



ID Design 2012/DOOEL Skopje
Open Access Macedonian Journal of Medical Sciences.
<http://dx.doi.org/10.3889/oamjms.2015.023>
Basic Science

Prediction of Gut Wall Integrity Loss in Viral Gastroenteritis by Non-Invasive Marker

Hala G. Elnady¹, Lobna S. Sherif¹, Maysa T. Saleh¹, Inas R. El-Alameey^{1*}, Mai M. Youssef¹, Amal I. El Shafie², Iman Helwa³, Haiam Abdel Raouf³, Ahmed N. EL-Taweel⁴

¹Child Health Department, National Research Centre, Cairo, Egypt; ²Health Radiation Research Department, National Centre for Radiation Research and Technology, Cairo, Egypt; ³Immunogenetics Department, National Research Centre, Cairo, Egypt; ⁴Environment Virology Laboratory and Water Pollution Department, National Research Centre, Cairo, Egypt

Abstract

Citation: Elnady HG, Sherif LS, Saleh MT, El-Alameey IR, Youssef MM, El Shafie AI, Helwa I, Abdel Raouf H, EL-Taweel AN. Prediction of Gut Wall Integrity Loss in Viral Gastroenteritis by Non-Invasive Marker. OA Maced J Med Sci. <http://dx.doi.org/10.3889/oamjms.2015.023>

Key words: Viral Gastroenteritis; Gut Wall Integrity Loss; Non Invasive Marker; Prediction.

***Correspondence:** Dr. Inas R. El-Alameey. National Research Centre, Child Health Department, Dokki, Cairo. Cairo P.O:12622, Egypt. Affiliation ID: 60014618. Phone: 0201001858378. E-Mail: inasno@hotmail.com

Received: 12-Jan-2015; **Revised:** 01-Feb-2015; **Accepted:** 02-Feb-2015; **Online first:** 05-Mar-2015

Copyright: © 2015 Hala G. Elnady, Lobna S. Sherif, Maysa T. Saleh, Inas R. El-Alameey, Mai M. Youssef, Amal I. El Shafie, Iman Helwa, Haiam Abdel Raouf, Ahmed N. EL-Taweel. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Competing Interests: The authors have declared that no competing interests exist.

BACKGROUND: Intestinal fatty acid binding proteins (I-FABPs) are mainly expressed in the intestinal villi, which are the initial site of destruction in viral gastroenteritis.

AIM: This study was designed to assess serum I-FABPs as a predictor of gut wall integrity loss in viral gastroenteritis.

PATIENTS AND METHODS: This case-control cross-sectional study was conducted on 93 cases of acute viral gastroenteritis. Twenty-eight healthy children matching in age were recruited as control group. Serum I-FABPs were measured using ELISA technique. Viral detection and typing were done by PCR for adenovirus, and by Reverse transcriptase PCR for rotavirus, astrovirus and norovirus.

RESULTS: Serum I-FABPs level was significantly higher in the cases compared to the controls and was also higher in the 46 rotavirus gastroenteritis cases compared to other viral gastroenteritis cases. Serum I-FABPs level was significantly higher in severely dehydrated cases as compared to mildly dehydrated ones ($P=0.037$).

CONCLUSION: Serum I-FABPs could be used as an early and sensitive predictor marker of gut wall integrity loss in children with viral gastroenteritis and its level can indicate case severity.

Introduction

Acute viral gastroenteritis (GE) is one of the commonest causes of morbidity and mortality in very young aged children [1-3]. This is definitely due to the dehydrating diarrhea and might be also due to secondary blood stream infection [4]. Four major viral pathogens associated with AGE are three RNA viruses (rotavirus, norovirus, and astrovirus) and one DNA virus (enteric adenovirus) [4-6]. Co-infections with these viruses have been frequently reported especially rotavirus and norovirus co-infections [7-9].

Rotavirus remains the most common cause of

severe childhood diarrhea worldwide and of diarrheal mortality in developing countries [10]. The World Health Organization (WHO) estimates that 527,000 children under the age of five years die of rotavirus disease each year [11]. Children in the poorest countries account for 82% of rotavirus deaths in children, fewer than five years of age [12, 13].

Loss of gut wall integrity, and intestinal ischemia are consequence of low flow states associated with viral gastroenteritis. Prolonged periods of intestinal ischemia reperfusion result in severe damage to the intestinal barrier, thereby allowing translocation of bacteria from the intestinal lumen to the systemic circulation [14]. As early

detection of intestinal ischemia reperfusion is essential to improve clinical outcome, Derikx et al., [15], and Grootjans et al., [16] have studied the diagnostic value of biomarkers to detect enterocyte membrane integrity loss, an early phenomenon during intestinal ischemia.

Intestinal fatty-acid binding protein (I-FABP) is a newly sensitive plasma marker of for early enterocyte membrane integrity loss. Several studies have described the use of I-FABP for the early detection of intestinal injury due to decreased perfusion of the small bowel, inflammation, or intestinal ischemia [17, 18]. It is part of a family of nine different FABP types, each named after the tissue of its first detection. I-FABPs are small (15 kDa) low molecular weight cytosolic proteins specifically present in mature enterocytes at the tip of the villus of small intestine and constitute 2% of enterocyte proteins. They are released upon enterocyte membrane integrity loss into the circulation, which makes them useful as plasma markers for enterocyte damage [19]. Assessment of gut wall integrity in clinical practice is still a challenge, as it is difficult to evaluate the condition of the gut non-invasively with currently available diagnostic tools. Moreover, non-invasive, rapid diagnostic means to assess intestinal condition are needed to evaluate the effects of treatment of intestinal disorders. Therefore, the aim of this current study is to assess serum intestinal fatty acid binding protein (I-FABP) as an early and sensitive predictor for the evaluation of gut wall integrity loss in GE particularly rotavirus gastroenteritis (RV-GE) in Egypt.

Subjects and Methods

Subjects

This cross sectional case control study was conducted on 93 cases of acute gastroenteritis. They were 55 males and 38 females. Their ages ranged from newborn up to 5 years old. They belonged to different social classes and were taken from Cairo, Giza and Kalyoubia governorates in Egypt. Thirty-two of these cases were hospitalized while the other sixty-one cases were not. Of these, eleven cases just entered the hospital to receive I.V. fluids and then were dismissed. The remaining 50 cases were taken from the outpatient clinics of the same hospitals.

Inclusion Criteria

1. Age range (newborn up to 5 years old).
2. Boys and girls of the same social classes are included.

Exclusion Criteria

1. Infants receiving Rota virus vaccine.
2. Children on corticosteroids or immunosuppressive drug therapy.
3. Children complaining of systemic autoimmune disease, immunodeficiency, chronic renal disease, or cancer.
4. Previous blood or any blood products transfusion.
5. History of chronic allergic diseases.

The control group comprised 28 apparently healthy children who did not have diarrhea during the 2 weeks period preceding enrollment in the study. They belonged to the same regions and were of the same age range. They were 13 males and 15 females.

Ethical approval was obtained from the Medical Ethical Committee of the National Research Centre. Written informed consent was obtained from the parents after explanation of the aim of the study and its possible benefits for identifying the cause of the acute viral gastroenteritis of their children.

Methods

All cases were subjected to full history taking, thorough clinical examination, and collection of clinical data. Clinical data included disease manifestations as fever, vomiting, abdominal pain or bloody diarrhea. Severity criteria like duration of the diarrhea, number of stool motions or bouts of vomiting, range of body temperature, and degree of dehydration were determined for all hospitalized children.

Blood samples approximately 3 ml were taken from all cases and controls for measuring intestinal fatty acid binding protein (I-FABP). Serum was immediately separated from each blood sample and stored frozen at -20°C till the measurement of I-FABP. Serum I-FABP levels were analyzed using the human I-FABP-specific enzyme-linked immunosorbent assay (ELISA) commercial test kit (EIAab®, China), according to the following steps. Firstly, 100 μl of standard, blank, and samples were added per well that wells were pre-coated with biotin-conjugated polyclonal antibody specific to human iFABP. Then, the plate covered with the plate sealer was incubated for 2 hours at 37°C . After incubation 100 μl of avidin conjugated was added to each well then plate covered and incubated for 1 hour at 37°C then each well aspirated and washed three times. After washing, 100 μl of horseradish peroxidase (HRP) was added to each microplate well then the plate incubated for 1 hour at 37°C . After incubation each well was aspirated and washed. This wash step was followed by addition of 90 μl TMB substrate solution to each well then the plate incubated for 30 minutes at 37°C and protected

from light. The enzyme-substrate reaction was terminated by the addition of 50 μ l sulphuric acid solution and the color change was measured using microplate reader at a wavelength of 450 nm [20].

Stool analysis was done by using Polymerase Chain Reaction (PCR) and by Reverse transcriptase PCR (RT-PCR) for viral detection and typing. Stool samples were collected in dry sterile clean plastic cups. Collected samples were diluted and prepared for PCR, and RT-PCR for detection of viral gastroenteritis agents in the collected samples. Viral gastroenteritis was detected in all collected stool samples were subjected to extraction of both viral RNA and DNA in the samples using Axygen® Kit (Axygen biosciences, Cat. No AP-MN-BF-VNA-250) to facilitate the detection of both RNA gastroenteritis viruses (such as rotaviruses, noroviruses, and astroviruses) and DNA gastroenteritis viruses (such as adenoviruses).

Viral genomes were extracted from 10% diluted stool samples using Axygen® Kit (Axygen biosciences, Cat. No. AP-MN-BF-VNA-250) according to manufacturers instructions and prepared for detection of viral gastroenteritis by both RT-PCR and PCR using sets of oligonucleotide primers specific to each type of virus Universal viral gastroenteritis oligonucleotide primers sequences were obtained from Bioneer Company, as purified lyophilized primers. Universal primers for G typing rotaviruses were selected for amplification of segment 9 as previously described [21]. These primer pairs are semi nested primers designed to amplify the highly conserved region among all known rotaviruses strains and specific for gene segment 9, as. Universal primers for P typing rotaviruses were selected for amplification of VP7 gene segment [22]. For detection of astroviruses sets of primers were selected and used as described by [23], and noroviruses sets of primers were used [24]. For adenoviruses PCR the primer sets described by [25] and designed to amplify the highly conserved region of hexon gene which is very similar in many types of adenoviruses.

Reverse transcriptase-polymerase chain reaction (RT-PCR):

All collected stool samples were subjected to extraction of both viral RNA and DNA in the samples to facilitate the detection of both RNA gastroenteritis viruses such as rotaviruses, noroviruses, and astroviruses and DNA gastroenteritis viruses such as adenoviruses. Viral genomes were extracted from 10% diluted stool samples and prepared for detection of viral gastroenteritis by both RT-PCR and PCR using sets of oligonucleotide primers specific to each type of virus as follow:

Rotaviruses

cDNA synthesis and PCR amplification: Viral RNA was extracted from 200 μ l aliquot of 10% stool

sample, using Axygen® Kit (Axygen biosciences, Cat. No. AP-MN-BF-VNA-250), according to the instructions of the supplier. Reverse transcription (RT) was carried out using a mixture of 10 μ l of extracted viral RNA, 5 μ l random primer (25 pmol/ μ l), and 20 μ l DEPC treated water. The reaction mixture, 35 μ l, was heated at 65°C for 5 min. and chilled on ice for 2 min. 35 μ l of the reaction mixture was added to 1 μ l of 200 U/ μ l M-MLV Reverse Transcriptase (Promega, Cat. # M1701), 10 μ l RT-buffer, 4 μ l of 10mM each dNTPs (Promega, Cat. # U1511). The mixture was heated at 42°C for 60 min and 37°C for 30 min, for cDNA synthesis, followed by 95°C for 5 min. Nested RT-PCR the reaction takes place in two rounds; of The first-round PCR was carried out in 50 μ l reaction mixture containing 10 μ l of the synthesized cDNA, 5 μ l 10 x PCR Buffer, 4 μ l of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5 μ l of 5 U/ μ l Go Taq DNA Polymerase (Promega, Cat. # M8305), 1 μ l (50 pmol/ μ l) of sense primer, 1 μ l (50 pmol/ μ l) of antisense primer, 4 μ l of 10 mM each dNTPs (Promega, Cat. # U1511) and 24.5 μ l DEPC treated water. For PCR amplification of G type rotaviruses (Table 1), an initial denaturation of 5min at 95°C was applied and 35 cycles for first round and 30 cycles for 2nd round were used. The PCR profile for first round was 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and an additional extension at 72°C for 7min was applied using a PCR machine (BioMetra, UK). The amplified RT-PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100 bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

Table 1: PCR reaction profile for G type Rotaviruses.

Step	Reaction			
	1st round		2nd round	
	Temp (°C)	Time (min)	Temp (°C)	Time (min)
Denaturation	94	1 min	94	1 min
Annealing	52	1 min	42	2 min
Extension	72	1 min	72	1 min
No. of cycles	35		30	
Expected Product	881 bp		175-754 bp	

For the second round; PCR amplification was carried out from the first round PCR reaction in 50 μ l reaction mixture containing 2 μ l of 1st round PCR product, 5 μ l 10 x PCR Buffer, 4 μ l of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5 μ l of 5 U/ μ l Go Taq Flexi DNA Polymerase (Promega Cat. No. M8301), 1 μ l downstream primer (50 pmol/ μ l), 1 μ l of 8 cocktail upstream primers (50 pmol/ μ l each), 4 μ l of 10 mM each dNTPs (Promega, Cat. # U1511) and 25.5 μ l DEPC treated water. The PCR profile for the second round was an initial denaturation of 5 min at 95°C followed by 30 cycle at 94°C for 1 min, 42°C for 2 min, and 72°C for 1 min, and an additional extension at 72°C for 7 min was applied using a PCR machine (BioMetra, UK). The amplified RT-PCR products were

electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

For PCR amplification of P type rotaviruses (Table 2), an initial denaturation of 5min at 95°C and 35 cycles for first round and 30 cycles for 2nd round. For 1st round PCR cycles profiles was at 94°C for 1min, 50°C for 2 min, and 72°C for 1 min, and an additional extension at 72°C for 7 min. Profile was applied using a PCR machine (BioMetra, UK). The amplified RT-PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

Table 2: PCR reaction profile for P type Rotaviruses.

Step	Reaction			
	1st round		2nd round	
	Temp (°C)	Time (min)	Temp (°C)	Time (min)
Denaturation	94	1 min	94	1 min
Annealing	50	2 min	45	2 min
Extension	72	1 min	72	1 min
No. of cycles	35		30	
Expected Product	876 bp		276-583 bp	

For the second round; PCR amplification was carried out from the first round PCR reaction in 50 µl reaction mixture containing 2 µl of 1st round PCR product, 5µl 10x PCR Buffer, 4 µl of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5 µl of 5 U/µl Go Taq Flexi DNA Polymerase (Promega Cat. No. M8301), 1 µl of a 7 cocktail downstream primers (50 pmol/µl each), 1 µl upstream primer (50 pmol/µl), 4 µl of 10 mM each dNTPs (Promega, Cat. # U1511) and 26.5 µl DEPC treated water. The PCR profile for the second round was an initial denaturation of 5min at 95°C followed by 30 cycle at 94°C for 1 min, 45°C for 2 min, and 72°C for 1 min, and an additional extension at 72°C for 7 min was applied using a PCR machine (BioMetra, UK). The amplified RT-PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

Astroviruses

cDNA synthesis and PCR amplification: Viral RNA was extracted from 200 µl aliquot of 10% stool sample, using Axygen® Kit (Axygen biosciences, Cat. No. AP-MN-BF-VNA-250), according to the

instructions of the manufacturer. Reverse transcription (RT) was carried out using a mixture of 10 µl of extracted viral RNA, 5 µl random primer (25 pmol/µl), and 20 µl DEPC treated water. The reaction mixture, 35 µl, was heated at 65°C for 5 min and chilled on ice for 2 min. 35 µl of the reaction mixture was added to 1 µl of 200 U/µl M-MLV Reverse Transcriptase (Promega, Cat. # M1701), 10 µl RT-buffer, 4 µl of 10 mM each dNTPs (Promega, Cat. # U1511). The mixture was heated at 42°C for 60 min and 37°C for 30 min, for cDNA synthesis, followed by 95°C for 5 min. The RT-PCR reaction was carried out in 50 µl reaction mixture containing 10 µl of the synthesized cDNA, 5 µl 10 x PCR Buffer, 4 µl of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5 µl of 5 U/µl Go Taq DNA Polymerase (Promega, Cat. # M8305), 1 µl (50 pmol/µl) of sense primer, 1µl (50 pmol/µl) of antisense primer, 4 µl of 10 mM each dNTPs (Promega, Cat. # U1511) and 24.5 µl DEPC treated water. For PCR amplification for astroviruses (Table 3), was start by an initial denaturation of 5min at 95°C, followed by 40 thermal cycles. The PCR profile for thermal cycles was 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and an additional extension at 72°C for 7 min was applied using a PCR machine (BioMetra, UK). The amplified RT-PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100 bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

Table 3: PCR reaction profile for Astroviruses.

Step	Reaction	
	Temp (°C)	Time (min)
Denaturation	94	1 min
Annealing	50	2 min
Extension	72	1 min
No. of cycles	40	
Expected Product	449 bp	

Noroviruses genogroup I (GI)

cDNA synthesis and PCR amplification: Viral RNA was extracted from 200 µl aliquot of 10% stool sample, using Axygen® Kit (Axygen biosciences, Cat. No. AP-MN-BF-VNA-250), according to the instructions of the manufacturer. Reverse transcription (RT) was carried out using a mixture of 10 µl of extracted viral RNA, 5 µl random primer (25 pmol/µl), and 20 µl DEPC treated water. The reaction mixture, 35 µl, was heated at 65°C for 5 min. and chilled on ice for 2 min. 35 µl of the reaction mixture was added to 1 µl of 200 U/µl M-MLV Reverse Transcriptase (Promega, Cat. # M1701), 10 µl RT-buffer, 4 µl of 10mM each dNTPs (Promega, Cat. # U1511). The mixture was heated at 42°C for 60 min and 37°C for 30 min, for cDNA synthesis, followed by 95°C for 5 min. The RT-PCR reaction was carried out in 50 µl reaction mixture containing 10 µl of the synthesized

cDNA, 5 µl 10 x PCR Buffer, 4 µl of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5µl of 5U/µl Go Taq DNA Polymerase (Promega, Cat. # M8305), 1 µl (50 pmol/µl) of sense primer, 1 µl (50 pmol/µl) of antisense primer, 4 µl of 10mM each dNTPs (Promega, Cat. # U1511) and 24.5 µl DEPC treated water. For PCR amplification for Noroviruses GI (Table 4), the reaction started by an initial denaturation of 5 min at 95°C, followed by 40 thermal cycles. The PCR profile for thermal cycles was 94°C for 45 sec, 50°C for 45 sec, and 72°C for 1 min, and an additional extension at 72°C for 7 min was applied using a PCR machine (BioMetra, UK). The amplified RT-PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100 bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

Table 4: PCR reaction profile for Noroviruses GI.

Step	Reaction	
	Temp (°C)	Time (min)
Denaturation	94	45 sec
Annealing	50	45 sec
Extension	72	1 min
No. of cycles	40	
Expected Product	329 bp	

Noroviruses genogroup II (GII)

cDNA synthesis and PCR amplification: Viral RNA was extracted from 200 µl aliquot of 10% stool sample, using Axygen® Kit (Axygen biosciences, Cat. No. AP-MN-BF-VNA-250), according to the instructions of the manufacturer. Reverse transcription (RT) was carried out using a mixture of 10 µl of extracted viral RNA, 5 µl random primer (25 pmol/µl), and 20 µl DEPC treated water. The reaction mixture, 35 µl, was heated at 65°C for 5 min and chilled on ice for 2 min. 35µl of the reaction mixture was added to 1µl of 200 U/µl M-MLV Reverse Transcriptase (Promega, Cat. # M1701), 10 µl RT-buffer, 4 µl of 10mM each dNTPs (Promega, Cat. # U1511). The mixture was heated at 42°C for 60 min and 37°C for 30 min, for cDNA synthesis, followed by 95°C for 5 min. The RT-PCR reaction was carried out in 50 µl reaction mixture containing 10 µl of the synthesized cDNA, 5 µl 10 x PCR Buffer, 4 µl of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5 µl of 5 U/µl Go Taq DNA Polymerase (Promega, Cat. # M8305), 1 µl (50 pmol/µl) of sense primer, 1 µl (50 pmol/µl) of antisense primer, 4 µl of 10 mM each dNTPs (Promega, Cat. # U1511) and 24.5 µl DEPC treated water. For PCR amplification for Noroviruses GII (Table 5), the reaction started by an initial denaturation of 5min at 95°C, followed by 40 thermal cycles. The PCR profile for thermal cycles was 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, and an additional extension at 72°C for 7 min was applied

using a PCR machine (BioMetra, UK). The amplified RT-PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100 bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

Table 5: PCR reaction profile for Noroviruses GII.

Step	Reaction	
	Temp (°C)	Time (min)
Denaturation	94	30 sec
Annealing	50	30 sec
Extension	72	1 min
No. of cycles	40	
Expected Product	343 bp	

DNA Viruses (Adenoviruses)

PCR amplification: Viral DNA was extracted from 200µl aliquot of 10% diluted stool samples, with sterile PBS pH 7.2, using Axygen® Kit, (Axygen biosciences, Cat. No. AP-MN-BF-VNA-250), according to the instructions of the manufacturer. Nested PCR was carried out where the reaction takes place in two rounds; of The first-round PCR was carried out in 50 µl reaction mixture containing 10 µl of extracted DNA, 5 µl 10 x PCR Buffer, 4 µl of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5 µl of 5 U/µl Go Taq DNA Polymerase (Promega, Cat. # M8305), 1 µl (50 pmol/µl) of sense primer (Adv-HEX1DEG-F10), 1 µl (50 pmol/µl) of antisense primer (Adv-HEX2DEG-R10), 4 µl of 10 mM each dNTPs (Promega, Cat. # U1511) and 24.5 µl DEPC treated water. For PCR amplification of adenoviruses (Table 6), an initial denaturation of 5 min at 95°C was applied and 35 cycles for first round and 35 cycles for 2nd round were used. The PCR profile for first round was 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and an additional extension at 72°C for 7 min was applied using a PCR machine (BioMetra, UK). The amplified PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100 bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

For the second round; PCR amplification was carried out from the first round PCR reaction in 50 µl reaction mixture containing 5 µl of 1st round PCR product, 5 µl 10 x PCR Buffer, 4 µl of 25 mM MgCl₂ (Promega, Cat. # A3511), 0.5µl of 5U/µl Go Taq Flexi DNA Polymerase (Promega Cat. No. M8301), 1 µl of (Adv-HEX3DEG-F10) downstream primer (50 pmol/µl), 1 µl of (Adv-HEX4DEG-R10) upstream primers (50 pmol/µl each), 4 µl of 10 mM each dNTPs (Promega, Cat. # U1511) and 28.5 µl DEPC treated water. The PCR profile for the second round was an

initial denaturation of 5 min at 95°C followed by 35 cycle at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and an additional extension at 72°C for 7 min was applied using a PCR machine (BioMetra, UK). The amplified PCR products were electrophoresed through 1.5% agarose gels in Tris acetate/EDTA buffer against 100 bp DNA ladder (Promega, Cat. # G2101) and visualized by staining with ethidium bromide. Bands were visualized under UV light using UV transilluminator. The obtained band size is determined with the reference of the DNA ladder.

Table 6: PCR reaction profile for Adenoviruses.

Step	Reaction			
	1st round		2nd round	
	Temp (°C)	Time (min)	Temp (°C)	Time (min)
Denaturation	94	30 sec	94	30 sec
Annealing	55	30 sec	55	30 sec
Extension	72	1 min	72	1 min
No. of cycles	35		35	
Expected Product	301 bp		171 bp	

Oligonucleotide primers

Universal viral gastroenteritis oligonucleotide primers sequences were obtained from Bioneer Company, as purified lyophilized primers. Universal primers for G typing rotaviruses was selected for amplification of segment 9 as previously described by Iturriza-Gomara, et al., [21]. These primer pairs are semi nested primers designed to amplify the highly conserved region among all known rotaviruses strains and specific for gene segment 9, as listed and shown in Table 7. Universal primers for P typing rotaviruses was selected for amplification of VP7 gene segment as described by Gentsch et al., [22] and also listed and shown in Table 7. For detection of astroviruses sets of primers were selected and used as described by Noel et al., [23] (Table 7), and noroviruses sets of primers were used as described by Kojima et al., [24] (Table 7). For adenoviruses PCR the primer sets listed in Table 7, described by Allard et al., [25] and designed to amplify the highly conserved region of hexon gene which is very similar in many types of adenoviruses.

Analysis of PCR products

PCR products were analyzed by electrophoresis using 1.5% Agarose gel with Ethidium bromide, as follow: 1.5 g umltrapure Agarose powder (BDH), was dissolved in 100 ml Tris-Borate-EDTA (TBE) and cooked at microwave for 2 min. Agarose was cooled down to about 42-45°C and 8 µl of Ethidium bromide (10 mg/ml) was added. The melted agarose was poured into horizontal electrophoresis chamber with 20 whole comb and left to dry at room temperature. 10 µl of PCR products were mixed with 2 µl of loading buffer (1% sodium dodecyl sulphate, 0.25% bromophenol blue and 0.1M EDTA, pH 8.0),

and applied into agarose gel and electrophoresed at 100V for 1-1.5 hr. Presumptive and confirmed product bands were visualized and compared with standard 100 bp DNA marker (Promega, USA, Cat. # G2101). Bands were visualized under UV light using UV transilluminator and gel documentation system.

Table 7: The list of primers sets used to detect viruses.

Primer	Sequence	Polarity	Location
G genotype of rotaviruses			
1st round			
Rota VP7-F10	ATGTATGGTATTGAATATTACCAC	+	51
Rota VP7-R10	AACTTGCCACCATTITTTTCC	-	932
2nd round			
Rota G1-aBT1-10	CAAGTACTCAAATCAATGATGG	+	314
Rota G2-acT2-10	CAATGATATTAACACATITTTCTGTG	+	411
Rota G- 10	ACGAACCTCAACACAGAGAGG	+	250
Rota G4-2aDT4-10	CGTTTCTGGTGAGGAGTTG	+	480
Rota G8-aAT8-10	GTCACACCATTGTAAATTCG	+	178
Rota G9-10	CTTGATGGACTAYAAATAC	+	757
Rota G10-10	ATGTCAGACTACARATACTG	+	666
Rota G9-VP7aFT9d-10	CTTGATGTRACTAYAAAMTAC	+	757
Rota VP7-R10	AACTTGCCACCATTITTTTCC	-	932
P genotype of rotaviruses			
1st round:			
Con3-10	TGGCTTCGCCATTTTATAGA	+	11
Con2-10	ATTTTCGGACCATTATAACC	-	885
2nd round:			
Con3-10	TGGCTTCGCCATTTTATAGA	+	11
Rota P(8)-1T1-10	TCTACTTGGATAACGTGC	-	356
Rota P(4)-2T1-10	CTATTGTAGAGGTTAGAGTC	-	494
Rota P(6)-3T1-10	TGTTGATTAGTTGGATTCAA	-	278
Rota P(9)-4T1-10	TGAGACATGCAATTGGAC	-	402
Rota P(10)-5T1-10	ATCATAGTTAGTAGTCGG	-	594
Rota P(11) -10	GTAACATCCAGAATGTG	-	323
Rota P(8)-1T-1D-10	TCTACTGGRTRACNTGC	-	356
Astroviruses			
Astro-mon-269-F-10	CAACTCAGGAACAGGGTGT	+	4526
Astro-mon-270-R-10	TTAGTGAGCCACCAGCCATC	-	4974
Noroviruses Genogroup I (GI)			
Noro GISKF-10	CTGCCCGAATTGTAAATGA	+	5342
Noro GISKR-10	CCAACCCARCCATTRTACA	-	5671
Noroviruses Genogroup II (GII)			
Noro GII SKF-10	CNTGGGAGGGCGATCGCAA	+	5058
Noro GII SKF-10	CCRCCNGCATRHCCRTTRTACAT	-	5401
Adenoviruses			
1st round			
Adv-HEX1 DEG10	GCCSCARTGGKWTACATGCACATC	+	21
Adv-HEX 2 DEG10	CAGCACSCCICGRATGTCAA	-	321
2nd round			
Adv-HEX3 DEG- F10	GCCCGYGCMACIGAIACSTACTTC	+	68
Adv-HEX4 DEG-R1	CCYACRGCCAGIGTRWAICGMRCYTTGTA	-	239

Statistical analysis

Statistical analysis was performed using the SPSS statistical package software for windows version 21 (SSPS Inc, Pennsylvania, and USA). Parametric variables are expressed as the mean ± SD. Differences between parametric variables among the obese and overweight versus control groups were evaluated using 2-tailed unpaired t-test. Pearson's correlation coefficients were used to evaluate correlations between the data exhibiting parametric distribution. P value <0.05 was considered significant difference and p < 0.005 was considered highly significant difference.

Results

Serum levels of I-FABP were higher in GE cases group compared to control group (1026.4 pg/ml

vs 267.9 pg/ml, respectively). Table 8 showed serum I-FABP level in gastroenteritis cases group versus control group. Serum levels of I-FABP were higher in the 46 Rotavirus - GE cases compared to the 9 adenovirus – GE cases. Also these levels were higher in the 46 Rotavirus- GE cases compared to the 4 Astrovirus-GE cases. There were 3 cases with mixed Rotavirus and Adenovirus co-infections whose serum I-FABP levels shouted to 1425 pg/ml. Similarly, the other 3 cases of mixed Rotavirus and Astrovirus co-infections had those levels reaching 1217 pg/ml. Although those cases were few in number but their importance will be stated in the discussion.

Table 8: Serum I-FABP level in gastroenteritis cases group versus control group.

Serum I-FABP conc. (pg/ml)	Number	Mean \pm SD	P- value
GE cases	93	1026.4 \pm 494.4	P<0.001
Control	28	267.9 \pm 200.4	P<0.001

**highly significant difference at p<0.001.

Table 9 showed the serum I-FABP level according to type of virus.

Table 9: Serum I-FABP level according to type of virus.

Variable	Rotavirus cases	Adenovirus cases	Astrovirus cases	Rotavirus and Adenovirus cases	Rotavirus and Astrovirus cases	Total GE cases	Control
No. of cases	46	9	4	3	3	93	28
Serum I-FABP	1017.4 \pm 456*	653 \pm 419	408 \pm 210	1425 \pm 871	1217 \pm 453	1217 \pm 453	267.9 \pm 200.4*

*Significant difference at p<0.05.

Paired Samples Test revealed statistically significant difference in I-FABP level when mild and severe degrees of dehydration were compared. On the other hand, statistically insignificant difference in I-FABP level between mild and moderate degrees of dehydration is observed in Table 10.

Table 10: Comparisons between serum I-FABP levels according to degree of dehydration in the studied groups.

Serum I-FABP level	t	P value
Mild vs Moderate cases	0.318	0.760
Mild vs Severe cases	2.564	0.037*

*Significant difference at p<0.05.

Significant positive correlations were found between hospitalization and serum level of I-FABP as well as dehydration (P=0.000). Furthermore, a significant positive correlation was found out between hospitalization and the degree of fever (P=0.002) as shown in Table 11.

Table 11: Correlations between hospitalization and other parameters in the study group.

Hospitalization		Serum I-FABP	Dehydration severity	Fever
	Pearson correlation	0.223*	0.713**	0.322**
Sig. (2-tailed)	0.045	0.000	0.002	

*Significant difference at p<0.05, **highly significant difference at p<0.001.

Discussion

Loss of gut wall integrity is consequence of low flow states associated with viral gastroenteritis. It is involved in the development of various inflammatory syndromes, including sepsis, and multiple organ failure. The delay in diagnosis is a major factor associated with the high morbidity and mortality of patients with intestinal ischemia [26]. Although diagnostic accuracy of intestinal ischemia has improved with the development of computed tomography (CT) [27] a laboratory test for clinical use is necessary [28]. Intestinal fatty acid-binding protein (I-FABP) is a useful marker in the detection of intestinal ischemia. These are mainly expressed in the intestinal villi which are the initial site of destruction in acute intestinal inflammation, injury, and ischemia all of which are present in viral gastroenteritis pathophysiology [29]. It has emerged as a valuable marker in the diagnosis of intestinal damage at an early stage [30, 31]. The current study provides additional information concerning the clinical utility of serum I-FABP levels during intestinal ischemia reperfusion.

Our present study dealt with the promising issue of resorting to serum I-FABPs in the early prediction of intestinal injury in viral GE particularly Rota viral GE. This is very crucial to Egypt and other developing countries because Rota virus vaccination is still fighting against poor sanitation in general and the sewage polluted River-Nile water in particular. Our study worked upon proving that, I-FABP could be made use as early predictor of the gut injury in viral GE.

Several studies showed that plasma I-FABP levels were significantly increased in patients suspected with intestinal ischemia, as compared with patients with other diagnoses [32]. It increases within the early stages of intestinal ischemia [33]. In our present study, serum I-FABP levels were significantly higher in GE cases compared to control group.

Many recent studies have also reported the existence of coinfections by different viruses whether in Egypt or in other countries [34-36]. Some studies have confirmed that in RV-GE cases, viral co-infections (and even bacterial and parasitic) aggravated the clinical picture of the disease and caused an extension of the hospital stay period [37, 38]. Some recent studies reported the predominance of Adenovirus and Astrovirus GE in certain countries in particular months of the year [39-40]. In our present study, we did have serious conditions which necessitated hospitalization, and serum I-FABPs levels were also higher in RV-GE cases compared to Adeno and Astro viruses GE cases. In addition, their levels were also higher in GE cases infected with mixed viruses (coinfections). Similar to our results, Ishimura et al., [41], and Pelsers et al., [42] found an

elevation in serum levels of I-FABP in cases of intestinal inflammation, injury and ischemia. In addition, their levels were not detectable in serum of healthy subjects.

Guthmann et al., [17] ensured that, I-FABP was a very specific marker for detection of intestinal injury which led to severe stage III. In accordance with our results, some studies emphasized that individuals who suffered from necrotizing enterocolitis (NEC), and acute ischemic diseases had their serum I-FABP concentrations applicable for detection of intestinal injury [20]. Again, in agreement with our study, Panl et al., [43] assumed that I-FABP might constitute a useful marker for acute intestinal failure in critically ill patients.

Machado et al., [36] found out that levels of I-FABP were early marker of intestinal necrosis after acute pancreatitis and after liver surgery and aortic surgery. In this study, critically ill patients had an evaluation of their intestinal failure via evaluating the circulating I-FABP values. Many studies also described I-FABP as plasma markers for intestinal injury [42, 43]. Although they are relatively old ones, still very important because all those research studies agreed upon that finding and the rarity of similar recent studies does not be little the crucial importance of old ones.

In our study, we observed a significant elevation in serum I-FABP level on comparing severely dehydrated groups with mild dehydrated cases. There was a positive correlation between the serum level of I-FABP and hospitalized patients, taking into consideration that severely dehydrated children needed hospitalization.

In conclusions, this study provides new insight into the significance of serum I-FABP following human intestinal injury. The results demonstrate that the extent of intestinal tissue damage is related to the duration of injury. In addition, systemic I-FABP levels can very well differentiate between mild, reversible damage and more extensive irreversible intestinal damage. Future studies should be conducted using I-FABP in the decision-making during the diagnostic workup of patients suspected for intestinal injury, to integrate I-FABP in daily clinical practice.

Acknowledgements

The authors are grateful to the patients and their caregivers, who were helpful and cooperative while participating in the study.

References

- Zlomy M, Kofler S, Orth D, Würzner R, Heinz-Erian P, Streng A, Prelog M. The impact of Rotavirus mass vaccination on hospitalization rates, nosocomial Rotavirus gastroenteritis and secondary blood stream infections. *BMC Infect Dis.* 2013;13:112.
- Giaquinto C, Van Damme P. Age distribution of pediatric rotavirus gastroenteritis cases in Europe: the REVEAL study *Scand J Infect Dis.* 2010; 42: 142-147.
- Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD; WHO-coordinated Global Rotavirus Surveillance Network. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *Lancet Infect Dis.* 2012;12(2):136-41.
- Zaghoul MZ, El-Sahn SF, Galal ZA. Confection of Rotavirus group A, Norovirus and Adenovirus in Egyptian children with Gastroenteritis. *Life Science Journal.* 2013; 10 (2): 848-852.
- Liu IJ, Liu W, Liu YX, et al. Identification of norovirus as the top enteric viruses detected in adult cases with acute gastroenteritis. *Am J Trop Med Hyg.* 2010; 82 (4): 717-22.
- Dey RS, Ghosh S., Chawla – Sarkar M, Panchalingam S., Nataro JP, Sur D, Manna B Ramamurthy T. Circulation of a Novel pattern of Infections by Enteric Adenovirus Serotype 41 among children below 5 years of age in Kolkata, India. *Journal of Clinical Microbiology.* 2011; 49 (2): 500-505.
- Tran A, Talmud D, Lejeune B, et al: Prevalence of rotavirus, adenovirus, norovirus and astrovirus infections and coinfections among hospitalized children in northern France. *J Clin Microbiol.* 2010; 48 (5): 1943-6.
- Levidiotou S, Gartzonika C, Papaventsis D, et al. Viral agents of acute gastroenteritis in hospitalized children in Greece. *Clin Microbiol Infect.* 2009; 15: 596-598.
- Junquera CG, de Baranda CS, Mialdea OG, et al. Prevalence and clinical characteristics of norovirus gastroenteritis among hospitalized children in Spain. *Pediatr Infect Dis.* 2009; 28: 604-607.
- Widdowson MA, Steele D, Vojdani J, Wecker J, Parashar UD. Global rotavirus surveillance: preparing for the introduction of rotavirus vaccines. *J Infect Dis.* 2009; 200(Suppl 1):S1-S8.
- Parashar UD, Burton A, Lanata C, Boschi-Pinto C, Shibuya K, Steele D, Birmingham M, Glass RI. Global mortality associated with rotavirus disease among children in 2004. *J Infect Dis.* 2009; 200(Suppl 1): S9-S15.
- Diggle L. Rotavirus diarrhoea and future prospects for prevention. *Br J Nurs.* 2007; 16(16):970-974.
- Parashar UD, Gibson CJ, Bresse JS, Glass RI. Rotavirus and severe childhood diarrhea. *Emerg Infect Dis.* 2006; 12(2):304-306.
- Fink MP, Delude RL. Epithelial barrier dysfunction: A unifying theme to explain the pathogenesis of multiple organ dysfunction at the cellular level. *Crit Care Clin.* 2005; 21:177–196.
- Derikx JP, Vreugdenhil AC, Van den Neucker AM, et al. A pilot study on the noninvasive evaluation of intestinal damage in celiac disease using I-FABP and L-FABP. *J Clin Gastroenterol.* 2009;43:727–733.
- Grootjans J, Thuijls G, Verdam F, et al. Non-invasive assessment of barrier integrity and function of the human gut. *World J Gastrointest Surg.* 2010; 2:61–69.

17. Guthmann F, Borchers T, Wolfrum C, Wustrack T, Bartholomaeus S, Spener F. Plasma concentration of intestinal- and liver FABP in neonates suffering from necrotizing enterocolitis and in healthy preterm neonates. *Mol Cell Biochem.* 2002; 239:227–34.
18. Cronk DR, Houseworth TP, Cuadrado DG, Herbert GS, McNutt PM, Azarow KS. Intestinal fatty acid binding protein (I-FABP) for the detection of strangulated mechanical small bowel obstruction. *Curr Surg.* 2006; 63(5):322-5.
19. Pelsers MM. Fatty acid-binding protein as plasma marker for tissue injury. Thesis Maastricht University ISBN 90-9018161-X. Printed by Ponsen and Looijen BV, Wageningen the Netherlands © M.M.A.L Pelsers: Maastricht, the Netherlands, 2004.
20. Thuijls G, Van Wijck K, Grootjans J, Deriks JP. Early Diagnosis of intestinal ischemia using urinary and plasma fatty acid binding proteins. *Ann Surg.* 2011; 253 (2): 303-8.
21. Iturriza-Gomara M, King G, Gray J. Rotavirus genotyping keeping up with an evolving population of human rotaviruses. *J Clin Virol.* 2004; 31: 259-265.
22. Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, Das BK, Bhan MK. Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J Clin Microbiol.* 1992; 30: 1365-1373.
23. Noel JS, Lee T, Kurtz, JB, Glass, RI, Monroe SS. Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing. *J Clin Microbiol.* 1995; 33: 797-801.
24. Kojima S, Kageyama T, Fukushi, S., Hoshino FB, Shinohara M., Uchida K, Natori K, Takeda N, Katayama K. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J Virol Methods.* 2002; 100: 107-114.
25. Allard A, Albinsson B, Wadell G. Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. *J Clin Microbiol.* 2001; 39: 498-505.
26. Oldenburg WA, Lau LL, Rodenberg TJ, et al. Acute mesenteric ischemia: a clinical review. *Arch Intern Med.* 2004; 164:1054–1062.
27. Menke J. Diagnostic accuracy of multidetector CT in acute mesenteric ischemia: systematic review and meta-analysis. *Radiology.* 2010; 256:93–101.
28. Acosta S, Nilsson T. Current status on plasma biomarkers for acute mesenteric ischemia. *J Thromb Thrombolysis.* 2011; 33: 355–361.
29. Pelsers MM, Hermens WT, Glatz JF. Fatty acid-binding proteins as plasma markers of tissue injury. *Clin Chim Acta.* 2005;352: 15-35.
30. Schellekens HS, Grootjans J, Dello SA, van Bijnen WG, Annemarie A, van Dam RM, Joep PM, Buurman WA. Plasma Intestinal Fatty Acid-Binding Protein Levels Correlate With Morphologic Epithelial Intestinal Damage in a Human Translational Ischemia-reperfusion Model. *Journal of Clinical Gastroenterology.* 2014; 48 (3): 253-260.
31. Ramig RF. The Pathogenesis of Intestinal and Systemic Rotavirus Infection. *J Virol.* 2004; 78 (19): 10213-10220.
32. Kanda T, Tsukahara A, Ueki K, et al. Diagnosis of ischemic small bowel disease by measurement of serum intestinal fatty acid-binding protein in patients with acute abdomen: a multicenter, observer-blinded validation study. *J Gastroenterol.* 2011; 46: 492–500.
33. Vermeulen Windsant IC, Hellenthal FA, Derikx JP, et al. Circulating intestinal fatty acid-binding protein as an early marker of intestinal necrosis after aortic surgery: a prospective observational cohort study. *Ann Surg.* 2012; 255: 796–803.
34. Ferreira CE, Raboni SM, Pereira LA, et al. Viral acute gastroenteritis: Clinical and epidemiological features of coinfecting patients. *Braz J Infect Dis.* 2012; 16 (3): 267-72.
35. Tamura T, Nishikawa M, Anh DD, et al. Molecular epidemiological study of rotavirus and norovirus infections among children with acute gastroenteritis in NhaTrang, Vietnam, December 2005 – June 2006. *Jpn J Infect Dis.* 2010; 63(6): 405-11.
36. Machado MCC, Barbeiro HV, Da Silva FP, De Souza HP. Circulating fatty acid-binding proteins as a marker of intestinal failure in septic patients. *Critical Care.* 2012; 16:455
37. Hung TY, Liu MC, Hsu CF, Lin YC. Rotavirus infection increases the risk of bacteremia in children with nontyphoid Salmonella gastroenteritis. *Eur J Clin Microbiol Infect Dis.* 2009;28(4):425-8.
38. Sinclair MI, Hellard ME, Wolfe R, Miakais TZ, Leder K, et al. Pathogen causing community gastroenteritis in Australia. *J Gastroenterol Hepatol.* 2005; 20 (11): 1685-90.
39. Shimizu H, et al. An outbreak of Adenovirus serotype 41 infection in infants and children with acute gastroenteritis in Maizura City, Japan. *Infect Genet Evol.* 2007; 7: 279-284.
40. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis *N Engl J Med.* 2009; 361 : 1776-1785.
41. Ishimura S, Furuhashi M, Watanabe Y, Hoshina K, Fuseya T, Mita T, Okazaki Y, Koyama M, Tanaka M, Akasaka H, Ohnishi H, Yoshida H, Saitoh S, Miura T. Circulating levels of fatty acid-binding protein family and metabolic phenotype in the general population. *PLoS One.* 2013;8(11):e81318.
42. Pelsers MM, Namiot Z, Kisielewski W, Namiot A, Janusz-Kiewicz M, et al. Intestinal-type and liver-type fatty acid-binding protein in the intestine. Tissue distribution and clinical utility. *Clin Biochem.* 2003; 36 (7): 529-35.
43. Panl, WX, Li W, Li N, Li J. the intestinal fatty acid-binding proteins diagnosing dysfunction in acute pancreatitis: A pilot study. *Pancreas.* 2010; 39: 633-638.