

## Increase in the detection rate of viral and parasitic enteric pathogens among Egyptian children with acute diarrhea

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### Abstract

**Introduction:** Acute diarrhea continues to be a major cause of morbidity and mortality in children from developing countries. Determination of the frequency of diarrhea in an area, along with the proportion of disease caused by specific enteric agents of different origins, is considered the first step in controlling diarrheal diseases.

**Methodology:** From 2005 to 2007, a hospital-based surveillance was conducted in two locations in Egypt to determine the causes of acute diarrhea in children younger than 5-years seeking treatment. Five additional enteric viral and parasitic pathogens were tested using commercially-available enzyme immunoassays (EIA) to re-evaluate the prevalence of diarrheal pathogens in undiagnosed cases.

**Results:** Adenovirus, astrovirus, norovirus and *G. lamblia* were detected as the sole pathogen in 2% (n=34), 3% (n=56), 9% (n=191) and 7% (n=146) of the cases, respectively. *E. histolytica* was never detected as the sole pathogen. The percentage of diarrheal cases with a known cause increased significantly, from 48% (n=1,006) to 74% (n=1,568) (P<0.0001).

**Conclusion:** In our study, the incorporation of immunoassays yielded useful data in identifying pathogens in previously pathogen-negative diarrhea cases.

**Key words:** enteric viruses; enteric parasites; ELISA; Egyptian children; acute diarrhea

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### Introduction

Acute diarrhea continues to be a major cause of morbidity and mortality in children from developing countries [1,2]. Determination of the frequency of diarrhea in an area, along with the proportion of disease caused by specific enteric agents of different origins, is considered the first step in controlling diarrheal diseases [3]. Of the bacterial infectious agents causing watery diarrhea, enterotoxigenic *Escherichia coli* (ETEC) has repeatedly been found in longitudinal and cross-sectional epidemiological surveillance studies to be one of the major pathogens afflicting children aged less than 5 years [4-7]. In Egypt, other common bacterial pathogens associated with diarrhea include *Campylobacter* spp. and *Shigella* spp. [5].

Detection of antigens specific for enteric viruses and parasites in stool using enzyme immunoassay (EIA) technology has demonstrated that these microbes are also commonly associated with childhood diarrhea although data has historically been lacking. Viruses, including rotavirus (RV), norovirus (NoV), enteric adenovirus 40/41 (AdV) and astrovirus (AsV) have been shown to be important causes of acute diarrhea in children during the early years of life [8-11]. RV and NoV are considered the most important causative agents of viral gastroenteritis [12,13] and surveillance studies have demonstrated that RV is one of the most common causes of diarrhea among children seeking hospital care for their illness [14]. RV infections are responsible for millions of childhood hospitalizations and thousands of potentially preventable deaths in developing countries each year [15].

**Table 1.** Sole pathogen detection rates from stools of children less than 5 years before and after addition of additional parasite and viral EIA testing, Egypt, 2005-2007

Pathogen	Initial testing (%) <sup>a</sup>	After subsequent testing (%)	p-value
ETEC	282 (13)	140 (7)	<0.0001
<i>Campylobacter spp.</i>	85 (4)	37 (2)	<0.0010
<i>Shigella spp.</i>	59 (3)	23 (1)	<0.0001
<i>Salmonella spp.</i>	2 (0.1)	2 (0.1)	0.6000
Rotavirus	286 (14)	171 (8)	<0.0001
<i>Cryptosporidium spp.</i>	96 (5)	53 (3)	<0.0010
Adenovirus	N/A*	34 (2)	N/A
Astrovirus	N/A	56 (3)	N/A
Norovirus	N/A	191 (9)	N/A
<i>Giardia lamblia</i>	N/A	146 (7)	N/A
Multiple pathogens	196 (9)	715 (34)	<.0001
Undiagnosed	1106 (52)	544 (26)	<.0001

<sup>a</sup>Total number of stool specimens screened was 2,112; with the exception of the multiple pathogens entry, the numbers refer to the detection of the organism as the sole pathogen in the sample.

\* N/A: not assayed in this group.

\*\**E. histolytica* cases were only found as a mixed infection.

**Table 2.** Comparison of pathogens detected, undiagnosed cases and mixed infection, Abu Homos site and Manshayet Nasser hospitals, 2005-2007

Pathogen	Abu Homos (n = 1,194)	Manshayet Nasser (n = 918)	p-value
ETEC	71 (6)	70 (8)	0.1
<i>Campylobacter spp.</i>	13 (1)	24 (3)	0.008
<i>Salmonella spp.</i>	2 (0)*	0 (0)	
<i>Shigella spp.</i>	7 (1)	16 (2)	0.01
Rotavirus	112 (9)	59 (6)	0.01
Norovirus	109 (9)	82 (9)	0.9
Astrovirus	35 (3)	21 (2)	0.4
Adenovirus	21 (2)	13 (1)	0.6
<i>Cryptosporidium</i>	45 (4)	8 (1)	< 0.0001
<i>Giardia lamblia</i>	75 (6)	71 (8)	0.2
Mixed	459 (38)	256 (28)	< 0.0001
Total number of undiagnosed cases	245 (21)	298 (32)	<0.0001
Total number of cases diagnosed with enteric pathogens	949 (79.5)	620 (67.5)	

\* Less than 1 %

*Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium parvum* are the major parasitic organisms causing childhood diarrhea in developing countries [16-18]. Although these parasites do not usually cause life-threatening disease, they are still important infectious agents, especially in infants. A previous surveillance study conducted in the Nile Delta of Egypt reported a high incidence of *Cryptosporidium* in children [19].

Surveillance for diarrheal disease in children aged younger than 5 years seeking medical care for diarrhea has been actively performed in a rural community in the Nile Delta and an urban slum area

in Cairo, Egypt, for over a decade. For the majority of these studies, the detection of enteric pathogens was limited to bacteria, and in some studies, *Cryptosporidium* and RV [5,7,19]. Overall pathogen identification in diarrheal stools was almost 50%. Since we observed a large number of undiagnosed diarrheal cases based on the initial testing scheme, the aim of this current study was to assess the prevalence of other enteric pathogens within these communities, by re-testing samples using commercially available EIA kits for enteric viral (NoV, AsV and AdV) and parasitic (*Giardia* and *E. histolytica*) pathogens.

**Table 3.** Distribution of sole identified pathogens from diarrheal children seeking for help during the period 2005 – 2007, according to age and seasonality

Age (months)	Pathogen										
	ETEC n = 141	<i>Campylobacter</i> n = 37	<i>Shigella</i> n = 23	<i>Salmonella</i> n = 2	<i>Cryptosporidium</i> n = 53	<i>Giardia</i> n = 146	Adenovirus n = 34	Astrovirus n = 56	Norovirus n = 191	Mixed n = 715	Undiagnosed n = 544
0 – 12	69 (49)	26 (70)	3 (13)	1 (50)	22 (42)	41 (28)	26 (76)	32 (57)	126 (67)	328 (46)	294 (54)
13 – 24	34 (24)	8 (22)	9 (39)	1 (50)	25 (48)	36 (25)	4 (12)	19 (34)	37 (20)	242 (34)	109 (20)
25 – 36	21 (15)	2 (5)	4 (17)	0 (0)	3 (6)	25 (17)	2 (6)	2 (4)	12 (7)	81 (11)	64 (12)
37 – 60	16 (11)	1 (3)	7 (30)	0 (0)	2 (4)	44 (30)	2 (6)	3 (5)	14 (7)	63 (9)	72 (13)
Warm season	107 (76)	29 (78)	13 (57)	2 (100)	47 (90)	85 (58)	20 (59)	42 (75)	111 (59)	545 (76)	310 (57)

All numbers are expressed as n (%)

Cases may not add up to n (%) due to missing answers in age or date

\* Warm season represents months from May to October

## Methodology

### *Study definitions and sample sources*

From 2005 to 2007, stool and rectal swab specimens were collected from children with diarrhea ( $n = 2,112$ ) presenting to two referral outpatient hospital clinics, one in Manshayet Nasser, Cairo, the second, Abu Homos General Hospital in the Nile Delta, using previously described methods [7]. Diarrhea was defined as the occurrence of at least three non-formed stools (or a minimum of one, if bloody) in a 24-hour period. In addition, if the child was breastfed and the stool was not bloody, the mother had to report an increase in frequency or a reduction in consistency of the stools, compared with what she considered to be normal.

Stool samples were divided into aliquots and stored frozen at  $-70^{\circ}\text{C}$  until use; bacterial pathogens were cultured from rectal swabs and one aliquot of frozen stool sample per patient was tested for the presence of RV and *Cryptosporidium*. A second aliquot of frozen stool was tested to detect other enteric viruses (NoV, enteric AdV and AsV) and enteric protozoa (*G. lamblia* and *E. histolytica*).

### *Bacterial pathogen detection*

Rectal swabs were cultured for detection of the presence of common enteric bacterial pathogens (*Aeromonas*, *Campylobacter* spp., *E. coli*-like, *Salmonella*, *Shigella*, *Vibrio cholerae*) using standard laboratory methods. In addition, five individual lactose colonies with typical *E. coli* morphology were isolated from the primary MacConkey agar plate and kept frozen in trypticase soy broth with 15% glycerol at  $-70^{\circ}\text{C}$  until further tested. *Campylobacter* spp. was isolated under microaerophilic conditions on modified Skirrow's medium. Putative colonies were confirmed by Gram stain, oxidase production, and catalase activity. Hippurate hydrolysis was used to differentiate *C. jejuni* from *C. coli*. SS medium was used for the isolation of *Shigella* and *Salmonella* and thiosulfate citrate bile salt medium for the isolation of *Vibrio* spp. The API 20E system (Analytab Products, New York, NY, USA) was used for species confirmation of Enterobacteriaceae. Commercially available antisera (Difco Laboratories, Detroit, MI) were used to speciate recovered *Shigella* isolates. Antimicrobial susceptibility testing (AST) was performed using the Kirby and Bauer disc diffusion method. All AST was performed and interpreted in accordance with Clinical and Laboratory Institute Standards (CLIS) guidelines.

### *Parasitic and viral pathogen detection*

Frozen stools were tested using commercially available enzyme immunoassay (EIA) kits targeting the following specific pathogens: RV, (Rotaclone, Meridian Bioscience, Inc., Cincinnati, OH, USA); AdV, AsV, NoV, (IDEIA Oxoid [Ely] Ltd, Denmark House, Angel Drove, UK). Compared to electron microscope (EM) analysis, the sensitivity for the RV, AdV and, AsV kits was 100%, 90%, and 100%, and the specificity values were 99%, 99%, and 98.3% respectively, while for the NoV kit the sensitivity was 72.8% and the specificity was 100% when compared with polymerase chain reactions.

All enzyme immunoassays for *Cryptosporidium* spp., *E. histolytica* and *G. lamblia* (*Cryptosporidium* II, *Giardia* II and *E. histolytica* II kits, Techlab, Blacksburg, VA, USA) were performed according to the kit manufacturer's instructions for antigen detection of each pathogen. The sensitivity values were 97.7%, 100% and 100% and the specificity values are 100%, 94.7% and 100%, respectively, compared to EM.

### *Statistical analysis*

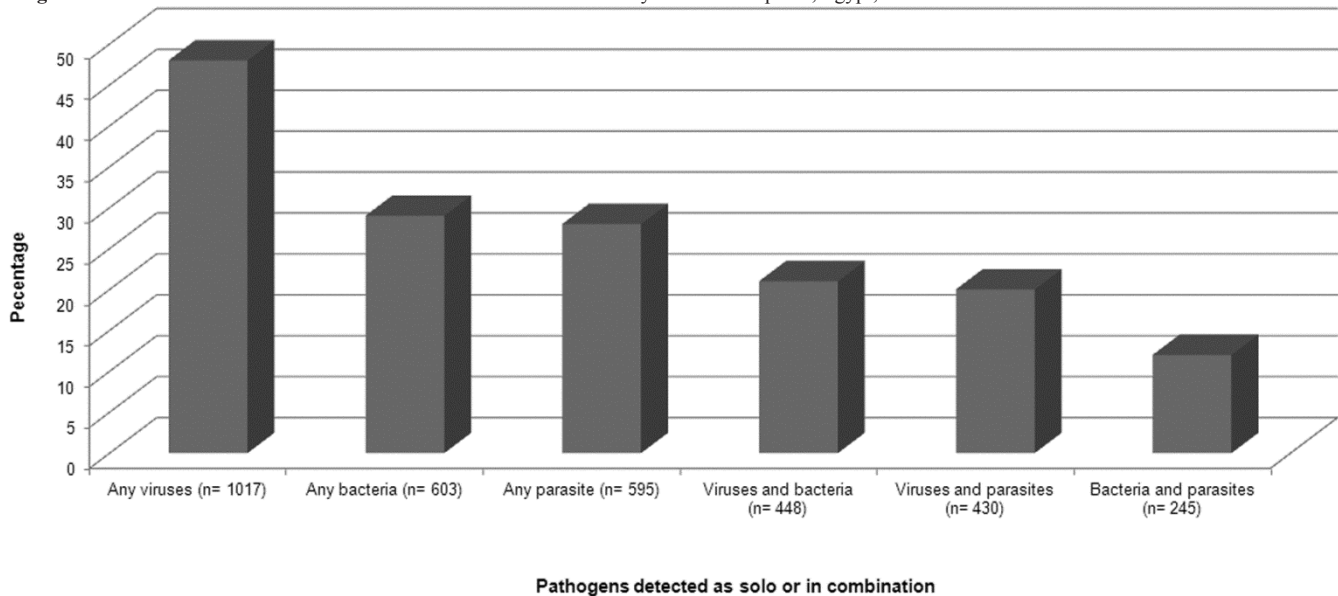
Survey data were entered and verified using Epi Info version 6 (CDC, Atlanta, GA, USA. and WHO, Geneva, Switzerland). Statistical analyses were performed using SAS software (version 9.1, SAS Institute Inc, Cary, NC, USA). Chi-square or Fisher's exact test were used to determine statistical significance and compare initial and subsequent test results.

## Results

Initial testing of stool samples from 2,112 children seeking medical treatment for diarrhea identified a bacterial pathogen, *Cryptosporidium* spp, and RV in 48% ( $n = 1,006$ ) of the cases. Bacteria were identified as a sole pathogen in 20% ( $n = 428$ ), RV in 14% ( $n = 286$ ) and *Cryptosporidium* in 5% ( $n = 96$ ) of the cases. Samples containing two or more pathogens were relatively common, representing 9% ( $n = 196$ ) of the samples (Table 1). Using a traditional enteric panel for laboratory testing, no pathogen was identified in 52% ( $n = 1,106$ ) of the stool specimens obtained from diarrheal cases seeking medical attention.

The testing of these samples via the five enzyme immunoassays reduced the number of cases without an identifiable pathogen by a factor of 2 (52% to 26%, ( $p < 0.0001$ )), so pathogens were detected in 74% (1,568/2,112) of the cases (Table 1). A single causative organism was identified in 40.4% ( $n =$

**Figure 1.** Distribution of all enteric infection at Abu Homos and Manshayet Nasser hospitals, Egypt, 2005-2007



853/2,112) of the tested specimens, including 21% viral, 9.5% bacterial, and 9.3% parasitic agents; in an additional 34% (n = 715/2,112) more than one pathogen was identified. Interestingly, NoV was the most commonly detected pathogen; it was identified in 26% (n = 560) of the cases, as a sole pathogen or in association with another organism. The percentage of mixed infection cases with pediatric diarrhea significantly increased from 9.3% (n = 196/2,112) to 34% (n = 715/2,112, p < 0.0001).

Of the 1,568 diagnosed cases, 3,338 enteric pathogens were identified; viruses were the most commonly identified pathogens (48%, n = 1,017/2,112) followed by bacteria (29%, n = 603/2,112). The percentage of mixed infection due to a combination of viruses and bacteria (21%, n = 448/2,112) or viruses and parasites (20%, n = 430/2,112) was very similar (Figure).

Comparing the distribution of enteric pathogens over the two study sites, isolation rates of bacterial pathogens (*Shigella* and *Campylobacter* spp.) were higher in stools from children residing in the urban slum of Manshayet Nasser while the isolation rates of viral, parasitic and mixed pathogens were higher in stools from children residing in rural villages of Abu Homos. Overall, Abu Homos had the highest prevalence of all detectable enteric pathogens (Table 2) although the samples were transferred from the sample collection point to the NAMRU-3 laboratory only twice weekly and not daily as was the case for

Manshayet Nasser. The reason for this sample transfer procedure was the location of the Abu Homos site, which is over 200 km from Cairo.

Most of the identified pathogen cases, whether sole or mixed infections, were detected during the warm season (May to October) (Table 3). Stratified analysis by age (months) revealed that the most common enteric infections in the 0-12 months age group were NoV, RV, ETEC, AsV, *Campylobacter* spp. and AdV (Table 3). When identified, *Shigella* was more commonly identified during the second year after birth (13-24 months). The rates of infection for *Cryptosporidium* spp. and *G. lamblia* were similar among children of both age groups.

**Discussion**

Although diarrheal diseases result in significant morbidity in the Middle East and in Egypt specifically, there has been relatively little published research to quantify the disease burden from enteric viruses and other parasites in this region. Enzyme immunosorbent assays have been used to study diarrheal infection for years and commercial kits with high sensitivity and specificity are readily available [20,21]. Despite this, only a few studies report the burden of enteric viruses in North Africa [13,22]. This report, the first comprehensive surveillance describing etiologies of enteric viruses and parasites from children with diarrhea seeking medical care, demonstrated that inclusion of additional laboratory tests significantly



decreased the number of undiagnosed cases of pediatric diarrhea.

The previous publication by our colleagues [7] characterized the frequency of bacterial etiologies of diarrhea in the same region, but in our study, additional analyses illustrated the significance of enteric viral infection in the first year of life in Egyptian children, both as a sole pathogen and as part of what may be a true polymicrobial infection. Also, low prevalence of enteric viruses was observed among children older than a year, which could be partly due to acquired immunity through previous exposures. The application of commercial EIA kits for enteric viral and parasitic pathogens demonstrated that enteric virus infections, whether as the sole or a co-pathogen, were responsible for almost half of the diarrheal episodes observed in our study.

Importantly, the dominant etiology resulting in pediatric diarrheal disease was affected by the application of the additional tests. Rotavirus-induced diarrhea has previously been reported as a significant cause of pediatric diarrhea in Abu Homos [7]; in our study, however, the re-evaluation of the stool samples has shown that other viruses causing diarrhea, notably NoV, was more common in diarrhea cases. We found that NoV, RV, AsV and AdV were present in 26.5%, 19%, 11.7% and 4.7% respectively of cases as a single or co-pathogen with seasonal distribution from May to October. A similar finding was reported by Sidiri-Loulizi *et al.* [22] from Tunisia, where RV was slightly more dominant than NoV (22.5 v 17.4%), followed by AsV (4.7%) and AdV (2.7%) in children hospitalized because of acute diarrhea. Distribution of viral gastroenteritis showed both a winter peak and a summer/autumn peak (May to October) in Tunis. Meanwhile, in a similar study performed in northern Ghana, RV was found as the most common pathogen (55%) in children younger than 12 years with acute diarrhea followed by AdV 28% and NoV 10% [23]. Therefore, there may be important regional and seasonal variations in Africa on the predominant enteric viruses associated with pediatric morbidity.

Cryptosporidiosis was previously shown to affect children younger than five years of age in Egypt [19]. In this study, we also identified almost 28% of diarrheal cases caused by infection with *G. lamblia* and/or *Cryptosporidium* spp.; the highest percentage of cases with intestinal parasites was identified during the warm season (May to October). Our results agree with those of Wongstitwilairoong *et al.* [24], who found in a case-control study conducted in Sangkhlaburi, Thailand (n = 472 pre-school children,

236 children with diarrhea, and 236 asymptomatic children) 17% of cases (n = 41) and 28% of controls (n = 66) were infected with intestinal parasites (*G. lamblia* and/or *Cryptosporidium* spp), while the highest proportion of intestinal parasites occurred during the same period of time (June to October, rainy season). However, due to the possibility of asymptomatic carriage of parasites (and lack of a control group) one cannot state definitively whether the detection of these parasites by enzyme immunoassay represents its association with disease in our study (regardless of whether it was identified as a sole pathogen or as part of a presumed polymicrobial infection).

Enterotoxigenic *Escherichia coli* diarrheal episodes remain a leading cause of bacterial infectious diarrhea in children, particularly those younger than two years of age [6,7,25,26] regardless of location. However, in this study, shigellosis was identified more often from urban rather than rural children. *Shigella* infections may be transmitted from person to person [27]. Although shigellosis was prevalent in Egyptian children with diarrhea [28], currently the rate of *Shigella* spp. isolation is lower in cases with pediatric diarrhea [7,28]. In rural areas, national projects for the control of diarrhea issued by local authorities resulted in the availability of potable water and in the improvement of sanitation and personnel hygiene [29].

The presence of mixed infections in more than a third of the samples clearly demonstrates one of the challenges to understand the relative role (if any) each pathogen plays in the overall pathogenesis of diarrhea. This is especially true in developing countries where typical pathogens can sometimes be found in the stools of asymptomatic children. Use of a vaccine targeting a single, apparently dominant pathogen may be of reduced value in these settings. Current vaccine strategies targeting bacterial and viral pathogens may not be effective in the future because of the changing patterns of pathogen etiology. In this study, testing of fecal specimens was performed in two stages. Although the cost of using additional commercially available diagnostic enzyme immunoassays for the detection of viral and protozoal pathogens seems to increase the cost per sample by more than four times, the importance of a comprehensive diarrheal surveillance for diarrheal pathogens has been emphasized by the National Control of Diarrheal Diseases projects organized in developing countries. The goals of these programs are to establish a surveillance system estimating the true burden of disease and to target specific vaccine development and

intervention, which will result in long-term cost savings. These observations are especially important as countries actively pursue mechanisms to decrease the mortality in children younger than age 5 by two thirds by 2015, as agreed to through the World Health Organization Millennium Development Goals Declaration [30-32].

### Disclaimer

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