International Journal of Multidisciplinary Research and Development
www.allsubjectjournal.com
Online ISSN: 2349-4182, Print ISSN: 2349-5979
Received: 02-06-2021, Accepted: 17-06-2021, Published: 01-07-2021
Volume 8, Issue 7, 2021, Page No. 34-40

Identification of rotavirus G-P genotypes among Iraqi children in Diyala province by multiplex semi-nested RT-PCR

Ali R Hameed[1, 2], Bakri Y Mohamed[1], Adam D Abakar[1], Karim S Ali[2]
[1] Faculty of Lab Medical Sciences, University of Gezira, Sudan
[2] Faculty of Veterinary Medicine, University of Diyala, Iraq

Abstract
Background: Rotavirus is a much more popular cause of viral diarrhea. and it has several genotypes.
Objectives: The aim of this study is to molecular identification of the Rotavirus G and P genotypes by detecting the VP7 and VP4 genes and to evaluate the relationship between the clinical presentation and rotavirus genotypes.
Material and Methods: This study was conducted on children with diarrhea under (5) years at Batool Hospital in Diyala province during 2019-2020. RNA extraction of 142 stool samples for children with rotavirus virus and G-P genotypes identified by Multiplex semi-nested RT-PCR.
Results: The results showed, prevalent G-genotypes were G1, G4, G2, G9, G1/G2 and G1/G4 at 37.32%, 29.58%, 21.83%, 2.11% and 0.70% respectively, whereas prevalent P-genotypes were P[8], P[4], P[6] and P[10] at 56.34% 13.38%, 8.45%, and 4.23% respectively. The G and P-type combinations were P[8]G4, P[8]G1, P[4]G1, P[6]G1, P[8]G2, P[4]G2, P[6]G4, P[6]G4 and P[4]G1/G4 at 24.65%,23.94%, 8.45%, 4.93%, 4.23%. 4.23%, 2.82%. 2.11% and 0.70% respectively. P[8]G1 the more predominant G-P genotype combination in children with fever and severe dehydration, while P[8] G4 most common in children with vomiting and some dehydration. Our study did not demonstrate any relationship between genotypes and clinical presentation.
Conclusion: There are different Rotavirus genotypes in Diyala province. The current study predicts that the Rotavirus vaccine used in Iraq is still protective for humans. On the other hand, there is no assortment between the human and animal rotavirus strains and our rotavirus strains in this study are similar to Russian strains.

Keywords: rotavirus, g-p genotypes, genotypes combination, multiplex semi-nested RT-PCR

Introduction

The Reoviridae family includes Rotavirus. It is a non-enveloped virus and double-stranded RNA (dsRNA) that has been classified into nine types based on phylogenetic analysis [1, 2]. More than 90% of rotavirus human infections are caused by Rotavirus A, the most common strain [3]. The genome of rotavirus is made up of eleven dsRNA segments that code for six structural proteins (VP1-VP4, VP6, VP7), as well as VP4 and VP7, which are essential for viral adherent proteins and neutralization antigens [4]. VP7 and VP4, which are encoded by RNA segments 9 and 4, respectively, make up the outer layer of rotaviruses. These proteins induce neutralizing antibody responses and are the basis for group A rotavirus classification into G (VP7) and P (VP4) groups, where G represents for glycoprotein and P represents for protease-sensitive protein [5]. Just a few G and P types play a significant role in human infections, despite the fact that there are at least 32 G and 47 P types. G1P [8], G2P [4], G3P [8], G4P [8], G9P [8], and G12P [8] are the names of the proteins [2]. A complete rotavirus genotyping system was developed, which was used to find out where uncommon strains came from [6]. In children under the age of five, rotavirus remains the most frequent cause of acute gastroenteritis. Rotavirus diarrhea kills nearly 702,000 children worldwide per year. Because of the widespread effects of rotavirus infection, the production of rotavirus vaccines has been accelerated. To prepare for the introduction of a vaccine, it is necessary to first know the spread and types of rotaviruses in each area [7]. In 2003, the mortality rate for children under the age of five in Iraq was registered to be 130 per 1,000 for males and 120 per 1,000 for females [8]. Acute dehydration diarrhea in men and livestock is caused by rotavirus A. Rotavirus has a high degree of variability, and a number of human strains share genetic and antigenic characteristics with rotavirus strains of animal sources. Rotavirus has a lot of variation, and a number of human strains share genetic and antigenic characteristics with Rotavirus strains of animal sources [9]. Rotaviruses are spread through the fecal-oral way, which involves contact with contaminated hands, surfaces, and objects, as well as the respiratory mode of transport [10, 11] and Rotavirus replicates mostly in the alimentary canal and infects enterocytes in the villi of the small intestine [12, 13]. In Iraq, there are few rotavirus molecular and genotyping studies. During the 2008 pre-vaccination period, a survey of children with acute gastroenteritis in three separate cities in Iraq found that the PCR detection rate of rotavirus nucleic acid was 40.0 percent [14]. Since the latest rotavirus vaccine (RV5; RotaTeq, Merck) was developed to provide immunity against the most common rotavirus genotypes, it is indeed protective for human vaccines (G1, G2, G3, G4, G9) and currently, it is the vaccine used in Iraq [15]. Enzyme immunoassay, electron microscopy, and polymerase chain reaction are used to diagnose rotavirus infection. [16, 17]. Rotaviruses species and genotypes can be detected and identified using reverse transcription-polymerase chain

reaction (RT-PCR) [18].

Material and Methods

RNA Extraction

TRIzol Reagent was used to extract RNA from the samples since it takes less time and the whole protocol can be done in less than two hours [19].

Reverse transcription (RT)

A mixture of extracted viral RNA, random primer, and Nuclease-free water was used for reverse transcription (RT). The reaction mixture was heated to 65°C for 5 minutes and then cooled for 2 minutes on ice. Reverse transcriptase, RT-buffer, and dNTPs were added to the reaction mixture (Promega, USA). For cDNA synthesis, the mixture was heated for 60 minutes at 42°C and 30 minutes at 37°C, then for 5 minutes at 95°C.

Polymer chain reaction (PCR)

I. Detect (VP7) and (VP4)

To detect (VP7), a reaction mixture containing synthesized cDNA, PCR Buffer, MgCl2, GoTaq DNA Polymerase (Promega, USA), each of the VP7-F and VP7-R primers described in
Table 1, dNTPs (Promega, USA), and nuclease-free water was used. The detection methods for (VP4) are the same as for (VP7), but we used VP4-F and VP4-R primers instead of VP7-F and VP7-R primers [19], described also in Table 1.

Detect G-P genotypes

To detect G-genotypes, PCR amplification was performed in a mixture containing 1st round PCR product, Buffer, MgCl2 (Promega, USA), Go Taq Flexi DNA Polymerase (Promega, USA), VP7-R primer and cocktail primers, dNTPs (Promega, USA), and nuclease-free water. Table 1 displays the PCR profile from the second round. The methods for detecting P-genotypes are the same as for detecting G-genotypes, with the exception of the VP7-F primer and cocktail primers as seen in Table 1, Nuclease-free water and dNTPs (Promega, USA). The second round's PCR profile consisted of a 1-minute denaturation at 95°C, followed by 30 cycles of 95°C for 1 minute, 45°C for 2 minutes, and 72°C for 1 minute, plus a 1-minute extension at 72°C [20].

Gel electrophoresis

In a Tris acetate and EDTA buffer, the amplified RT-PCR products were electrophored to 100 bp DNA ladder (Promega, USA) in 1.5 percent agarose gel and Ethidium bromide stain for visualization. A UV transilluminator was used to see the bands under UV light. The obtained band size is calculated using the DNA ladder as a guide [20].

Sequencing of DNA

Macrogen Company performed the sample sequencing (Korea). Blast software was used to compare the sequencing results to the reference DNA sequence.

Data Analysis

All data was statistically analyzed with the help of a computerized program known SPSS (Statistical Package for Social Science).

Pearson's chi-square (X2) was used to determine the relationship between variables, with a P-value of less than or equal to 0.05 considered significant.

Table 1: The primers used to identify rotavirus genotypes G and P are mentioned below [21, 22]

Primer		Sequence (5′ – 3′)	Position	PCR (bp)
G-typing (VP7) first amplification	VP7-F	ATG TAT GGT ATT GAA TAT ACC AC	nt 51-71	881
	VP7-R	AAC TTG CCA CCA TTT TTT CC	nt 914-932	
P-typing (VP4) first amplification				
	VP4-F	TAT GCT CCA GTN AAT TGG	nt 132-149	663
	VP4-R	ATT GCA TTT CTT TCC ATA ATG	nt 775-795	
G-typing (VP7) second amplification				
G1	aBT1	CAA GTA CTC AAA TCA ATG ATG G	nt 314-335	618
G2	aCT2	CAA TGA TAT TAA CAC ATT TTC TGT G	nt 411-435	521
G3	mG3	ACG AAC TCA ACA CGA GAG G	nt 250-269	682
G4	aDT4	CGT TTC TGG TGA GGA GTT G	nt 480-498	452
G8	aAT8	GTC ACA CCA TTT GTA AAT TCG	nt 178-198	754
G9	mg9	CTT GAT GTG ACT AYA AAT AC	nt 757-776	179
P-typing (VP4) second amplification				
P[4]	2T-1	CTA TTG TTA GAG GTT AGA GTC	nt 474-494	362
P[6]	3T-1	TGT TGA TTA GTT GGA TTC AA	nt 259–278 146	146
P[8]	1T-1	TCT ACT TGG ATA ACG TGC	nt 339 –356 224	146
P[9]	4T-1	TGA GAC ATG CAA TTG GAC	nt 385-402	270
P[10]	5T-1	ATC ATA GTT AGT AGT CGG	nt 575–594	462

Results

Detect G-P genotypes

The results revealed six different G-genotypes (G1, G2, G4, G9, G1/G2, and G1/G4) according to PCR production size in the second amplification was (179 bp-618 bp) as described in Figure 1. The results showed that 133 of the 142 specimens were identified as G-genotypes. G1 most dominant 53(37.32%), followed by G4 42(29.58%), G2 31(21.83%), G9 3(2.11%), G1 and G2 Mixed 3(2.11%), G1and G4 mixed 1(0.70%) and unknown genotypes 9(6.34%). As shown in Figure 1 and Table 2.
Four different P-genotypes (P [4], P [6], P [8] and P [10]). However, G-unknown rotavirus genotypes were detected in 9 of 142 specimens and P-unknown rotavirus genotypes were detected in 25 of 142 specimens according to PCR production in second amplification was (146 bp-462 bp) as described in Figure 1 as explained in the below in Table 2. The results showed that 117 of the 142 specimens were identified as P-genotypes. P [8] most dominant 80(56.34%), followed by P [4] 19(13.38%), P [4] 12(8.45%), P [4] 6(4.23%) and unknown genotypes 25(17.61%), G1and G4 mixed 1(0.70%) and unknown genotypes 9(6.34%) as described in Figure 1 and Table 2.

Table 2: The prevalence of G-P genotypes of Rotavirus in children in Diyala governorate

Genotypes		NO.	%
G- Genotypes	G1	53	37.32%
	G2	31	21.83%
	G4	42	29.58%
	G9	3	2.11%
	G1/G2	3	2.11%
	G1/G4	1	0.70%
	ND	9	6.34%
Total		142	100.00%
P- Genotypes	P[4]	19	13.38%
	P[6]	12	8.45%
	P[8]	80	56.34%
	P[10]	6	4.23%
	ND	25	17.61%
Total		142	100.00%

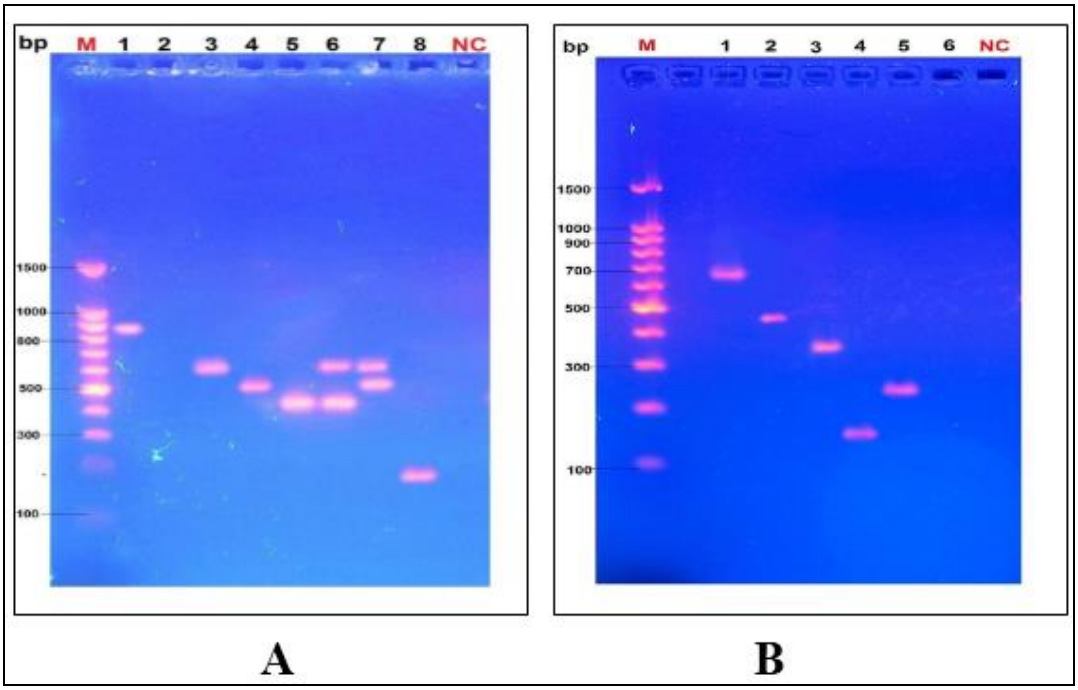

Fig 1: A: G-genotypes of rotavirus by multiplexed semi-nested PCR. M: Molecular marker (100bp); lane 1: VP7 RT-PCR as control positive (881bp): lane 2: No detected; lane 3: G1 (618bp), lane 4: G2 (521bp); lane 5: G4 (482bp); lane 6: G1 and G4 (618bp and 482bp); lane7: G1 and G2 (618bp and 521bp) and lane 8: G9 (179bp) and lane NC: Negative Control. B: P-genotypes of rotavirus by multiplexed semi-nested PCR. M: Molecular marker (100bp); lane 1: VP4 RT-PCR as control positive (633bp); lane 2: P [10] (462bp); lane 3: P [4] (362bp), lane 4: P [6] (164bp); lane 5: P [8] (224bp); lane 6: No detected and lane NC: Negative Control.

Rotavirus G and P Genotype Combinations

There are different G and P-type combinations (P[8]G1, P[4]G1, P[6]G1, P[4]G2, P[8]G2, P[6]G4, P[8]G9 and P[4]G1/G4). P[8]G4 formed most of the rotavirus strains at 35(24.65%), followed by P[8]G1 at 34(23.94%), P[4]G1 12(8.45%), P[6]G1 7(4.93%), P[4]G2 and P[8]G2 at 6(4.23%) for each one, P[6]G4 4(2.82%), P[8]G9 3(2.11%) and P[4]G1/G4 1(0.70%). However, as shown in Figure 2, 6 (4.23%) of P [10], 2 (1.41%) of P [8], and 1 (0.70%) of P [10] are associated with unknown G-types, while 19 (13.38%) of G1, 3 (2.11%) for each G1/G4 and G4 are associated with unknown P-types.

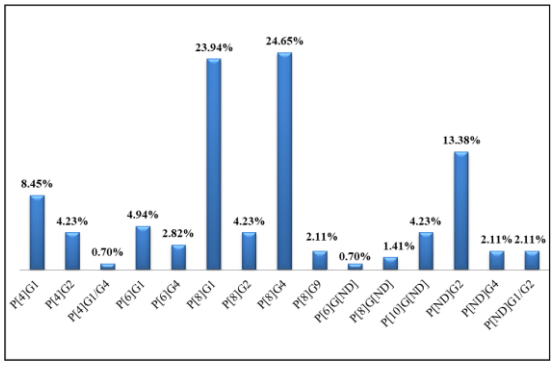

Fig 2: Rotavirus P-G Genotype Combinations

Association of G-P genotype combinations with clinical features

There was variation in the G-P genotype distribution. P[8] G1 was the most common G-P genotype combination detected in children with fever (16 cases, 28.57%), but lower genotype combinations detected P[6] G4, P [8] G[ND], and P[ND] G1/G2 (1 case, 1.79%).P[8] G4 was the more predominant G-P genotype combination detected in children with vomiting (14 cases, 24.14%), but lower genotype combinations detected P [4] G1/G4 and P [ND] G4 (1 case, 1.72%). P [8] G4 was the more predominant G-P genotype combination detected in children with some dehydration (16 cases, 25.00%) and severe dehydration (22 cases, 28.21%), but lower genotype combinations detected P [4] G1/G4 and P [8] G2 at (1case, 1.56%) in children with some dehydration and P [6] G [ND] and P [ND] G4 at (1case, 1.28%) in severe dehydration. The association of G-P genotype combinations with clinical features did not reach the statistical significance (P > 0.05) as displayed in Figure 3.

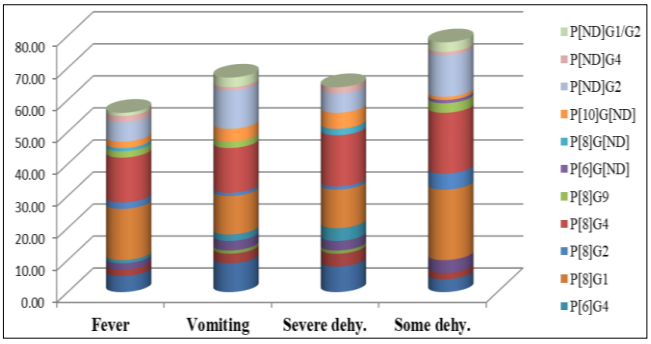

Fig 3: Distributions of rotavirus P-G genotype combinations with clinical features

Sequences

By using Blast software for all local samples of vp7derived from diarrheic human samples derived nucleotide sequences were aligned for matching with the reference database (Gen Bank), revealing the local human samples revealed completely match for VP7 human sample with reference VP7 in Gen Bank sequence accession ID: MN577099.1 and MN551959.1 as in Figures 4, 5, 6 and 7. The phylogenetic tree shows that our rotavirus strains are closely related to human strains Russian, as shown in Figure 8.

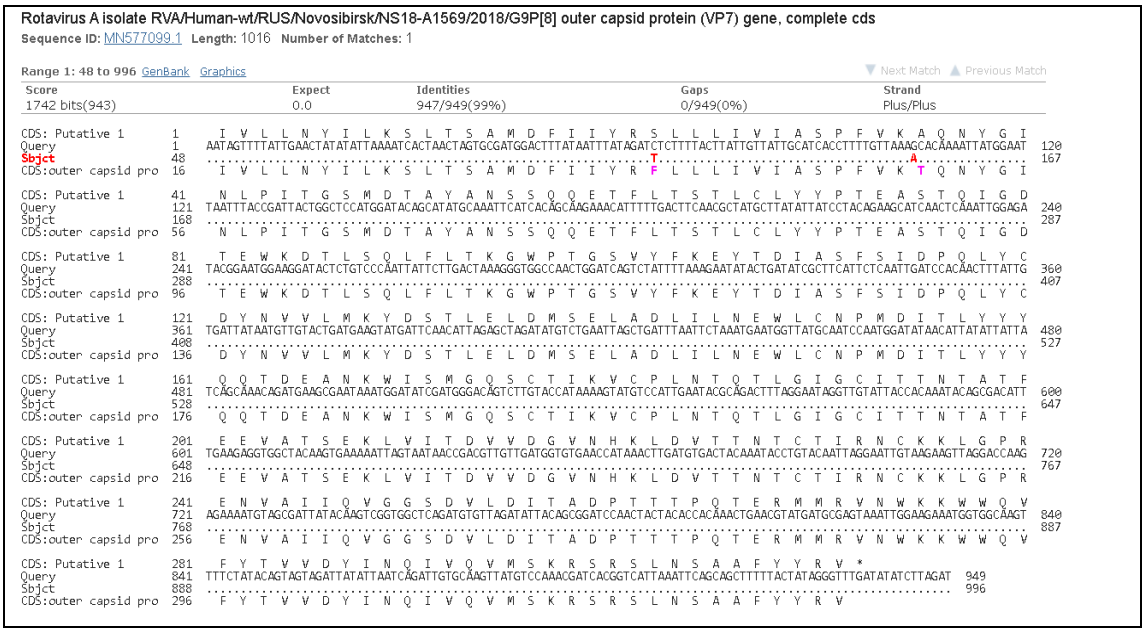

Fig 4: GenBank database alignment of VP7 gene sequence from a local human sample showing 99.79% match with match with reference human Rotavirus, using Blast

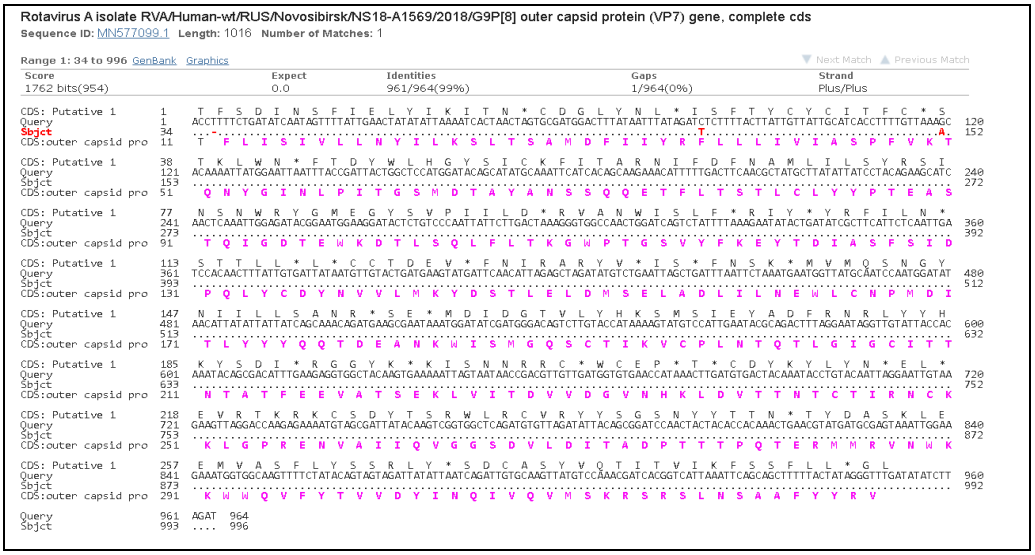

Fig 5: GenBank database alignment of VP7 gene sequence from a local human sample showing 99.69% match with reference human Rotavirus, using Blast

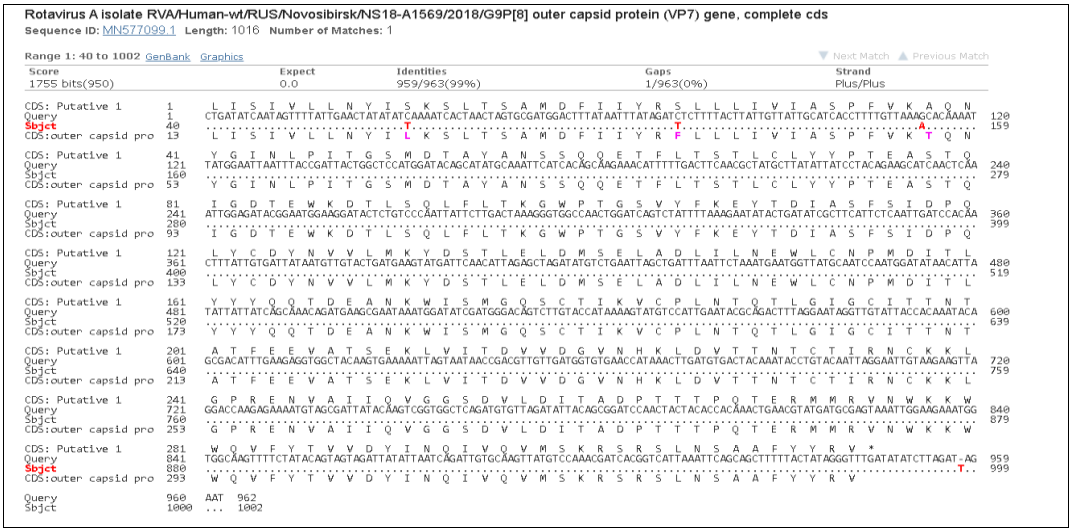

Fig 6: GenBank database alignment of VP7 gene sequence from local a human sample showing 99.58% match with reference human Rotavirus, using Blast

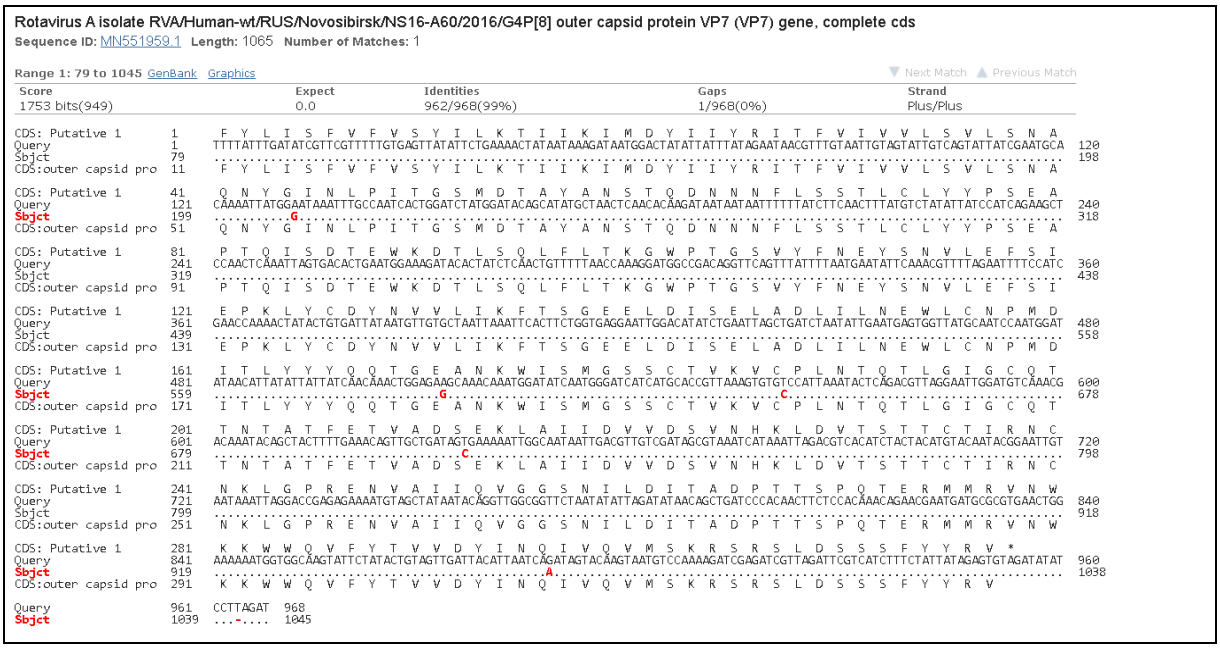

Fig 7: GenBank database alignment of VP7 gene sequence from a local human sample showing and 99.38% match with reference human Rotavirus, using Blast

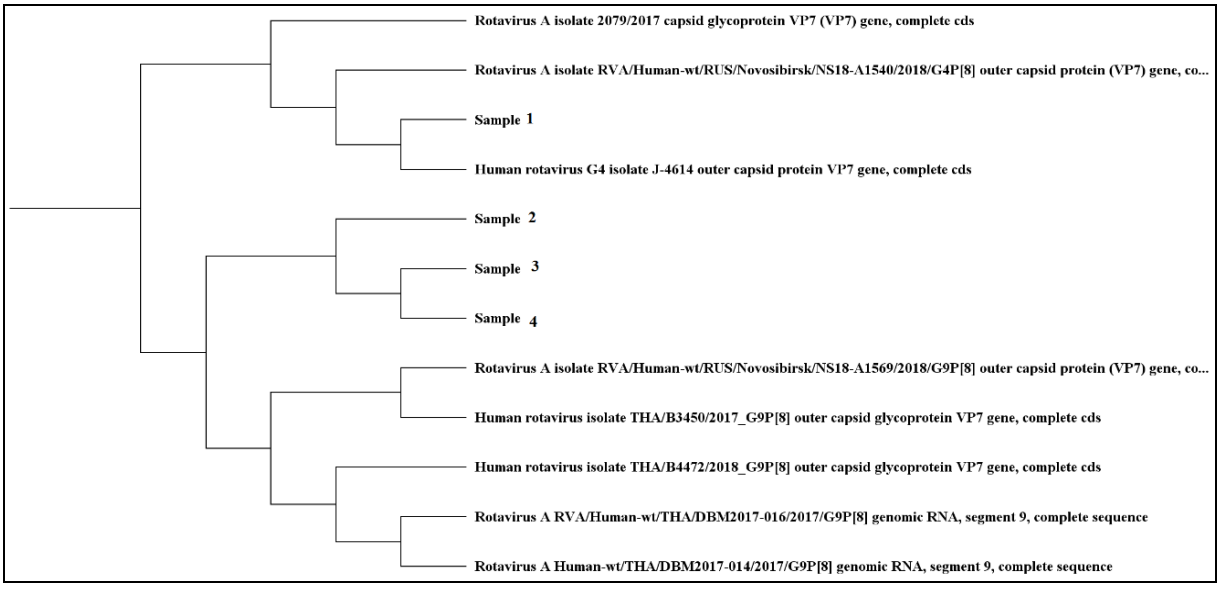

Fig 8: Phylogenetic Tree between samples and isolates were registered in the NCBI database. The Local Rotavirus A isolate (sample 1) was found to be closely related to the NCBI Blast Rotavirus A (j-4614) VP7gene, whereas the Local Rotavirus A isolates (samples 3, 4, and 2) were found to be less distinct and out of the tree.

Discussion

In this study, out of 142 samples tested for RT-PCR, 9 samples did not amplify as G- Genotypes, while 25 samples did not amplify as P- Genotypes; this may be due to degradation of RNA while transportation or low viral load at the time of specimen collection. According to the reports recorded all over the world, the main genotypes G1, G2, G3, G4, G9 that exist with P [4], P [6], P [8] [23]. There are other studies consonant with our results. Younis and Hawraa reported that the most common G-P genotypes in Iraq-Babylon province were G1 (30%), P [8] (36.25%), G4 (17.5%), P [8] (23.75%), and P [8] G1 predominant genotypes combination [23]. The Rotavirus infected 96 children in Iraqi Kurdistan (37%). G1, G4, G2, G9, P [8], P [6], and P [4] were found to be the most common genotypes by RT-PCR. There were eight G/P combinations discovered, but P [8] G1 and P [4] G2 accounted for more than half of the strains [24]. In the zone of Mid Iraq, the predominant G type was G1 (48.57%), followed by G2 (22.14%), G9 (11.42%), G3 (2.14%), G4 (0.71%). Only P [8] (61.4%), P [4] (11.4%) and P [6] (5.7%) genotypes were found [25]. Although, G3 and G12 genotypes were present in Iraq, they were not proven in our study. Our results matched the results of researchers in neighboring countries such as Iran, The researchers found that G1, G4, G8 were the more predominant, while P [8] was the more predominant, followed by P [4] [24]. and our results are similar to the search results in Kuwait that showed that G1 and P[8] are the predominant serotypes in Kuwait [26].

Our findings contradict the findings of a study conducted in Turkey, which found that G4 [P8] is the most common, followed by G1 [P8], which includes more than two-thirds of the population in the study [27], as well as the findings of a study conducted in Pakistan, which found a high prevalence of G12 and G3 RVA strains in 2015 and 2016 [28]. The G1P

[8] was the most common circulating strain pre-vaccination [29]. According to the rotavirus genotypes discovered in Diyala governorate, the current study hypothesizes that the rotavirus vaccine currently in use in Iraq is still protective for human vaccines, as the vaccine currently in use was designed to protect against more prevalent rotavirus serotypes (G1, G2, G3, G4, G9) and to reduce the incidence of illness, hospitalization, and costs [30, 31].

Conclusion

In Diyala Province, there are six different Rotavirus G-genotypes G1, G2, G4, G9, G1/G2, and G1/G4. G1 is the most dominant, followed by four different P-genotypes, P [4,] P [6,] P [8], and P [10], with P [8] being the more dominant. There were 9 different combinations genotypes. P [8] G4 formed most of the rotavirus strains. There is no statistical significance between G-P genotype combinations and clinical features. Because the vaccine currently in Iraq is designed to provide protection and the ability to reduce disease severity, the current study believes that the Rotavirus vaccine used is still protective for humans. All the genotypes examined in this study did not demonstrate the occurrence of an assortment between the human and animal rotavirus strains in the Diyala governorate and our human rotavirus strains similar to Russian strains.

References

1. Matthijnssens J, Van Ranst M. Genotype constellation and evolution of group A rotaviruses infecting humans. Current opinion in virology. 2012 Aug 1;2(4):426-33.
2. Suzuki H. Rotavirus replication: Gaps of knowledge on virus entry and morphogenesis. The Tohoku journal of experimental medicine. 2019;248(4):285-96.
3. Bishop R. Discovery of rotavirus: Implications for child health. Journal of gastroenterology and hepatology,2009:24:S81-5.
4. Desselberger U. Rotaviruses. Virus research,2014: 22:190:75-96.
5. Hassine-Zaafrane M, Sdiri-Loulizi K, Salem IB, Kaplon J, Ayouni S, Ambert-Balay K *et al*. The molecular epidemiology of circulating rotaviruses: three-year surveillance in the region of Monastir, Tunisia. BMC Infectious Diseases,2011:11(1):1-6.
6. MIRANDA, Robyn C. Understanding and managing risk of norovirus contamination on frozen berries from farm to fork. PhD Thesis. Rutgers University-School of Graduate Studies, 2019.
7. Parashar, Umesh D. Global mortality associated with rotavirus disease among children in f, 200.Supplement,2004:1:S9-S15.
8. World Health Organization. Iraq. Statistics by country or region. 2003 [cited 2005 Oct 19].
9. Doro R, Farkas SL, Martella V, Bányai K. Zoonotic transmission of rotavirus: surveillance and control. Expert review of anti-infective therapy,2015:13(11):1337-50.
10. Butz AM, Fosarelli P, Dick J, Cusack T, Yolken R. Prevalence of rotavirus on high-risk fomites in day-care facilities. Pediatrics,1993:92(2):202-5.
11. Dennehy PH. Transmission of rotavirus and other enteric pathogens in the home. The Pediatric infectious disease journal,2000:19(10):S103-5.
12. Greenberg HB, Estes MK. Rotaviruses: from pathogenesis to vaccination. Gastroenterology,2009:136(6):1939-51.
13. Greenberg HB, Clark HF, of fit PA. Rotavirus pathology and pathophysiology. Current topics in microbiology and immunology,1994:185:255-283.
14. Hardiman O, Al-Chalabi A, Chio A, Corr EM, Logroscino G, Robberecht W *et al*. Amyotrophic lateral sclerosis. Nature reviews Disease primers,2017:5:3(1):1-9.
15. Velázquez FR. Protective effects of natural rotavirus infection. The Pediatric infectious disease journal,2009:8(3):S54-S56.
16. Angel J, Franco MA, Greenberg HB. Rotavirus immune responses and correlates of protection. Current opinion in virology,2012:2(4):419-25.
17. GASTROENTERITIS, Viral. Viral gastroenteritis: Causes, pathophysiology, immunology, treatment, and epidemiology, 2011.
18. Fischer TK, Gentsch JR. Rotavirus typing methods and algorithms. Reviews in medical virology,2004:14(2):71-82.
19. Chavan N. Detection and Characterization of Human G Type Rotavirus Among Children With Diarrhoea In *Rural Hospital* (Doctoral dissertation, Datta Meghe Institute of Medical Sciences).
20. Elnady H, Abdelsamie O, Sallam S, Sherif L, Kholoussi N, Khoulousi S*et al*. Genotyping of rota virus causing gastroenteritis in Egyptian children. Research Journal of Pharmaceutical, Biological and Chemical Sciences,2016:7(5):512-21.
21. Sudarmo SM, Shigemura K, Osawa K, Wardana OP, Darma A, Ranuh R *et al*. Genotyping and clinical factors in pediatric diarrhea caused by rotaviruses: one-year surveillance in Surabaya, Indonesia. Gut pathogens,2015:7(1):1-0.
22. World Health Organization (WHO). Manual of rotavirus detection and characterization methods. No. WHO/IVB/08.17. Geneva: World Health Organization, 2009.
23. Santosham M, Nelson EA, Bresee JS. Implementing rotavirus vaccination in Asia. Vaccine,2007:1:25(44):7711-6.
24. Ahmed HM, Coulter JB, Nakagomi O, Hart CA, Zaki JM *et al*. Molecular characterization of rotavirus gastroenteritis strains, Iraqi Kurdistan. Emerging Infectious Diseases,2006:12(5):824.
25. Abood WS. The molecular epidemiology of rotavirus strains causing gastroenteritis in infant in the region of Mid Iraq. Al-Qadisiyah Journal of Veterinary Medicine Sciences,2013:30:12(1):121-7.
26. Marmash RW, Dalwai AK, Szucs G, Molla AM, Pacsa AS, Al-Nakib W *et al*. Genotypic characterization of rotaviruses and prevalence of serotype-specific serum antibodies in children in Kuwait. Epidemiology & Infection,2007:135(8):1331-7.
27. Cataloluk O, Iturriza M, Gray J. Molecular characterization of rotaviruses circulating in the population in Turkey. Epidemiology & Infection,2005:133(4):673-8.
28. Sadiq A, Bostan N, Bokhari H, Matthijnssens J, Yinda KC, Raza S *et al*. Molecular characterization of human group A rotavirus genotypes circulating in Rawalpindi, Islamabad, Pakistan during 2015-2016. PloS one,2019:14(7):e0220387.
29. Almalki SS. Circulating rotavirus G and P strains post

rotavirus vaccination in Eastern Mediterranean Region. Saudi medical journal,2018:39(8):755.
30. Velázquez FR. Protective effects of natural rotavirus infection. The Pediatric infectious disease journal,2009:28(3):S54-6.
31. Burnett E, Parashar U, Tate J. Rotavirus vaccines: effectiveness, safety, and future directions. Pediatric Drugs,2018:20(3):223-33.