diarrhoea. Forty eight percent of samples were positive for enteric pathogens. Enteric bacterial pathogens were isolated from 25% of samples. Parasites 29% and mixed bacterial and parasitic infections were detected in 6% of samples. Enterotoxigenic *E.coli* (ETEC) was the most common bacterial isolate detected in10% of samples, followed by *Salmonella* (8%), *Shigella* (6.67%) *Campylobacter* (5%) and *Vibrio parahaemolyticus* (1.33%). *Cryptosporidium* was the most commonly identified parasite (13%) followed by *Giardi lamblia* (11%), *Entamoeba histolytica* (8%) and *Cyclospora cayetanensis* (3%). *Ascaris lumbricoides* and *Haeminolipus nana* were only identified in 1% of samples, each. The gold standard for evaluation of faecal screening tests was positive culture for invasive bacterial pathogens and/or positive *E. histolytica* on microscopic examination of stool samples. Leuko test had the highest sensitivity (85.54%), specificity (73.73%), positive predictive value (55.47%), negative predictive value (93.02%) and accuracy (77%). False positive results of the Leuko-test were significantly higher in the breast-fed children than non breast-fed ones (26.7%, 11.7% respectively, $p < 0.01$). Better sensitivity, specificity, positive and negative predictive values of the Leuko-test was recorded in the non breast-fed children than in the breast-fed ones. The recorded values in the first group were: 91.11%.83.64%, 69.5% and 95.83%, respectively compared to 78.95%, 63.55%, 43.48% and 89.47%, respectively in the second group. The study concluded that, Leuko test is the best applicable faecal screening test in differentiation of invasive and non invasive diarrhoea but is better avoided in breast-fed infants as many false positive results might be interpreted.

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INTRODUCTION:

Diarrhoea is defined as the passage of three or more loose or watery stools per day or a single loose stool containing blood, pus or mucus (invasive diarrhoea).[1,2]

Diarrhoea is still one of the leading causes of morbidity and mortality in children worldwide, causing one billion episodes of illness and 3-5 million deaths annually.[3]. The incidence of diarrhoeal episodes in developing countries ranges from 2-5 illnesses per year. [4]. In Egypt it was estimated that 10 million Egyptian children, under five, suffer 30 million episodes of diarrhoea annually and that diarrhoea is responsible for about 14.64 % of deaths in this age group and is the leading cause of malnutrition ;impairing the quality of life of Egyptian children.[5,6]

The main bacterial causative agents of pediatric diarrhoea include: *Shigella, Salmonella, Campylobacter, E. coli* and vibrios.[7,8] Notable parasitic infections include those of Giardia *lamblia, Entamoeba histolytica and Cryptosporidium.(9)*

Unlike most diarrhoeal illnesses which are self-limited, invasive diarrhoea necessitates extensive diagnostic studies and specific antimicrobial therapy to shorten the clinical course, decrease excretion of multiresistant organisms or to prevent complications.[3,10,11] .

Stool culture is the standard method for diagnosis, however it is tedious, laborious and time consuming. Faecal screening methods (as detection of faecal leuocytes, faecal lactoferrin and faecal occult blood), have diagnostic and therapeutic implications in the provisional diagnosis of invasive diarrhea, as they are rapid and simple to perform.[2,12]

Examination of methylene blue-stained smears is the traditional method for detection of leucocytes in stool samples.[13] To be interpreted as positive; ≥5 leucocytes HPF must be identified in the majority of fields or >10 leucocytes in a

single field should be detected.[14]

Test for occult blood in stool depends on the oxidative capacity of hemoglobin.[10,15] HEMA SCREEN™ slide is an electrophoresis paper impregnated with natural guaiac resin. When occult blood is present in stool, its hemoglobin portion incorporated in the slide comes in contact with guaiac incorporated in the slide. On addition of HEMA SCREEN™ peroxide developing solution, a guaiac-peroxidase like reaction occurs. The chemical reaction is visualized by the appearance of a blue-green colour within 30 seconds-2 minutes.[15]

Leuko test is a semiquantitative method for detection of faecal leucocytes using an anti lactoferrin latex bead agglutination assay. When lactoferrin is present in elevated levels in stool sample, it cross links the anti-lactoferrin antibody coated latex beads giving visible agglutination. Latex beads coated with normal IgG serves as a negative control to identify non specific reactions.[16]

Aim of the study:

1. To outline the bacterial and parasitic profile of acute pediatric diarrhoea.
2. To evaluate three faecal screening tests in preliminary diagnosis of invasive diarrhoea**.**

SUBJECTS AND METHODS:

The present study involved 300 infants and children, under five years of age, suffering from acute diarrhoea (< 4 days) and attending the out-patient clinic of El Shatby Children's University Hospital in Alexandria, over a period of one year. All cases didn't receive any antibiotic therapy at least within the 48 hours prior to sampling.

Stool samples were collected from the children in sterile containers and were subjected to bacteriological examination, parasitological examination and 3 faecal screening tests to distinguish invasive (inflammatory) from non invasive (non inflammatory) diarrhoea.

1. Bacteriological examination:

Each sample was directly cultured on the surface of Mac Conkey agar, xylose lysine deoxycholate (XLD) agar, thiosulphate citrate bile salt (TCBS) agar, sorbitol Mac Conkey (SMAC) agar, Salmonella shigella (SS) agar and Modified Skirrow's agar. All samples were also inoculated on selenite broth, Gram negative (GN) broth and alkaline peptone water (APW) for primary enrichment of *Salmonella, Shigella* and vibrios; respectively. The tubes were incubated and were then subcultured on XLD and SS agar for the first two and on TCBS agar for the third. Media were incubated according to standard methods and isolated colonies were identified according to the methods described by Bailey and Scott's.[17] Suspected enterobactericae were confirmed using API 20E (BIO Merieux sa 008040-06/00).[18] Diarrhoeagenic *E.coli* were identified using *Ecoli* O 157 latex test (Oxoid DR 620 M) [19] and GM1-ELISA for detection of enterotoxigenic *E.coli.*[20]

2. Parasitological examination:

Each stool sample was subjected to direct microscopic examination for identification of motile trophozoites, formol ether concentration for detection of protozoan cysts and helminthic ova and larvae and modified Ziehl-Neelsen stain for detection of *Cryptosporidium* and *Cyclospora* oocysts.[21]

3. Faecal screening tests:

I. Direct microscopic examination:

Faecal smears were thoroughly mixed with 2 drops of methylene blue on glass slides. A cover-slide was placed on the stained suspension and 2-3 minutes were allowed for good nuclear staining of leucocytes. The test was considered positive if ≥5 leucocytes were detected in the majority of 20 fields or >10 leucocytes were detected in 1 field; examined by the 40X objective lens.[22,23-24]

II. Detection of occult blood in stool using HEMA SCREEN™ test:

A very thin portion or several drops of a faecal suspension were applied inside the oval locations indicated with Roman numeral I and II on the HEMA SCREEN™ test slide. Through the perforated section on the back of the slide, a drop of HEMA SCREEN™ developing solution (stabilized mixture of hydrogen peroxide < 6% and 75% denatured ethyl alcohol in aqueous solution) was added directly on the control area between positive and negative performance standards. Two or more drops of the developer were applied to oval I and II. Any trace of blue colour within 2 minutes was considered as a positive result indicating presence of occult blood in sample.[15]

II. Detection of faecal lactoferrin using Leuko- Test:

Fifty µl of the tested stool sample were diluted 1:50 and thoroughly mixed using a vortex mixer. For each sample, two circles on the agglutination card were used: one for testing the sample itself and the other used as a negative control. A positive control was done for each group of tested samples. A drop of sensitized latex was placed on each tested sample and on the positive control reagent. A drop of negative control latex was tested with a drop of the diluted sample simultaneously. Cards were rotated gently for 3 minutes. Both the positive control and positive samples showed easily visible agglutination with a clearing background. The negative control and the negative samples showed no visible agglutination. [25, 26]

The collected data was statistically analyzed and a number of correlations were found. [27]

RESULTS:

The present study included 300 children (180 females and 120 males) ≤5 years of age with a mean age of 1.98 years. All cases suffered from acute diarhoea (< 4 days).

Out of the 300 examined samples; only 144 (48%) were positive for enteric pathogens. Enteric bacterial pathogens were isolated from 75 samples (25%) of all samples. Parasites were identified in 87 samples (29%). Mixed bacterial and parasitic infections were detected in 18 samples (6%). (Table I)

Diarrhoegenic *E.coli* strains were the most common bacterial isolates detected in 30 samples (10%): [27 enterotoxigenic *E.coli* (ETEC) and 3 *E.coli* O157 strains].This was followed by 24 *Salmonella* spp (8%): [3 *S.typhi,* 15 *S. enteritidis* and 6 *S.typhimurium*], 20 *Shigella* spp (6.67%): [15 *S.flexneri* and 5 *S.dysenteriae*], 15 *Campylobacter* spp (5%): [9 *C.jejuni* and 6 *C.coli*] and 4 *V.parahaemolyticus* isolates (1.33%). *Cryptosporidium* was the most commonly identified parasite (13%) followed by *Giardia lamblia* 33 (11%), *E.histolytica* 24 (8%) and *Cyclospora cayetanensis* 9 (3%). *Ascaris lumbricoides* and *H.nana* were only identified in 3samples (1%), each.(Table II)

The gold standard for evaluation of faecal screening tests was positive culture for invasive bacterial pathogens and/or positive *E. histolytica* on microscopic examination of stool samples. The 3 samples positive for *E.coli* O157 were negative for the 3 faecal screening tests.

Leuko test had the highest sensitivity (85.54%), specificity (73.73%), positive predictive value (55.47%), negative predictive value (93.02%) and accuracy (77%). (Table III)

Leuko test was positive in 18 samples positive for *Shigella* (90%), 21 samples positive for *E. histolytica* (87.5%), 13 samples positive for *Campylobacter* (86.67%) and 19 samples positive for *Salmonella* (79.17%). (Table IV)

The observed agreement between the results of the faecal leucocytic count and both of occult blood and Leuko-tests was good (68% and 62% respectively) and this

was statistically significant (p=0.00) (TableV)

False positive results of the Leuko-test were recorded in 39 of the breast-fed children (26.71%) compared to only 18 of the non breast-fed ones (11.69%). The difference between both groups was statistically significant (p<0.01). On the other hand, false negative results were recorded in 8 of the breast-fed children (5.48%) and in 4 of the non breast-fed ones (2.6%). The difference between both groups was not statistically significant (p>0.05). (Table VI)

Better sensitivity, specificity, positive and negative predictive values of the Leuko-test was recorded in the non breast-fed children than in the breast-fed ones. The recorded values in the first group were: 91.11%.83.64%, 69.5% and 95.83%, respectively compared to 78.95%, 63.55%, 43.48% and 89.47%, respectively in the second group. (Table VI)

Table 1: Distribution of the enteric pathogens identified in stool culture and parasitological examination of the 300 stool samples.

Identified enteric pathogens	Frequency of identification	
	Nº	**%**
Bacterial isolates:		
• Single pathogen	39	13.00
• Polymicrebial	18	6.00
Subtotal	57	19.00
Identified parasites:		
• Single species	45	15.00
• Multiple parasites	24	8.00
Subtotal	69	23.00
Mixed infection (ba*cteria& parasites)*	18	6.00

NB: Total number of positive stool cultures =57+18=75 (25%)
Total number of samples positive for intestinal parasites=69+18=87 (29%)
Total number of samples with polymicrobial profile= 18+24+18=60 (20%)

Table 2: Frequency distribution of the different types of enteric pathogens recovered from the 300 stool samples.

Enteric pathogens	Frequency of identification	
	No	**%**
Bacteria:		
Diarrhoeagenic *E.coli*:	30	10
ETEC	27	9
E.coli O157	3	1
***Salmonella* spp :**	24	8
S.typhi	3	1
S.enteritidis	15	5
S.typhimurium	6	2
***Shigella* spp:**	20	6.67
S.flexneri	15	5
S.dysenteriae	5	1.67
***Campylobacter* spp:**	15	5
C.jejuni	9	3
C.coli	6	2
***V.parahaemolyticus*:**	4	1.33
Parasites:		
G.lamblia	33	11
E.histolytica	24	8
Cryptosporidium	39	13
C.cayetanensis	9	3
A.lumbricoides	3	1
H.nana	3	1

Table 3: Comparative evaluation of three faecal screening tests in the preliminary diagnosis of invasive diarrhoea.

Faecal screening test	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %	Accuracy %
• **Faecal leucocytes**	74.70	70.50	49.20	87.93	71.67
• **Occult blood test**	73.50	71.89	50.00	71.89	72.33
• **Leuko test**	85.54	73.73	55.47	93.02	77.00

N.B: The gold standard for evaluation of faecal screening tests was positive culture for invasive bacterial pathogens (*Salmonella, Shigella,* and *Campylobacter*) and/ or positive *E. histolytica* in microscopic examination of stool samples.

Table 4: Relationship between the positive results of the three faecal screening tests and the identification of invasive enteric pathogens.

Positive result / Invasive Enteric pathogens	Faecal leucocytic count		Occult blood test		Leuko test	
	No.	%	No.	%	No.	%
***Salmonella* spp(n=24)**	17	70.83	14	58.33	19	79.17
***Shigella spp* (n=20)**	15	75.00	16	80.00	18	90.00
***Campylobacter* spp (n=15)**	10	66.67	11	73.33	13	86.67
***E. histolytica* (n=24)**	20	83.33	20	83.33	21	87.50

NB: The 3 samples positive for *E.coli* O:157 were negative for the three faecal screening tests

Table 5: Agreement between the results of the faecal leucocytic count and both occult blood test and Leuko-test.

Faecal leucocytes	Occult blood test		Leuko test	
	Positive	Negative	Positive	Negative
• Positive	76	50	70	56
• Negative	46	128	58	116
• Observed agreement		0.68	0.62	
• Kappa coefficient		0.34	0.22	
• Z of Kappa		5.90*	3.84*	
• p value		0.000	0.000	

* Significant

Table 6: Effect of breast-feeding on the results of the Leuko-test.

Leuko-test results	Breast-fed (n=146)		Non breast-fed (n=154)	
	No	%	No	%
• True Positive	30	20.55	41	26.62
• True Negative	68	46.57	92	59.74
• False Positive	39	26.71	18	11.69*
• False Negative	8	5.48	4	2.60**
• Sensitivity	78.95%		91.11%	
• Specificity	63.55%		83.64%	
• Positive predictive value	43.48%		69.50%	
• Negative predictive value	89.47%		95.83%	

N.B: Breast-fed children include both the exclusively breast-fed and those who are supplemented with external diet in addition to breast milk.

* $Z=3.350$, $p<0.01$

**$Z=1.265$, $p>0.05$

DISCUSSION:

Diarhoeal diseases continue to be a major global burden. They accounted for 21% of all deaths in children under five years of age from 1990 to 2000; amounting for 2.5 million deaths. By the year 2005, diarrhoeal diseases accounted for 13% of all childhood deaths amounting to 1.4 million deaths per year.[28]

In the present study enteric pathogens were recovered from 48% of the studied cases of diarrhoea. In Egypt and worldwide, recovery of enteric pathogens from cases of acute pediatric diarrhoea ranged from 41% to 64%. [29-35] Failure to identify enteropathogens may be due to clearance of pathogens from the gut at the time of sampling which was only done once in this work, infection with *Yersinia enterocolitica* and *C.difficile* which is missed due to lack of suitable culture techniques or diagnostic kits and excluding viral causes of diarrhoea.

Infection with a single enteric pathogen was recorded in 28% of the studied cases while polymicrobial infection was recorded in 20% of which. Polymicrobial infection was recorded in similar studies as well. [36-38] this is reflection of environmental contamination. In fact, it may be that multiple pathogens act synergistically to produce diarrhoea.

Positive stool culture was recorded in 25% of examined samples in the current work. Several studies conducted worldwide, reported a positive stool culture that varied widely from 3% to 64%.[13,31,36,39-41] Low isolation rates could be attributed to intake of antibiotics, insufficient stool samples provided at time of sampling and performance of stool culture only once.

ETEC was the most common bacterial pathogen isolated in this study from 9% of samples. Other Egyptian studies reported ETEC as most common enteropathogen, isolated at a rate ranging from 10% to 27%.[42-45] Intestinal parasites were detected in 29% of studied cases. This

figure is consistent with what was previously reported by other investigators.[46-49].*Cryptosporidium* was isolated in 13% of cases in this study .Higher rates of identification were reported in other studies in Egypt in the range of 17% to 28%.[46-49]

Faecal screening tests were used in the current study to predict the presence of invasive pathogens in stool as a step in differentiation of inflammatory and non inflammatory diarrhoea. The faecal leucocytic count showed a sensitivity of 74.7% which agreed with results of previous studies in which a high sensitivity ranging from 75.5% to 85% was recorded. [24, 50, 51]. The recorded specificity of leucocytic count in this study was 70.5%, which coincided with previous reports ranging from 70% to 88 %.[50, 51, 52]. In the present study, the positive predictive value of this test was 49.2% and the negative predictive value was 87.93%. A positive predictive value that didn't exceed 60% and a negative predictive value ranging from 88% to 98.5% were reported in several studies. [12, 13, 51]

Evaluation of the occult blood test in the current work, it was found that it had a sensitivity of 73.5%. Previous studies reported a sensitivity ranging from 63% to 85 %.[24, 51] As regards specificity of this test, it was estimated in present investigation as 71.89%. Bardhan *et al.,* (2000), reported a specificity of 68% .[51] The positive and negative predictive values in the present work were 50% and 71.89%, respectively. In other studies as well, the positive predictive value didn't exceed 55% while the negative predictive value was sometimes as high as 91 %.[50, 51]

The Leuko test in the current study was proved to have a sensitivity of 85.54%, a specificity of 73.73%, a positive predictive value of 55.47% and a high negative predictive value of 93.02%. The potential of the Leuko test lies in its high negative predictive value. The absence of faecal

lactoferrin at a dilution of 1:50 makes the presence of an invasive pathogen unlikely.[12] The recorded accuracy for this test was 77%. The agreement between the results of the Leuko test and the leucocytic count was 62%. In previous studies carried out to evaluate Leuko test, a sensitivity ranging from 62% to 96.5%, a specificity ranging from 28% to 90%, a positive predictive value ranging from 11% to 76% and a negative predictive value ranging from 71% to 94% were recorded.[13,52-54] In agreement with the present findings, previous reports indeed suggested that the Leuko test is considerably more sensitive than the traditionally applied faecal leucocytic count and the occult blood test for diagnosis of inflammatory diarrhoea caused by invasive pathogens.[11, 13, 52, 55, 56] In the present study, the Leuko test was positive in 90% of *Shigella* positive samples, 87.5% of *E.histolytica* positive samples, 86.67% of *Campylobacter* positive samples and 79.17% of *Salmonella* positive samples. Several previous studies reported positive results of Leuko test in 85% to 100% of stool samples positive for invasive pathogens.[11, 56] In spite of this, positive results of the Leuko test in enteric infections caused by non invasive pathogens as ETEC were noticed in the present study and in other studies as well.[13, 57] False positive results of faecal screening tests in general could be explained by colonic inflammation caused by frequent exposure to infectious bacterial and parasitic agents in endemic areas. Other explanations include: associated non-infectious inflammatory enteritis, presence of invasive pathogens that are sometimes missed on culture or presence of *Yersinia enterocolitica*, *C.difficile* or enteroinvasive *E.coli* for which routine tests are usually not performed. In addition some non invasive pathogens like Rota virus, *V.parahaemolyticus* and *Cryptosporidium* may also produce mild inflammation in the intestinal mucosa.

In the current work it was noticed that the false positive results of the Leuko test were significantly more frequent among breast-fed children and better sensitivity, specificity, positive and negative predictive values were recorded among non breast fed children. The false positive results, the limited specificity and negative predictive value of the Leuko test among the breast-fed is attributed to the secretion of lactoferrin naturally in breast milk.[58] It was reported that lactoferrin titers ranging from 50-100 ng/µl could be detected in stool samples of healthy breast-fed infants. [16, 58]

From the present investigation it could be concluded that:

1. Parasitic agents are responsible for the majority of cases of acute pediatric diarrhoea in Egypt.
2. Diarrhoeagenic *E.coli* (mainly ETEC) are the most commonly encountered bacterial enteropathogens while *Cryptosporidium* is the most common parasite in cases of acute pediatric diarrhoea.
3. Leuko test is the best applicable faecal screening test in differentiation of invasive and non invasive diarrhoea. A negative result rules out the involvement of an invasive pathogen in the diarrhoeal attack.
4. Leuko test is better avoided in breast-fed infants as many false positive results may be interpreted.

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