



## Comparative evaluation of antigen ELISA technique and PCR for detection of Rotavirus in stool samples of pediatric patients with diarrhea

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### Abstract

**Background:** Acute diarrheal disease is a common infection of young children and adults which is characterized by rapid dehydration of body fluid. Identification of the causative agent and timely treatment can be life saving of children. The objective of this study was to detect Rotavirus (RV) in stool samples and to compare ELISA and RT-PCR techniques.

**Methods:** Hospitalised children less than 5 years admitted for acute diarrhea were examined and clinical profile of diarrhea in the children. Detection of Rotaviral antigen in stool samples was done by a commercial Enzyme Immuno Solvent Assay Premier Rotaclone kit (Meridian Bioscience, Inc.) and the samples were processed for extraction of nucleic acid using an automated extraction system and RT-PCR was performed.

**Results:** A total of 135 stool samples were tested by ELISA and RT-PCR. A total of 20/135 (14.81%) samples were found to be positive for RV infection by ELISA. Positivity of RV by RT-PCR was 33/135 (24.44%). The positivity by ELISA was significantly lower than by RT-PCR. The sensitivity and specificity of PCR for detection of RV was 100%.

**Conclusion:** RT-PCR is a better and sensitive technique as compared to ELISA for detection of Rotavirus.

### Introduction

Acute diarrheal disease is a common infection of young children and adults which is characterized by rapid dehydration of body and one of the most important worldwide causes of morbidity and mortality, accounting for an estimated 1.3 million deaths in children under five aged<sup>(1,2)</sup>. Clinically, rotavirus gastroenteritis is characterized by profuse diarrhea, mild fever, and vomiting, leading to mild to severe dehydration. Rotavirus is the major cause of acute gastroenteritis and severe dehydrating diarrhea in young children<sup>(3)</sup>. Rotavirus is estimated to cause about 40% of all

hospital admissions due to diarrhea among children under five years of age worldwide<sup>(4)</sup>

In India rotavirus is responsible for 40% of children diarrhea hospitalization and approximately 527,000 (475,000 - 580,000) deaths annually (5) or 29 to 45% of all death due to diarrhea among children  $\leq 5$  year of age globally<sup>(6,7)</sup>. Several techniques have been developed to detect rotavirus in stool samples, including electron microscopy, polyacrylamide gel electrophoresis (PAGE) of viral nucleic acid, various immuno assays, and PCR-based molecular methods<sup>(1)</sup>. Several rapid methods, such as the

immuno-chromatographic test and the latex agglutination test (LA), have been evaluated and compared to other methods, such as enzyme-linked immunosorbent assays (ELISA) and quantitative reverse transcription - polymerase chain reaction (RT-PCR) and they have shown a wide range of sensitivity and specificity<sup>(8)</sup>. The performance of those rapid tests indicated that they could be used for screening but not for confirmation of the disease. Therefore, a highly specific and sensitive test is a pressing need for use in confirming rotavirus infections<sup>(9)</sup>. Laboratory diagnosis of rotavirus infection is usually performed by antigen detection, using enzyme immunoassay or LA techniques, which have a sensitivity and specificity above 90%. Molecular techniques such as PAGE and RT-PCR are used to determine the RNA migration patterns and virus genotyping, respectively<sup>(10-13)</sup>.

ELISA and RT-PCR are being used in research settings. In recent years, ELISA has become the method of choice for screening. The sensitivity of routine diagnostics is high since the amount of virus excreted by a child with rotavirus diarrhea ( $\sim 10^{10}$  viruses/g of stool) far exceeds the level of detection ( $\sim 10^7$  viruses/g of stool)<sup>(14)</sup>. Early studies on strain surveillance identified rotavirus serotypes using neutralization assays. Monoclonal antibodies to specific serotypes were used. New methods have greatly improved data on circulating rotavirus strains and include multiplex RT-PCR based genotyping, hybridization assays and nucleotide sequencing<sup>(15)</sup>.

### Material and Methods

A total of 135 stool samples of children of age  $\leq 5$  years admitted in wards of the Department of General Medicine at J K Lone Hospital, Jaipur were enrolled in the study. All children were suffering with diarrhea, vomiting, headache, signs and symptoms fever, chills and abdominal pain. Approval by the institutional Ethics committee has been granted for the present study.

### Sample collection and transportation

Samples were collected from suspected or clinically diagnosed cases of gastrointestinal tract infections in to a sterile transport container. The stool samples were properly labeled & transported in cold chain (4-8°C) at the earliest to the laboratory and stored the samples -80°C until further investigations.

### Detection of rotavirus by ELISA

ELISA for RV was performed using Commercial kit, Premier Rotaclone EIA kit (Meridian Bioscience, Inc.) as per manufacturer's instructions. This kit is used to detect the presence of viral antigen (VP6) for RV was tested by in a solid phase sandwich ELISA. The antigen is sandwiched between anti RV monoclonal antibody and enzyme linked antibodies.

### Nucleic Acid Extraction

Viral nucleic acid was extracted from 400  $\mu$ l of the stool samples in a final elution volume of 110  $\mu$ l by using Nucli SENS Easy MAG automated nucleic acid extractor instrument (Biomeurix) as per the manufacturer's instructions. In brief, 400 $\mu$ l homogenized stool sample with 1600 $\mu$ l lysis buffer was add in column tube. 140 $\mu$ l of magnetic silica was added to the sample with internal control, mixed well by up & down followed by automated processing. The nucleic acid was eluted in a volume of 110 $\mu$ l and processed for multiplex PCR

### Master Mix preparation

Preparation of the master mix, primer probe mix was first prepared. For each reaction, PPM was prepared using 0.2  $\mu$ l (4 picomole) of forward primer, 0.2  $\mu$ l (4 picomole) of reverse primer and 0.2  $\mu$ l (2 picomole) of probe. Master mix for each reaction was prepared in a total reaction volume of 20  $\mu$ l volume which consisted of 12.5  $\mu$ l of 2X RT-PCR buffer, 0.5  $\mu$ l of 25X RT-PCR enzyme, 1.4  $\mu$ l of nuclease free water, 0.6  $\mu$ l of primer probe mix and 5  $\mu$ l of extracted nucleic acid. The thermal cycling profile for PCR was; 50°C for 30

minutes (1 cycle), 95°C for 10 minutes (1 cycle) followed by 90°C for 15 seconds and 55°C for 30 seconds (45 cycles). In each run, a positive control

and a negative control was included. The primers and probe sequences for detection of RV in PCR are given as follows.

Forward (5' - 3')	Reverse (5' - 3')	Probe (5' - 3') <sup>@</sup>	Reference
ACCATCTWCACRTRACCC TCTATGAG	GGTCACATAACGCCCT ATAGC	\$NED- AGTAAAAGCTAACACTGTCAAA	Liu <i>et al.</i> , 2013

**Statistical Analysis**

Statistics like sensitivity, specificity was performed between the two tests performed and one was gold standard (FTD)

**Results**

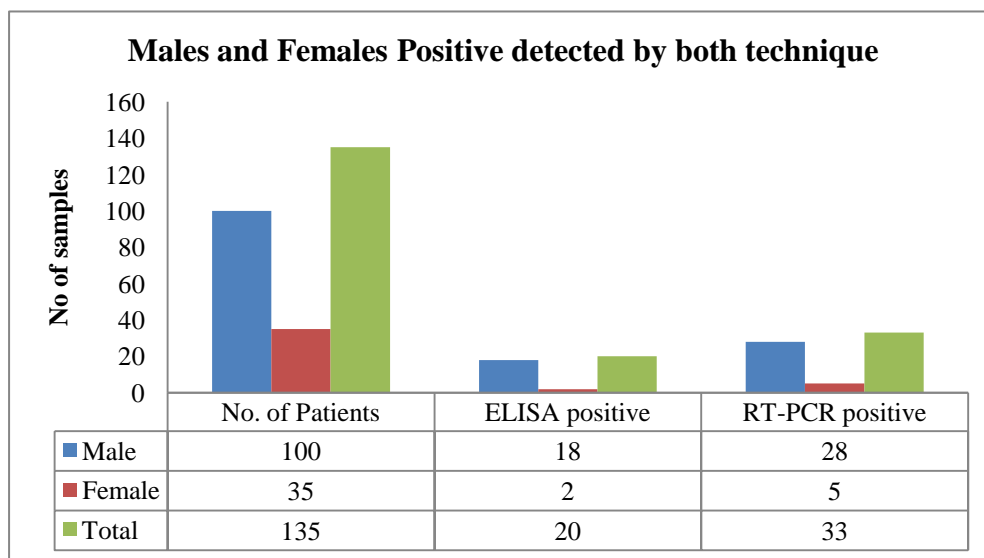
A total of 135 stool samples from hospitalized children of age ≤ 5 years were tested for Human RV infection by both ELISA and RT-PCR techniques, out of which, 100 (74.07%) were from

males and 35 (25.93%) were from females. A total of 20/135 (14.81%) samples were found to be positive for RV detected by ELISA technique and among these, 18 (18.00%) were from males and 2 (5.71%) were from females. And 33/135 (24.44%) samples were found to positive for RV detected by RT-PCR technique and among these, 28 (28.00%) were from males and 5 (14.28%) were from females.

**Table:-1** Sex wise distribution of positive cases of RV infection by detected both techniques

	No. of Patients	ELISA		RT-PCR	
		Positive	Negative	Positive	Negative
Male	100 (70.07%)	18 (18.00%)	82 (82.00%)	28 (28.00%)	72 (72.00%)
Female	35 (25.93%)	2 (5.71%)	33 (94.29%)	5 (14.28%)	30 (85.72%)
Total	135 (100%)	20 (14.81%)	115 (85.19%)	33 (24.44%)	102 (75.56%)

**Figure:-1** Sex wise distribution of sample collected and positivity detected by both techniques



Age wise distribution of RV infected samples detected by both techniques is shown in table 2. The maximum numbers of samples were collected from children with age group 1-6 months and 7-12 months as compared to 19-24 months age group from which minimum number of samples were

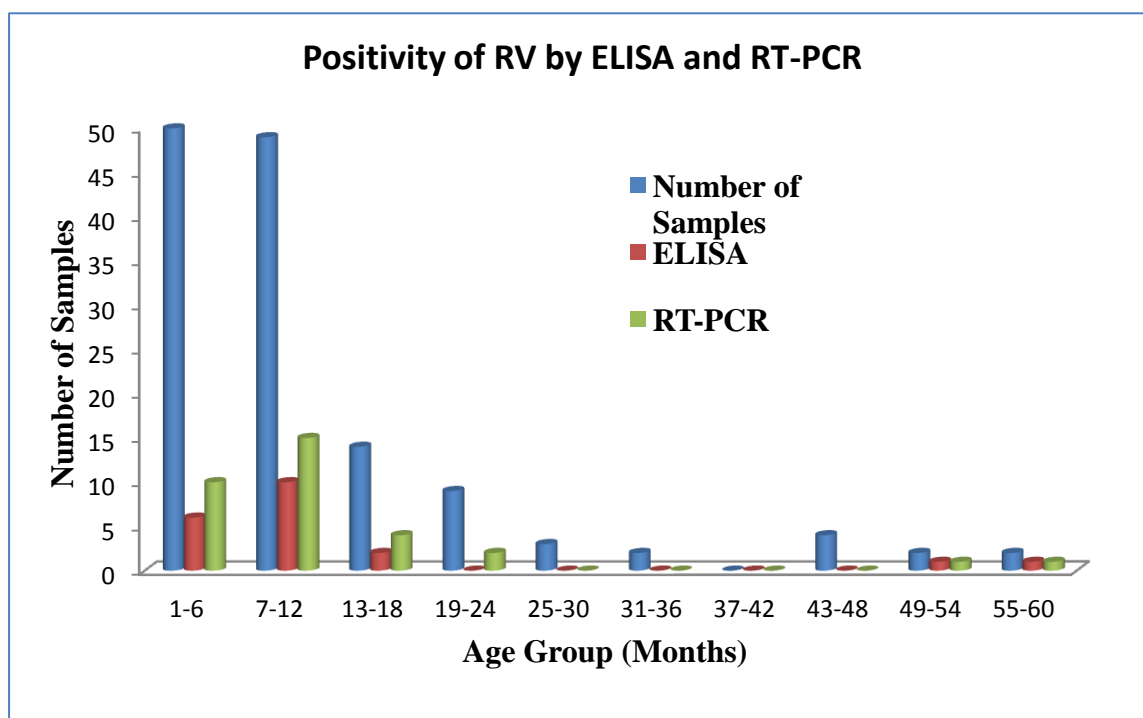
collected. Also children less than 2 year age were found to be infected more than in elder age groups.

The positivity of RV in different age grouped children using both techniques is shown in table 2. Higher positivity was seen with those tested by

PCR as compared to ELISA technique. The total positivity of RV by ELISA was found to be 14.81% only while by PCR was 24.44%.

**Table: 2** Age wise distribution of RV detected by both techniques

Age groups (months)	Number of Samples	ELISA		RT-PCR	
		Positive	Negative	Positive	Negative
1-6	50	6(12.00%)	44(88.00%)	10(20.00%)	40(80.00%)
7-12	49	10(20.41%)	39(79.59%)	15(30.61%)	34(69.39%)
13-18	14	2(14.28%)	12(85.72%)	4(28.57%)	10(71.43%)
19-24	9	0(0.0%)	9(100%)	2(22.22%)	7(77.78%)
25-30	3	0(0.0%)	3(100%)	0(0.0%)	3(100%)
31-36	2	0(0.0%)	2(100%)	0(0.0%)	2(100%)
37-42	0	-	-	-	-
43-48	4	0(0.0%)	4(100%)	0(0.0%)	4(100%)
49-54	2	1(50.00%)	1(50.00%)	1(50.00%)	1(50.00%)
55-60	2	1(50.00%)	1(50.00%)	1(50.00%)	1(50.00%)
<b>TOTAL</b>	<b>135</b>	<b>20(14.81%)</b>	<b>115(85.19%)</b>	<b>33(24.44%)</b>	<b>102(75.56%)</b>



**Figure:-2** Age wise distribution of RV detected by both techniques

**Comparison of RT-PCR and ELISA with regard to specificity and sensitivity**

We used two different diagnostic techniques in samples for the detection of RV using the same FTD standard. The following 2 × 2 tables result:

**Table:- 3** Comparison of RT-PCR and ELISA with regard to specificity and sensitivity

ELISA	RV(+)	RV(-)	PCR	RV(+)	RV(-)
Positive	17	2	Positive	33	0
Negative	14	66	Negative	0	68

Techniques	Sensitivity	Specificity
ELISA	54.83%	94.12%
PCR	100%	100%

The sensitivity of ELISA is ~55% while for PCR 100% (almost double). Similarly specificity of RT-PCR (100%) is greater than ELISA (94.12%). The above tables cleared that RT-PCR techniques is better than ELISA.

### Discussion

Several decades after the rotavirus description many studies have been carried out to compare detection techniques<sup>(11,16-21)</sup>. Currently, several commercial tests are available from less laborious based techniques that enable a quick diagnosis, e.g. antigen detection methods, to more complex tests such as the polymerase chain reaction<sup>(22-24)</sup>

In the present study we collected the samples of children under 5 year age locating around Jaipur (Rajasthan). One year aged children were more affected by RV according to data of our sample collection. When we see sex wise distribution, the positivity of Males was greater than Females. And this pattern is similar for both ELISA and RT-PCR techniques. We observed the positivity of RV by RT-PCR is greater than ELISA in age group from 1-24 months.

Presence of Human Rota virus antigen was detected in 135 stool samples by ELISA. A total of 20/135 (14.8 %) samples were found to be positive for RV infection by ELISA. Positivity of RV by PCR was 33/135 (24.44%). The data was found to be similar to studies made elsewhere. Studies conducted in Iran, reported that rotavirus was detected in 15.3-26.3% of children with gastroenteritis and the highest incidence of infection was in children aged 1-12 months (Khalili B, et al., 2004) However, studies from Iran also reported the PCR positivity of Rotavirus between 20.6%-32% (Modaress et al., 2010). The positivity of RV by ELISA was significantly lower than by PCR custom assay. The sensitivity and specificity of PCR for detection of RV was 100%, while for ELISA it was found to be 54.83% and 94.12% in our study. In Saudi Arabia, rotavirus is an important cause of severe diarrhea among children. The prevalence of rotavirus infection ranged from 10 to 46% with an average

of 30%. Most of the cases were among children less than 2 years of age, and particularly in the first year of life (Al- Bwardy et al., 1988; Al-Freihi et al., 1993; Ghazi et al., 2005; Kheyami et al., 2006). The ELISA positivity of Rotavirus from Tunisia was reported as 26.2% (Trabelsi et al., 2010). Because of the large quantities of rotavirus present in stool samples from children with gastroenteritis, the viral nucleic acid segments can be visualized directly after extraction from virus particles, by electrophoresis on acrylamide gels, and staining with ethidium bromide or silver nitrate. For Group A rotaviruses, most samples that are positive for rotavirus by EIA will be positive for the characteristic pattern of rotavirus RNA segments after electrophoresis and silver staining. In some cases, silver nitrate staining of viral nucleic acid has roughly the same sensitivity as EIA methods. Consequently, the PAGE method has sometimes been used to diagnose Group A rotavirus infections for surveillance studies. However, this method is very labor intensive and time consuming. A variety of sensitive conventional or real-time reverse transcription polymerase chain reaction (RT-PCR) methods have been developed based on primers specific for several different rotavirus genes. These methods have been particularly useful in detecting rotavirus in extra-intestinal tissues, in studies of the duration of viral shedding in stool and the correlation between disease severity and virus load. RT-PCR is also useful for verifying that RNA extracts contain intact rotavirus RNA.

### Conclusion

So we can conclude that RT-PCR technique is better than ELISA for the detection of RV from above discussion.

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