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Comparative study between Immunochromatography, Enzyme immunoassay and Real-time PCR for diagnosis of Rotavirus infection in infants and young children with acute winter diarrhea

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Abstract: Background: One of the most etiologies of mortality between newborns and children in developing countries is acute infectious gastroenteritis. It is frequently due to viral infection. Rotaviral infections in young children can result in severe, life threatening diarrhea. In developing countries, the impact of infection is further severe where about 600,000 deaths occur yearly. An easily, sensitive and rapid assay is required to offer timely detection of this viral agents for operative clinical controlling and employment of separation measures. **The aim of the study:** The aim of the present study was to compare between Lateral flow Immunochromatographic test (RIDAQUICK Rotavirus Test), Enzyme immunoassay test (RIDASCREEN Rotavirus Test) and Quantitative RT real-time PCR (VIASURE Rotavirus kit) in the diagnosis of Rotavirus infection in infants and young children with acute winter diarrhea. **Methodology:** The present study was carried out on 50 infants and young children, who were attending the Diarrhea and Nutrition Unit of Pediatric Department at Tanta University Hospitals in the period from December 2016 to March 2017 and were clinically diagnosed according to history, clinical signs, symptoms and using Vesikari scoring system as having acute gastroenteritis, those were included in the patients' group. In addition to 10 apparently healthy infants and young children were included as a control group. Stool samples were collected from the study group and the control group. The samples underwent testing by Lateral flow Immunochromatographic test (RIDAQUICK Rotavirus Test), Enzyme immunoassay test (RIDASCREEN Rotavirus Test) and Quantitative RT real-time PCR (VIASURE Rotavirus kit) for diagnosis of *Rotavirus* in stool samples. **Results:** Using the 3 different diagnostic methods on the patients' group revealed that 35 (70%) of the cases gave positive results with RIDAQUICK Immunochromatography kit and 41 (82%) were positive by RIDASCREEN ELISA and 49 (98%) were positive with real time RT-PCR. In addition to the control group they all gave negative results with the 3 tests. **Conclusion:** The *rotavirus* immunochromatographic test (RIDAQUICK) is a good substitute for the infrequent analysis of stool samples in ambulatory practice. It is rapid, inexpensive and useful for testing single specimen. However, it has minor sensitivity and not perfectly detect positive samples obtained post the sequence of clinical disease. ELISA test (RIDASCREEN *Rotavirus*) is more accurate than IC test. It is suitable as a routine diagnostic tool in the lab. and can display large numbers of samples. However, a major drawback of ELISA system is not costly effective in case of assaying a single sample. Quantitative real time PCR, can provide higher sensitivity and specificity. It also offers important benefits for the recognition of *rotavirus* nucleic acids in minimal levels in stool samples.

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Keywords: Comparative study; Immunochromatography; Enzyme immunoassay; Real-time PCR; diagnosis

1. Introduction:

One of the most prevalence etiologies of mortality through newly born and children in developing countries is acute infectious gastroenteritis [(1)]. It is utmost generally owing to viral infection [(1,2)]

The great risk is present in the younger child, where a great losses in the fluid and electrolytes will causing to dehydration. There are different types of dehydration, isotonic, hypotonic and hypertonic which is independent on the etiological agents. Diarrhea and vomiting resulting in losses of fluid from circulation nearly three times higher than the volume of circulating blood (80–125–250 mL/kg b.wt./day). To compensate the dehydration, the body extracts the fluid from the intracellular space to keep the blood volume constant, leading to dehydration. To avoid the

complications and morbidity of dehydration it is recommended to give as early as possible a rehydration solution (glucose-electrolyte solution) and adequate nutrients corresponding to the child's age [2-4].

Generally, rotavirus infections affecting mainly all ages of human being. Exposing to primary rotavirus infection in young children is accompanied with sever and life-threatening diarrhea. While in older subjects the signs may be a non-symptomatic or mildly enteric signs, probably due to growing cross-protective immunity resultant from recurrent infections, while in some cases, severe illness may also affecting old aged subjects. The consequence of infection is more sever in developing other than in other countries, where about 600,000 deaths take place yearly and living children still complaining from morbidity [5,6].

Even though in developed countries, the death rates are somewhat low, *rotavirus* infection is liked with 30–60% of morbidity as a result of acute gastroenteritis, thus donating a vital disorder burden to the healthcare system [6-8]

Therefore, easily, sensitive and rapid technique is urgently required to deliver timely diagnosis of these causative agents for efficient actual clinical control and application of identification techniques [9].

Accurate detection of *Rotavirus* particularly *Rotavirus A (RVA)* is critical for control and avoidance of disorder and observation of outbreaks [9,10].

Early studies, reported that, electron microscopy (EM) was used for the first time for the recognition of viral particles in samples of stool [10]. Though, EM surveillance is uncommonly applied as a predictable diagnostic method for its high expenses and the skill necessities and expensive instrumentation, in addition to a decreased sensitivity [11].

Monoclonal or polyclonal antibodies against the inner capsid protein VP6 were used commercially as a diagnostic tool beginning from the 80s, instead of costs and time consuming EM examination [11]. The estimation of VP6 protein in stool samples is commonly used as a biomarker of *RVA* infection, being the maximum copious viral protein, highly conserved and antigenically dominant between *RVAs* of various animal species. Several commercial tests including enzyme-linked immunosorbent (ELISA), latex agglutination assays, and immunochromatographic tests (ICT) were used for the detection of *RVA* infection. The advantageous of ICTs are easily, rapid and simple, and can give a result within half an hour, making them an desirable diagnostic method [12,13]. Recently, molecular methods, like Real-time PCR and reverse transcriptase polymerase chain reaction (RT-PCR) are specific and highly sensitive, representing the gold standard for genetic characterization, epidemiological studies and diagnosis of *RVAs* [13]

Molecular techniques using reverse transcription polymerase chain reaction (RT-PCR) have augmented the frequency of estimation of *rotaviruses* in contrast with enzyme immunoassays (EIA) [14,15].

One of the advances in the molecular technology is the introduction of real time PCR for diagnosis that has several presentations. The advantages of RT-PCR represented in highly sensitivity and specificity, faster turn-around time, superior accuracy, and minimization of cross-contamination due to the close-tube system [16]

Aim of the work:

The aim of the present study was to compare between Lateral flow Immunochromatographic test (RIDAQUICK Rotavirus Test), Enzyme immunoassay test (RIDASCREEN Rotavirus Test) and Quantitative RT real-time PCR (VIASURE Rotavirus kit) in the diagnosis of Rotavirus infection in infants and young children with acute winter diarrhea.

2. Patients, materials & methods:

a) Patients & control:

This study was conducted on 50 infants and young children suffering from acute diarrhea and attended to the Diarrhea and Nutrition Unit of Pediatric Department at Tanta University Hospitals in the period from December 2016 to March 2017. Written informed consent was obtained from the parents or guardian of the studied patients. In the present study 10 apparently healthy infants and young children with no history of diarrhea since 3 weeks ago were included as a control group.

b) Materials:

(i) Immunochromatographic Lateral-Flow Test (RIDA®QUICK Rotavirus Test) (R-Biopharm AG, Germany):

It is a quick immunochromatographic test for the qualitative determination of rotavirus antigen in stool samples.

(ii) Enzyme Immunoassay Test (RIDASCREEN® Rotavirus) (R-Biopharm AG, Germany)

(iii) RT real-time PCR targeting *Rotavirus A* in human stool:

- ✓ **RNA Extraction kit (QIAamp Viral RNA Mini Kit) (QIAGEN®co)**
- ✓ **Rotavirus Real Time PCR (VIASURE Rotavirus Real Time PCR Detection Kit) (CERTEST BIOTEC)**

c) Methods:

Stool samples were collected in clean containers without any additives from 50 patients with acute watery diarrhea defined as 3 or more loose stools without blood within 24 h period [27,28]. and from the

control group. They were transported as soon as possible with ice bags to the Medical Microbiology and Immunology Department and were subjected to the following diagnostic tests.

Immunochromatographic Lateral-Flow Test (RIDA®QUICK Rotavirus Test) (R-Biopharm AG, Germany)

1 ml of Extraction Buffer Diluent was placed in the test tubes indicated. 100 µl of the stool sample was pipetted with a disposable pipette and was suspended in the buffer placed in the tube. The sample was well homogenised. Then allowed to precipitate for at least **3 minutes** until a clear supernatant is formed from which **200 - 500 µl** were then transferred into another clean tube. The test strip was removed from the tube and immersed it in the prepared sample. The test result was read after **5 minutes**. **Rotavirus positive:** the **red** and **blue** bands were visible. **Rotavirus negative:** only the **blue** band was visible.

Enzyme Immunoassay Test (RIDASCREEN® Rotavirus) (R-Biopharm AG, Germany):

It employs monoclonal antibodies in a sandwich type method**.** It uses a solid-phase sandwich EIA format**.** A monoclonal antibody to the product of the 6th viral gene (VP6) is coated to the well surface of the microwell plate. This is a group specific antigen that is found in all *rotaviruses* that cause disease in humans. A pipette was used to place a suspension of the diluted stool sample to be examined as well as control specimens into the well of the microwell plate together with biotinylated monoclonal anti-rotavirus antibodies (Conjugate 1) for incubation at room temperature (20-25 °C). After a wash step, streptavidin poly-peroxidase conjugate (Conjugate 2) was added and it was incubated again at room temperature (20–25 °C). With the presence of rotaviruses in the stool sample, a sandwich complex was formed which consists of immobilized antibodies, the rotavirus antigens, and the antibodies conjugated with the biotin-streptavidin-peroxidase complex. Another wash step removed the unattached streptavidin poly-peroxidase conjugate. After adding the substrate, the attached enzyme changed the colour of the previously colourless solution in the wells of the microwell plate to **blue** if the test is positive. Addition of a stop reagent changed the color from blue to yellow. The extinction was proportional to the concentration of rotaviruses found in the specimen.

RT real-time PCR targeting *Rotavirus* in human stool:

Stool samples were collected in clean containers and processed as soon as possible to guarantee the quality of the test.

For longer storage, the samples were frozen at -20ºC. In this case, the sample was totally thawed and brought to room temperature before testing. stool sample was Homogenised as thoroughly as possible prior to preparation. Freezing and thawing cycles are not recommended.

Stool samples were recommended to be diluted before extraction. A pea-size stool (approx. 8mm) was collected and was put in a 1.5 mL microcentrifuge tube containing 100 µL of Phosphate Buffer Saline (PBS). Vortex intensely and centrifuged 10,000 rpm for 1min. 200 µL of supernatant were used to perform RNA extraction.

RNA Extraction Procedure:

The sample was first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions were then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample was loaded onto the QIAamp Mini spin column. The buffering conditions of the lysate were adjusted to provide optimum binding conditions for the viral RNA before loading the sample onto the QIAamp Mini column. Viral RNA was adsorbed onto the QIAamp silica membrane during two brief centrifugation steps. Salt and pH conditions in the lysate ensured that protein and other contaminants, which can inhibit enzymatic reactions, were not retained on the QIAamp membrane**.** The RNA binded to the membrane, and contaminants were efficiently washed away in two steps using two different wash buffers that improved the purity of the eluted RNA. Optimized wash conditions ensured complete removal of any residual contaminants without affecting RNA binding. High-quality RNA was eluted in a special RNase-free buffer that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases ready for direct use or safe storage. The purified RNA is free of protein, nucleases, and other contaminants and inhibitors. The special QIAamp membrane guaranteed extremely high recovery of pure, intact RNA in just 20 minutes without the use of phenol/chloroform extraction or alcohol precipitation.

Real Time RT-PCR Procedure:

The PCR primers were selected from a highly conserved region of the group A *rotavirus* non-structural protein 3 (NSP3) sequence, table (1). The size of the expected amplicon was 87 bp. The fluorogenic probe was labeled with a FAM reporter at the 5'end and a TAMRA quencher at 3'end material. VIASURE *Rotavirus* Real Time PCR Detection Kit contains in each well all the components necessary for real time PCR assay (specific primers/probes, dNTPS, buffer, polymerase, Reverse-transcriptase) in an stabilized format, as well as an internal control to monitor PCR inhibition. *Rotavirus* Positive Control contains high copies template, the recommendation was to open and manipulate it in a separate laboratory

area away from the other components. The lyophilized *Rotavirus* Positive Control (red vial) was reconstituted by adding 100 µL of Water RNAse/DNAse free (with vial) supplied and vortex thoroughly.

Table (1): Sequence And Location Of Oligonucleotide Primers And Probe In *Rotavirus* Non- Structural Protein 3 (NSP3) Region

Sequence and Location of Oligonucleotide Primers and Probe in Rotavirus non-Structural Protein 3 (NSP3) Region (Genebank Access Number X81436)

Primer and probe	Nucleotide sequence (5′– 3′)	Location
Rota NVP3-F	accatctacacatgaccctc	963–982
Rota NVP3-R	ggtcacataacgcccc	1,034–1,049
TagMen probe	atgagcacaatagttaaaagctaacactgtcaa	984–1016

Reconstitute the number of wells you need:

15 µL of Rehydration Buffer (blue vial) was added into each well.

Table (2): RNA real time PCR program conditions

Cycles	Step	Time	Temperature
1	Reverse transcription	15 min	45ºC
1	Initial denaturalization	2 min	95ºC
45	Denaturalization	10 seg	95ºC
	Annealing/Data collection*	50 seg	60ºC

Adding samples and controls:

5 µL of RNA sample, reconstituted *Rotavirus* Positive Control (red vial) or Negative Control (violet vial) were added in different wells and the wells were closed with the caps provided. Centrifuged briefly. Then loaded in the thermocycler.

The thermocycler was set up (Roche LightCycler ®96 Real-Time PCR System**): It was programmed with the following conditions shown in table (2) and then started the run.**

4) Result interpretation:

The use of positive and negative controls in each run, validated the reaction by checking the absence of signal in negative control well and the presence of signal for *Rotavirus* positive control well. Internal Control signal was checked to verify the correct functioning of the amplification mix. The analysis of the samples was done by the software Roche light cycler version 4.0.

The following table was used to read and analyze the results: Table (3):+ Amplification curve - No amplification curve.

Table (3) Sample interpretation

Rotavirus	Internal control	Negative control	Positive control	Interpretation
+	+/-	-	+	Rotavirus A Positive
-	+	-	+	Rotavirus A Negative
+	+	+	+	Experiment fail
-	-	-	-	Experiment fail

A sample was considered positive if the Ct value obtained was less than 40 and the internal control showed an amplification signal. A sample was considered positive if the sample showed an amplification signal less than 40 Ct value but the internal control was negative. Sometimes, the detection of internal control was not necessary because a high copy number of target could have caused preferential amplification of target-specific nucleic acids.

A sample was considered negative, if the sample showed no amplification signal in the detection system but the internal control was positive. An inhibition of the PCR reaction can be excluded by the amplification of internal control.

The result was considered invalid if there was signal of amplification in negative control or absence of signal in the positive well. It was recommended to repeat the assay again.

Real time measurements were taken and a threshold cycle (Ct) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit of 0.04.

3. Results:

In the present study there was a significant difference between the 3 age groups proposed. Age distribution in patients' group with 25 (50 %) of patients in the age Group (7-12) months,18 (36%) of patients in the age group (13-24) months & 7(14%) of patients in the age group (25-60) months.

With significant increase in number of patients in the age groups (7-12) & (13-24) (P-value =0.002). as seen in Table (4).

Table (4): Distribution of Age (months) in patients' group

Age (months)	Freq.	Percent	Cum.
7-12 months	25	50.00	50.00
13-24 months	18	36.00	86.00
25-60 months	7	14.00	100.00
χ^2	9.88		
P-value	0.002**		

Also there were 34 males (68%) and 16 females (32%). with a significant P-value =0.009. as seen in table (5)

Table (5): Distribution of Gender in patients' group

Gender	Freq.	Percent	Cum.
Male	34	68.00	68.00
Female	16	32.00	100.00
χ^2	6.48		
P-value	0.009**		

There were 74% of patient's group have rural residence and 26 % have urban residence with a significant p-value of **<0.001**. As well there were 11 (22%) of cases with exclusive breast feeding and 32 (64 %) with bottle milk feeding and 7 (14%) are weaned children with a significant P- value **<0.001**. most of the patients (38 %) presenting with both diarrhea and vomiting followed by (34%) presented with a triad of Diarrhea, Fever & Vomiting. With a significant P-value **<0.001.**

Among both groups (n=60) RIDAQUICK immunochromatography gave positive results in 35 (60%), RIDASCREEN ELISA detected 41 (68.3%) positive cases while real time PCR detected 49 (81.7%) positive cases for Rotavirus A infection in stool samples. Table (6).

Among the patients' group there was 35 (70%) positive cases with RIDAQUICK,41 (82%) positive cases with RIDASCREEN and 49 (98%) positive cases detected by real time PCR. Table (7).

Indicating that the control group (n=10) all gave negative results with the 3 tests used in the present study.

Table (6): Total number of positive and negative cases observed by different studied tests in both studied groups (patients & control)

Table (6): Total number of positive and negative cases observed by different studied tests in both studied groups (patients & control)

	Negative	Positive	Total
Immunochromatography	25(40%)	35(60%)	60(100%)
ELISA	19(31.7%)	41(68.3%)	60(100%)
PCR	11(18.3%)	49(81.7%)	60(100%)

Table (7): Total number of positive and negative cases observed by different studied tests in patients' group

	Negative	Positive	Total
Immunochromatography	15(30%)	35(70%)	50(100%)
ELISA	9(18%)	41(82%)	50(100%)
PCR	1(2%)	49(98%)	50(100%)

Table (8) Illustrates correlation between demographic and clinical data collected from patients' group (n=50) and their results with real time PCR as it showed the highest sensitivity, specificity & accuracy.

Table (8) correlation between demographic and clinical data collected from patients' group and their results with real time PCR.	Real time PCR		
	Negative	Positive	P-value
Age (months)			
7-12 months	0(0%)	25(51 %)	0.14
13-24 months	0(0%)	18 (36.7%)	
25-60 months	1(100%)	6(12.2%)	
Total	1(100%)	49 (100%)	
Gender			
Male	0(0%)	34 (69.4%)	0.320
Female	1(100%)	15 (30.6%)	
Total	1(100%)	49 (100%)	
Location			
Urban	1(100%)	12 (24.5%)	0.26
Rural	0(0%)	37 (75.5%)	
Total	1(100%)	49 (100%)	
Feeding Pattern			
Breast	0(0%)	11(22.4%)	0.16
Bottle	0(0%)	32(63.3%)	
Weaned	1(100%)	6(14.3%)	
Total	1(100%)	49 (100%)	
Clinical Features			
Diarrhea	0(0%)	8(16.3%)	0.12
Diarrhea & Fever	0(0%)	1(2%)	
Diarrhea & Vomiting	0(0%)	19(38.8%)	
Diarrhea & Dehydration	1(100%)	4(8.2%)	
Diarrhea & Fever & Vomiting	0(0%)	17(34.7%)	
Total	1(100%)	49 (100%)	
Stool consistency			
Watery	0(0%)	37(75.5%)	0.26
Semisolid	1(100%)	12(24.5%)	
Total	1(100%)	49 (100%)	
Stool odour			
Fecal	1(100%)	36(73.5%)	1
Offensive	0(0%)	13(26.5%)	
Total	1(100%)	49 (100%)	

Diagnostic efficacy of immunochromatography test when compared with ELISA is shown in Table (9)

Table (9): Diagnostic efficacy of immunochromatography test when compared with ELISA	
True Positive	33
False Positive	2
True Negative	7
False Negative	8
Sensitivity (%)	80.5
Specificity (%)	77.8
Positive Predictive Value (%)	94.3
Negative Predictive Value (%)	46.7
Accuracy (%)	80

Total number of positive and negative cases observed with immunochromatography test as compared to real time PCR are shown in Table (10).

Table (10): Total number of positive and negative cases observed with immunochromatography test as compared to real time PCR

	Negative	Positive	Total
Immunochromatography	**15(30%)**	**35(70%)**	**50(100%)**
PCR	**1 (2%)**	**49(98%)**	**50(100%)**

Diagnostic efficacy of immunochromatography test when compared with PCR is shown in Table (11)

Table (11): Diagnostic efficacy of immunochromatography test when compared with PCR	
True Positive	**35**
False Positive	**0**
True Negative	**1**
False Negative	**14**
Sensitivity (%)	**71.4**
Specificity (%)	**100**
Positive Predictive Value (%)	**100**
Negative Predictive Value (%)	**6.7**
Accuracy (%)	**72**

Total number of positive and negative cases observed with RIDSCREEN ELISA test as compared to real time PCR are shown in table (12)

Table (12): Total number of positive and negative cases observed with RIDSCREEN ELISA test as compared to real time PCR.

Table (12): Total number of positive and negative cases observed with RIDSCREEN ELISA test as compared to real time PCR

	Negative	Positive	Total
ELISA	**9(18%)**	**41(82%)**	**50(100%)**
PCR	**1 (2%)**	**49(98%)**	**50(100%)**

Diagnostic efficacy of RIDSCREEN ELISA test when compared with real time PCR is shown in Table (13)

Table (13): Diagnostic efficacy of RIDSCREEN ELISA test when compared with real time PCR	
True Positive	**41**
False Positive	**0**
True Negative	**1**
False Negative	**8**
Sensitivity (%)	**83.7**
Specificity (%)	**100**
Positive Predictive Value (%)	**100**
Negative Predictive Value (%)	**11.11**
Accuracy (%)	**84**

Diagnostic efficacy of immunochromatography (RIDAQUICK) test & (RIDSCREEN) ELISA test when compared with real time PCR shown in Figure (1)

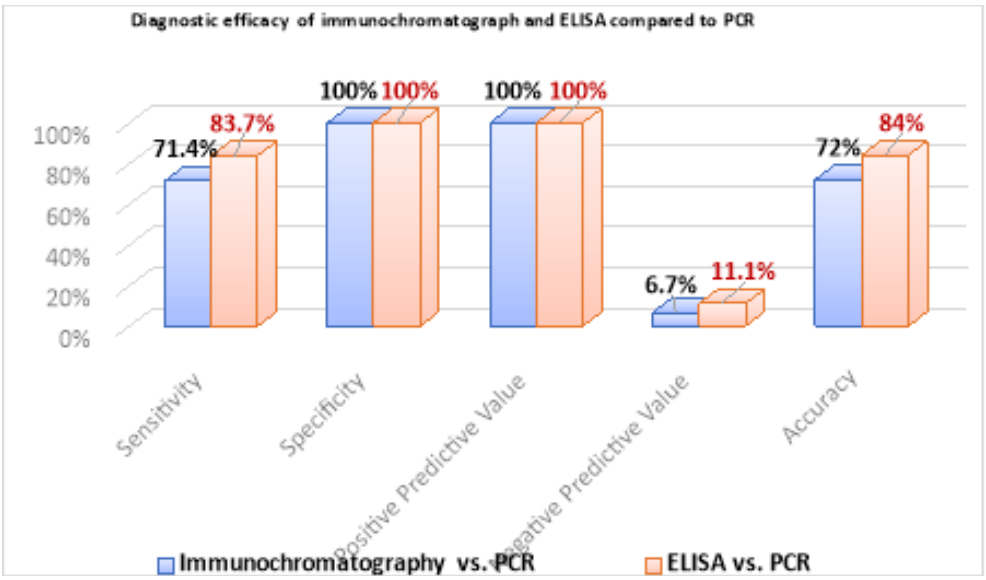


Figure (1): Diagnostic efficacy of immunochromatography & RIDSCREEN ELISA test when compared with real time PCR

Table (14) demonstrates diagnostic accuracy of Real time PCR for detecting Rotavirus.

Table (14) Diagnostic accuracy of Real time PCR for detecting Rota virus

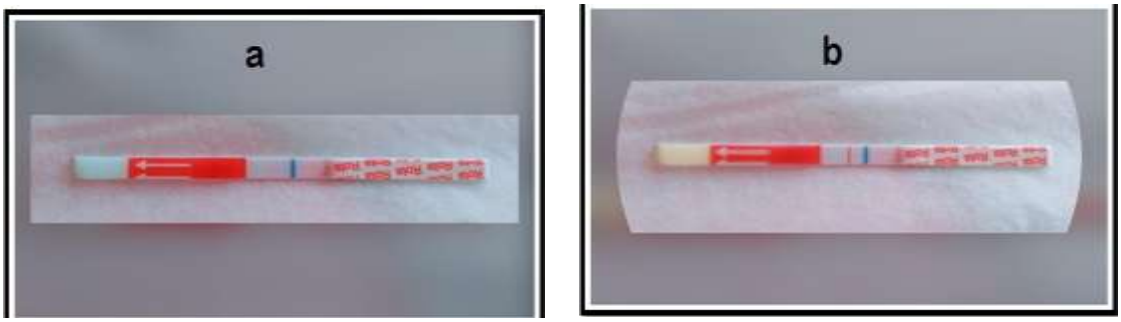
Clinically diagnosed patients	Real time PCR		
	Negative	Positive	Total
Negative	10(90.9%)	0(0%)	10(16.67%)
Positive	1(9.1%)	49(100%)	50(83.33%)
Total	11(100%)	49(100%)	60(100%)

Table (15) shows the diagnostic efficacy of real time PCR in detection of rotavirus A in stool samples.

Table (15) Diagnostic efficacy of real time PCR

	Sensitivity	Specificity	PPV	NPV	Accuracy
Real time PCR	98%	100%	100%	90.9%	98.3%

Photo (5-1) a: shows a negative ridaquick test strip with only the blue control line. while **photo (5-1) b** shows a positive RIDAQUICK test strip with a red line (positive test line)



a) Negative **b) Positive**

Photo (1): RIDAQUICK Rota (Lateral Flow Immunochromatography)

Photo (2): Stool samples diluted in Diluent 1

Photo (3): After adding 50 µl of the Stop reagent in order to stop the reaction & before reading

Instrument

Type	3 Ch.	Serial Numbe	302

2 Standard 116.69770000 (Absolute Quantification)

Settings

Channel	530	Color Compensatio	Off				
Program	amp			Method	Automated (F" max'	Units	

Results

Inc	Pos	Name	Type	CP	Concentratio	Standard
☑	1	Sample 1-	Unknown	29.21	3.52E4	
☑	2	Sample 2-	Unknown	[32.24]	[4.08E3]	
☑	3	Sample 3-	Unknown	28.51	5.71E4	
☑	4	Sample 4-	Unknown	28.18	7.09E4	
☑	5	Sample 5-	Unknown	34.74	6.24E2	
☑	6	Sample 6-	Unknown	26.77	1.61E5	
☑	7	Sample 7-	Unknown	30.92	1.07E4	
☑	8	Sample 8-	Unknown	28.96	4.18E4	
☑	9	Sample 9-	Unknown	27.47	1.14E5	
☑	10	Sample 10-	Unknown	27.72	9.67E4	
☑	11	Sample 11-	Unknown	24.72	6.79E5	
☑	12	Sample 12-	Unknown	25.57	3.93E5	
☑	13	Sample 13-DEPC WATER	Unknown			
☑	14	Sample 14-SAMPLE 5 REP	Unknown	31.50	7.01E3	
☑	15	Sample 15-SAMPLE 2-REP	Unknown	31.82	5.54E3	
☑	16	Sample 16-NTC	Unknown			
☑	17	Sample 17-ST	Standard	23.03	2.00E6	2.00E6

Rota virus-quant19-2-1! 2/18/2019 Page 2 of 3

Photo (4) Real time PCR results: Analysis of fecal samples for rotavirus by the VIASURE Rotavirus Real Time PCR Detection Kit.

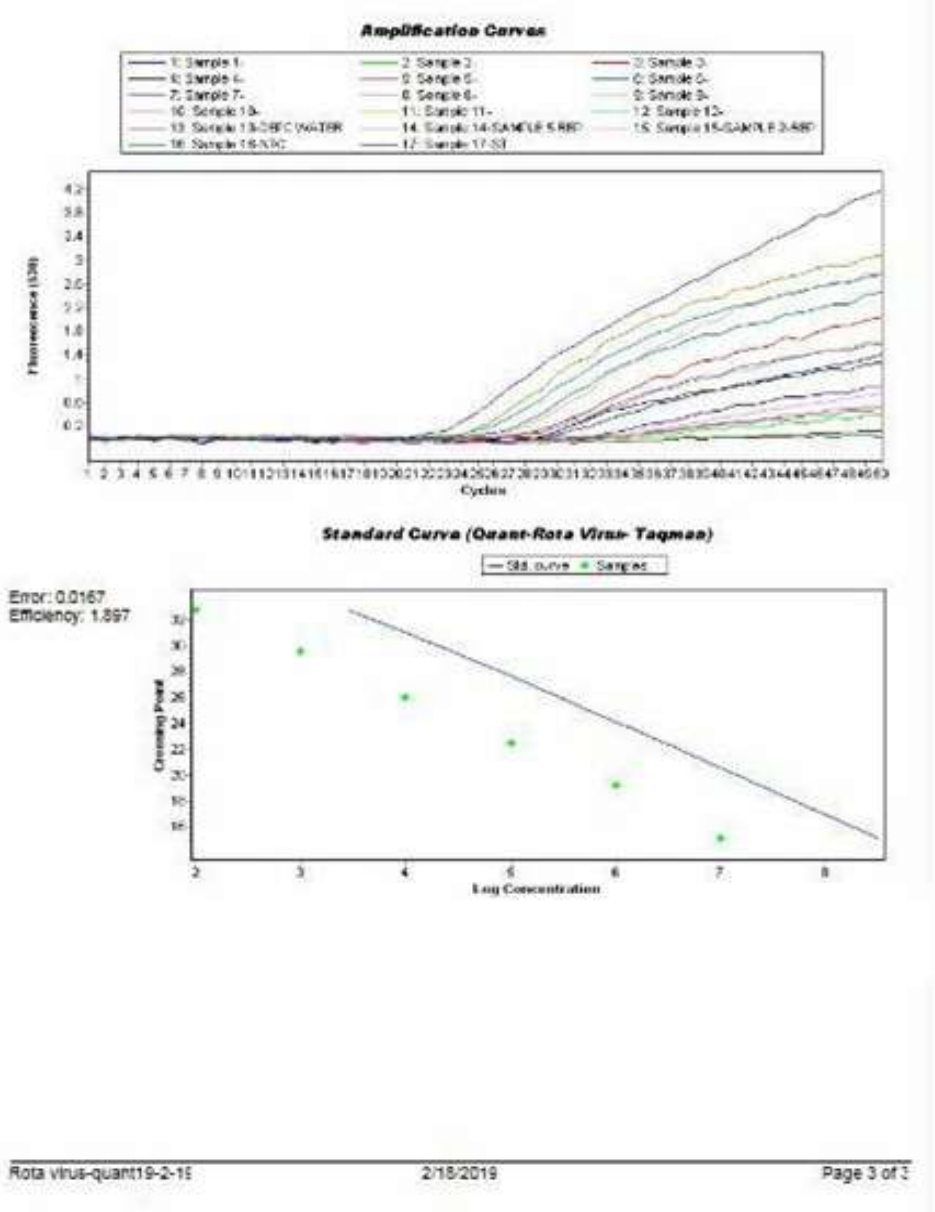


Photo (5) Real time PCR results: Analysis of fecal samples for rotavirus by the VIASURE Rotavirus Real Time PCR Detection Kit. Graph was obtained with the lightcycler version 4.0 software.

Discussion:

The present study was carried out on 50 infants and young children, who were attending the Diarrhea and Nutrition Unit of Pediatric Department at Tanta University Hospitals in the period from December 2016 to March 2017 and were clinically diagnosed according to history, clinical signs, symptoms and using Vesikari scoring system as having acute gastroenteritis, those were included in the patients' group. In addition to 10 apparently healthy infants and young children were included as a control group. Stool samples were collected from the study group and the control group.

Using the 3 different diagnostic methods on the patients' group revealed that 35 (70%) of the cases gave positive results with RIDAQUICK Immunochromatography kit and 41 (82%) were positive by RIDASCREEN ELISA and 49 (98%) were positive with real time RT-PCR. In addition to the control group they all gave negative results with the 3 tests.

Regarding the Lateral flow Immunochromato graphic test (RIDAQUICK *Rotavirus* Test 35 patients (70%) gave positive results whereas 15 patients (30%) gave negative cases. And when compared to Enzyme immunoassay test (RIDASCREEN *Rotavirus* Test) the

sensitivity was 80.5% & the specificity was 77.8 %, PPV was 94.3 %, NPV was 46.7 % and accuracy was 80 %. Besides when it was compared with real-time PCR the sensitivity was 71.4 % & the specificity was 100%, PPV was 100%, NPV was 6.7 % and accuracy was 72 %.

Our results were in agreement with studies done by ***S. De Graziaa et al., (2017) & de Rougemont A, et al. (2009)*** who found that sensitivity and specificity of the immunochromatographic test compared to ELISA were also strictly comparable and very good. Sensitivity was 83.0% & 79% and specificity was 81.6% & 80.5% respectively [13,17]

As well, ***Kim J, et al. (2014), Bruggink et al., 2015;*** results were in agreement with the present study and reported that patients showing signs of disease with higher viral loads were commonly separated as positive by ICTs [18,19].

In addition***, Shaveta Dhiman et al., 2015*** declared that because of limited availability and rather high cost of ELISA test for detection of *rotaviruses* they compared ICG to ELISA. They found that sensitivity was 95.24% and specificity was 97.47% of ICG matches with ELISA, in addition to performing the diagnosis in a simple manner, convenient, rapid and cost-effective [20]. their results were comparable but slightly higher than the results of the present study.

Those higher results are most probably because the antigen excretion in the stools differs during the course of the disease and they could have took their samples in the period of high viral excretion in stool, also the number of cases in those studies was higher than our study.

likewise a study by ***Khamrin P, et al., (2011***) reported that IC tests are relatively cheaper, rapid diagnosis, and carrying high specificity and sensitivity and when matched with RT-PCR, they found that the rates of sensitivity of IC test kit was 78.7% and their specificity was 100% which was very similar to the results of our study [21]

Also the results of the study by ***Moutelíková R., et al (2019)*** reported comparable but higher results than our results; the immunochromatography diagnostic sensitivity was assessed as 82.5% & the specificity was calculated as 96.4% also positive predictive value was determined to be 80.3% when compared with real time PCR [22].

However ***Ye et al., 2015*** & ***Izzo et al., 2012 & Maes et al., 2003*** reported that immunochromatographic method showed a low specificity 54.3% & 59 % & 60 % respectively when compared with real time PCR. They also reported a restriction of antibody-based examination for the estimation of enteric microorganisms. Which is not in concordance with the present study results [23,24,25].

The possible cause for this lower efficacy of IC assay in the detection of enteric pathogens could be due to the requirement of high concentration of free antigen in the stool sample to produce a positive response, the free antigen is declined greatly along the pathogenesis of disorder. Consequently, these tests possessing minor sensitivity and could be not detected some positive specimens taken lately in the development of pathogenesis of illness, when paralleled to real time RT-PCR [24,25].

Regarding ELISA test (RIDASCREEN Rotavirus) results 41 patients (82%) gave positive results and 9 patients (18%) revealed negative cases. And when compared to real-time PCR the sensitivity was 83.7% & the specificity was 100 %, PPV was 100 %, NPV was 11.11 % and accuracy was 84 %. which is more accurate than IC test.

A study by ***Rashi Gautam, et al., (2013)*** reported very similar results by using the gold standard method (RT-PCR) for example, the recital features of the RIDASCREEN Rotavirus kit were, 82.1% sensitivity, 100% specificity, PPV = 100%[26]. Furthermore, ***Moutelíková R., et al (2019)*** also reported comparable results with our study; Sensitivity & specificity for EIA were 84.2% & 97.8% respectively [22]

Other studies by ***Sukran Artiran, et al., (2017) & Mariet de Beer, et al., (1997)*** revealed similar findings EIA test showed 94%, 95 % sensitivity, 100%, 100% specificity, PPV = 100%., 100%, respectively. They also favoured the usage of Commercial ELISA kits to screen large numbers of samples as a routine laboratory diagnostic method where it characterized by simplicity, easy to perform, and both the specificity and sensitivity are high for diagnosis of rotavirus antigen in stool samples [27,28].

On the other hand, a study by ***Fruhwirth et al., (2000)*** didn't agree with our study results and reported low sensitivity of ELISA also the false positive cases were 12% of the samples when compared with real time PCR [29]

The sensitivity of ELISA varied depending on the time of stool collection relative to the onset of symptom also some specimens very rich in *rotavirus* particles could produce prozone effects that could passively affect the results of ELISA test [30,31]. Sensitivity of ELISA may drop throughout the course of the disease due to stimulation of immune system and formation of immunity against *rotavirus* and liberate mucosal antibodies that covering the virus and thus, hinder it's detection by ELISA method [32].

Concerning VIASURE Rotavirus Real Time PCR assay results 49 patients (98%) showed positive results and 1 patirnts (2%) was negative. with 98% sensitivity, 100% specificity, PPV 100%, NPV 90.9 %, accuracy 98.3 %. Showing superior results compared to both IC test and ELISA.

Results of our study were very similar to results of a study by ***C. Santiso-Bellon, et al., (2016)*** who reported that the sensitivity of the VIASURE Rotavirus was 97% by using Real Time PCR technique and it was much higher than IC and ELISA tests [(15)]

Also***, Ye.S., et al., (2015)*** agreed with our results declaring that real time PCR provided very high sensitivity and specificity 99% & 100% respectively. While the antigen tests were less sensitive than the real time PCR [(23)].

Additionally, ***Liu J, et al., (2014***) & ***Corcoran et al., (2014***) also reported that molecular methods are the most sensitive and accurate while in clinical samples estimation of antigen still only appropriate for rapid detection of infection by *rotavirus* and defined Real-time PCR as the standard tool for diagnosis owing to high specificity and sensitivity [(33,34)]

Bennett et al., (2015); Tate et al., (2013) reported similar results and stated that antibody based recognition methods are less sensitive by about 1000–10,000 fold than RT-PCR [(35,36)].

Moreover***, Yunjin Wang et al., (2013)*** reported that quantitative real time PCR provide a very high sensitivity and specificity. It also give additional important benefits for the detection of *rotavirus* nucleic acids in minimal levels [(37)]

Negative results obtained by real time PCR may be because the patient was not infected by *rotavirus* or misclinical diagnosis and may be the delicate Virus was distructed during transfer.

As regards age of the patients 25 patients (50%) were in the age group between 7-12 months & 18 patients (36%) were in the age group between 13-24 months and only 7 (14%) were in the age group between 25-60 months. with a significant increase in the age group more than 6 months and less than 24 months (p- value =**0.002**).

Out of these patients 49 (98%) with more positive results for rotavirus by real time PCR in the age group 7-12 months and 13-24 months (87.7%).

Similar results were reported by ***Joshua Gikonyo et al., (2019***) with children aged 13 to 24 months had the highest infection 41%, while the least common *rotavirus* infections were observed among the 3 years and above age group [(38)].

Furthermore, ***Shaveta Dhiman et al., (2015)*** observed that the highest age group was from 6 months to 24 months (85.71%) [(20)].

Similarly, ***Surajudeen A Junaid et al., (2011)*** revealed that the highest age prevalence was between 7-12 months ($P < 0.05$) [(39)].

Also similar findings were reported by other researchers ***Catherine Muendo et al., (2018) & Zarnani AH et al., (2004) & Morris O et al., (1986***) [(40,41,42)].

Additionally, other studies by ***Kang G et al., (2009) & Shariff M et al., (2003)*** done in Eastern Nepal and other countries showed similar results [***(43,44)***].

The proposed reasons for this age distribution is attributed to early exposure from contaminated sources as well as over-crowded homes in under developed regions, which result in appearance of the early peak of rotavirus diarrhea, Meanwhile nearly all humans practice at 3 years of age, at least one rotavirus infection and presence of rotavirus antibodies in the blood continue detectable forever giving an acquired active immunity by 24 months of age [(45)]. This may resultant in formation of antibodies against rotavirus infection and rising the titer in the circulation, which subsequently diminish the amplitude of symptoms of disease, which in older children decrease the incidence of rotavirus gastroenteritis [(45,46)]. Passive immunity acquired by the infants from their mothers play an important role in decreasing the frequency of rotavirus gastroenteritis during 0-6 months of age, these immunity disappear post 6 months of infant's age, and moreover, it is observed also a higher rate in the breast feeding which may play an important role in protection against gastroenteritis infection through passing of IgA antirotavirus antibodies to the newly born babies [(20,24)].

Regarding the gender of the patients in the present study the ratio of participating males to females were 68% (34) to 32% (16), respectively, with a significant difference between both groups (P-value =0.009).

All the males and 15 female patients gave positive results with real time PCR for *rotavirus*.

In our study *rotavirus* infection in males was significantly higher than in females. Similar findings were reported by ***Shaveta Dhiman et al.,2015***, who reported that males had higher significant incidence of *rotavirus* infection (90.5%) than females [(20)].

Also similar results were reported by ***Sally F. Lafta et al., 2019***, With males tended to be more effected by RV with 31 (62%) cases in comparison to females with 19 (38%) cases. Statistically, gender differences were significant ($p>0.05$) [(47)].

Some investigators tried to explore the high possibility of males to be infected with rotavirus than females to the tendency of parents to take care with males than females concerning treatment in the hospitals [(20)]. In addition, to the hypothesis that females are more resistance to infection than females due to hereditary factors represented in XX chromosomes in females. [(48)].

As regards residence of the patients 37 patients (74%) had rural residence while 13 patients (26 %) came from urban residences. 80 % of the patients having positive rotavirus detected by real time PCR

were from rural residence. with a significant increase in rural patients.

According to ***Shaveta Dhiman et al.,2015*** higher number of *rotavirus* positive cases were from rural areas [20]. This may be attributed to lower educational and socioeconomic status and less clean water supply.

Regarding feeding pattern in the patients' group there were 11 (22%) breast fed 32 (64%) bottle fed & 7 (14%) weaned patients with a significant P value < 0.001.

Those group were also positive for *rotavirus* by real time PCR with 20% breast fed, 66% bottle fed and 7% weaned. There is an obvious increase of *rotavirus* positive patients among the bottle fed group.

Similarly, ***Shaveta Dhiman et al., (2015)*** reported that there was a statistically significant linkage among feeding pattern anion of *rotavirus*. Bottle feeding was usually accompanied by a higher rate of diarrhea (52.38%) due to *rotavirus* infection, while in children on exclusive breast feeds having a decreased frequency of *rotavirus* diarrhea. [20].

Sally F. Lafta et al., (2019) results also revealed that the rate of *RV* gastroenteritis was the highest in children who used bottle feeding (56%) and least among breast fed children (18%). This variation was statistically significant ($p < 0.01$) [47].

Moreover, ***Nakawesi JS et al,. (2010)*** showed similar infection distribution according to the feeding type [49].

It can be justified that breast feeding diminishes gastrointestinal infections due to supplying milk with passive immunity from mothers (IgA) antibodies, immune cells and other defense agents like oligosaccharides, human milk glycans and lactoferrin that guard the intestinal epithelium against infections [50].

Regarding clinical presentations in the patients' group there were 19 patients (38%) presenting with diarrhea and vomiting and 17 patients (34 %) presented with a triad of diarrhea, fever and vomiting and also gave positive results with real time PCR. With a significant P-value ($p < 0.001$).

These results were in accordance with ***Shaveta Dhiman et al., (2015)*** they reported Maximum number of *rotavirus* positive cases presented with a harmony of fever, vomiting and diarrhea [20].

Also ***Surajudeen A Junaid et al., (2011)*** Reported that vomiting followed by fever or diarrhea seems to be more corporate with rotavirus diarrhea than being presented with diarrhea alone, the significant variation among rotavirus positive and rotavirus negative children was the occurrence of all 3 signs among positive cases: Diarrhoea, fever and vomiting (P <0.05) [39]. Similar findings were observed in the study conducted by ***Staat MA et al., (2002)*** [51].

On the other hand, ***Sally F. Lafta et al., (2019)*** & ***Kargar M, et al., (2012)*** results showed that diarrhea was the predominant symptom among *RV* infected children [47,52].

The predominance of vomiting, diarrhea and fever is justified with the fact that During *RV* infection, intestinal enterochromaffin cells release 5-HT, which interacts with 5-HT3 receptors and stimulates the vagal afferent nerve projecting to the vomiting center of the brain [53]. Watery diarrhea could be due to the effect of paracellular leakage is induced by NSP4 In enterocytes NSP4 results in disruption of tight junctions while in crypt cells it stimulates secretion [54] Fever occur as *Rotavirus* stimulates the release of several pyrogens, such as prostaglandins and interleukins, from infected cells. In addition to their temperature modulating effect of prostaglandins (PGE2) they may also stimulate water secretion [55].

Conclusion:

The *rotavirus* immunochromatographic test (RIDAQUICK) is a good substitute for the random analysis of fecal specimens in ambulatory field. It is rapid, inexpensive and useful for testing single specimen. However, it has minimal sensitivity and could miss positive specimens obtained lately in the course of clinical illness. ELISA test (RIDASCREEN *Rotavirus*) is more accurate than IC test. It is suitable for the routine diagnostic laboratory and to screen large numbers of samples. However, a major drawback of ELISA system are that it is not cost-effective for testing single specimens. Quantitative real time PCR, can provide higher sensitivity and specificity. It also offers significant benefits for the detection of *rotavirus* nucleic acids in minimal levels in stool samples.

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