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Molecular assay for rotavirus NP4 protein gene in infected children under five years in Hilla City

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Abstract---Rotavirus gastroenteritis is one of the most common causes of severe diarrhea in children under the age of five worldwide, with the majority of mortality in lower-income countries. A total of 150 children under the age of five year were involved. (100 child suffering from rotavirus diarrhea were enrolled at Babylon Maternity and Children's Hospital –Al Noor Children's Hospital and 50 child healthy as control), for RT-PCR and RNA extraction the cDNA for DNA sequencing method was carried out to identification genetic variation (substation Mutations) analysis in non-structural protein NSP4 (NSP4) gene of local Human Rotavirus A isolates (IQH1 - IQH.5) and NCBI-Blast related Human Rotavirus A isolates. The phylogenetic tree genetic relationship analysis was showed that local Human Rotavirus A isolates (IQH.1 - IQH.4) were showed closed related to NCBI-BLAST Human Rotavirus A strain RV1326 , the local Human Rotavirus A isolates (IQH.5) were showed closed related to NCBI-BLAST Human Rotavirus A strain G1P at total genetic changes (0.0080-0.0020%).The homology sequence identity between local Human Rotavirus A isolates (IQH1 - IQH.4) and NCBI BLAST related Human Rotavirus A strain RV1326 were showed genetic homology sequence identity ranged from (99.37-99.84%). local Human Rotavirus A isolates (IQH.5) and NCBI BLAST related Human Rotavirus A strain G1P were showed genetic homology sequence identity ranged from (99.52%).

Keywords---rotavirus, genetic homology, NP4 protein gene.

Introduction

Astrovirus, Norovirus, Sapovirus, Rotavirus, Adenovirus, and Torovirus infections are now widely diagnosed using polymerase chain reaction (PCR). (Ahmad Malla et al., 2020). These PCR assays are very sensitive, specific, and simple to operate. The most accurate sign of virus infection is the presence of viral nucleic acid in stool specimens. As a result, diarrhea patients' stool samples are the chosen specimen. To aid molecular research, amplification of the viral genome and sequencing of the amplification products should be done, and virus genotypes can be determined using sequence analysis. As a result, PCR assays and nucleic acid sequence analysis are frequently employed to detect and genotype viruses that cause gastroenteritis. For nearly two decades, these approaches have gradually displaced older immunological tests and have become the gold standard for diagnosing gastrointestinal viruses. In order to improve both sensitivity and specificity, nested PCR tests were designed. (Sidoti *et al.*, 2015). Multiplex RT-PCRs have been widely described in recent years. Multiplex RT-PCRs have been established for the detection of rotavirus groups A, B, and C, as well as the identification of G and P genotypes of group A rotaviruses. Multiplex molecular assays have the potential to consolidate laboratory workflow by reducing time to result, improving diagnostic accuracy, and allowing to simultaneously detect different pathogens. (Binnicker, 2015)

Methods

Viral RNA Extraction

Viral RNA was extracted from stool samples by using AccuZol™ Total RNA extraction kit (Bioneer, Korea) and done according to company instructions as following steps:

Estimation of extracted RNA from stool samples

The extracted RNA were estimated by using Nanodrop spectrophotometer that used to measurement the RNA concentration and purity at absorbance 260/280 nm at ratio (1.8-2.0) as pure RNA, and done as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipeted 2µl of free nuclease water and put on the surface of the lower measurement pedestal to blanking of Nanodrop.
3. After that, the pedestals are cleaned and pipette 1µl of total RNA sample for measurement.

Reverse Transcription PCR (RT-PCR)

RT-PCR was performed for direct detection Human Rotavirus based on specific amplification of NSP4 gene. The RT-PCR was done according to (Teimoori *et al.*, 2018)

DNA sequencing method

The RT-PCR products of positive Rotavirus NP4 protein gene were sent to MacroGen Company in Korea for performed the DNA sequencing by (AB DNA sequencing system). The DNA sequencing analysis was conducted by using phylogenetic tree UPGMA method (MEGA 6.0 version), Multiple alignment analysis based CrlustalW alignment analysis, and NCBI-BLAST for homology sequence identity.

Statistical analysis

Statistical analysis was carried out using SPSS version 16. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means $\pm$ SD). Pearson's chi square (X^2) and Fisher-exact tests were used to find the association between categorical variables. A p -value of ≤ 0.05 was considered as significant (Daniel and Cross, 2018).

Result

The results of rapid test and RT-PCR in patients with rotavirus and in control group

The results of rapid test and RT-PCR in patients with rotavirus and in control group are shown in table (1). Out of all patients detected by rapid test, 80 % showed positive results following RT-PCR and this suggested reduced specificity of rapid antigen test in comparison with RT-PCR, and this suggestion was further confirmed by table (2).

Table (1): The results of rapid test and RT-PCR in patients with rotavirus and in control group

Characteristic	Control group $n = 50$	rotavirus $n = 100$	p
Rapid Test Rotavirus			
Positive, n (%)	0 (0.0 %)	100 (100.0 %)	< 0.001 C **
Negative, n (%)	50 (100.0 %)	0 (0.0 %)	
RT-PCR-Rotavirus			
Positive, n (%)	0 (0.0 %)	80 (80.0 %)	0.001 C **
Negative, n (%)	50 (100.0 %)	20 (20.0 %)	

n is the number of instances; C denotes the chi-square test; ** denotes significance at p 0.01.

Table (2): Sensitivity test

		RT-PCR (gold standard test)	
		Positive $n = 80$	Negative $n = 70$
Rapid antigen test	Positive	80	20
	Negative	0	50

Sensitivity %	100.0		
Specificity %	71.4		
PPV %	80.0		
NPV %	100.0		
Accuracy %	86.7		

n: number of cases; PPV: positive predictive value; NPV: negative predictive value

The phylogenetic tree genetic relationship analysis was showed that local Human Rotavirus A isolates (IQH.1 - IQH.4) were showed closed related to NCBI-BLAST Human Rotavirus A strain RV1326 , the local Human Rotavirus A isolates (IQH.5) were showed closed related to NCBI-BLAST Human Rotavirus A strain G1P at total genetic changes (0.0080-0.0020%).. as showed in figure (1).

The homology sequence identity between local Human Rotavirus A isolates (IQH1 - IQH.4) and NCBI BLAST related Human Rotavirus A strain RV1326 were showed genetic homology sequence identity ranged from (99.37-99.84%). local Human Rotavirus A isolates (IQH.5) and NCBI BLAST related Human Rotavirus A strain G1P were showed genetic homology sequence identity ranged from (99.52%).

The genetic variation (substitution Mutations) analysis in non-structural protein NSP4 (NSP4) gene between local Human Rotavirus A isolates and NCBI-Blast related Human Rotavirus A isolate were find substitution mutations at total genetic variation percentage ranged (0.16-0.63%). Finally, the local Human Rotavirus A isolates (IQH1 - IQH.5) were submitted into NCBI Genbank and identified by accession numbers .

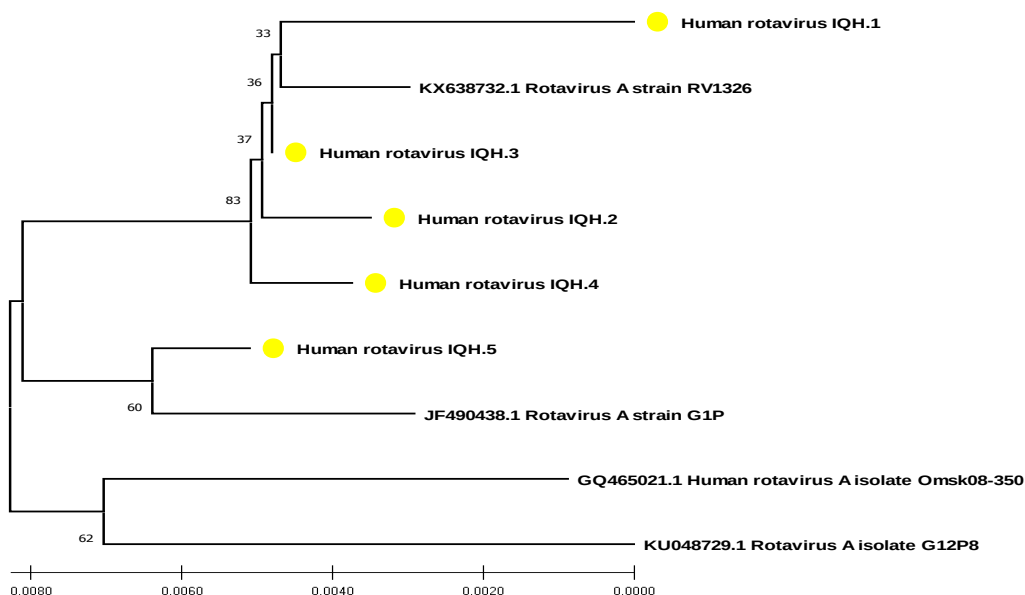


Figure (1): Phylogenetic tree analysis based non-structural protein NSP4 (NSP4) gene partial sequence in local Human Rotavirus A isolates that used for genetic

relationship analysis. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Human Rotavirus A isolates (IQH.1 - IQH.4) were showed closed related to NCBI-BLAST Human Rotavirus A strain RV1326 , the local Human Rotavirus A isolates (IQH.5) were showed closed related to NCBI-BLAST Human Rotavirus A strain G1P at total genetic changes (0.0080-0.0020%).

Discussion

In the current study, there was discrepancy in the results of rapid test and RT-PCR in such a way that 20 % of cases which were diagnosed as positive by rapid test were proved to be negative using RT-PCR. Rotavirus is a common cause of gastroenteritis in children younger than 3 years worldwide. Rapid Antigen Testing (RAT) is a quick and easy tool to detect virus antigen in stool samples and is more specific than sensitive (higher specificity and lesser sensitivity). Reverse transcription-polymerase chain reaction (RT-PCR) and PCR are more sensitive and specific than RAT. Sensitive and specific tools are required for true diagnosis (Barsoum, 2020).

In our study rapid antigen test has a sensitivity level of 100 %, but the specificity level was 71.4 %. Thus, it appears that the rapid test is specific but less sensitive when compared to results of RT-PCR. In one previous study, RAT kits detected 69, 68, and 63 of the 71 samples that were positive for RV by RT-PCR, suggesting 97.2, 95.8, and 88.7 percent sensitivity for RAT kits, with just one false positive result in one of the three RAT kits (specificity up to 100 percent) (Khamrin *et al.*, 2011).

When comparing the sensitivity of rapid antigen test for group A rotavirus detection, it is clearly demonstrated that among various immunochromatography kits, sensitivity and specificity for group A rotavirus infection were a bit different. In addition, it was observed that several rotavirus genotypes G1, G3 and G9 were reacted with these kits. Therefore, genotype variations of rotavirus may not be a problem for false negative results (Khamrin *et al.*, 2011). The RT-PCR assay was discovered to be rotavirus specific and widely reactive to rotavirus genogroups 1–4, 9, 10, and 12. (Jothikumar *et al.*, 2009). The assay's specificity testing revealed no cross-reactivity with a panel of 36 non-rotaviruses. Virus of the intestine specimens (Jothikumar *et al.*, 2009). For accurate viral diagnosis, highly sensitive and specific procedures such as one-step RT-PCR are still necessary gastroenteritis because of clinical suspicions about gastroenteritis and gastroenteritis rotavirus vaccine efficacy trials, as well as related problems (Barsoum, 2020).

Conclusion:

The sensitivity of the fast antigen test is 100 percent, but the specificity is 71.4 percent. When compared to RT-PCR data, the quick test appears to be more specific but less sensitive. RAT kits detected 69, 68, and 63 of the 71 samples that were positive for RV by RT-PCR in a prior study, indicating 97.2, 95.8, and 88.7% sensitivity for RAT kits, respectively, with only one false positive result in one of the three RAT kits.

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