(RESEARCH ARTICLE)

Immunological and molecular detection of rotavirus genotype in children with gastroenteritis in Diyala-Iraq

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Abstract

Aim: To explore the prevalence along with the molecular detection and genotyping of group A rotavirus (RVA) among children up to 5 years old complaining of gastroenteritis in Diyala province-Iraq.

Methods: This is a cross sectional study conducted in Diyala province-Iraq during the period of 2019-2020. One hundred children up to 5 years old with gastroenteritis were enrolled. 54% were males and 46% were females. They were attending hospitals or primary healthcare centers. All were vaccinated with rotavirus vaccine. Blood and fecal samples were collected from each subject. Serum anti-rotavirus IgM and IgG plus fecal rotavirus Ag were tested for using ELISA techniques. Stool samples positive for rotavirus Ag were submitted for conventional PCR and for G and P genotyping thereafter. Statistical analysis was done using SPSS version 25 and P values ≤ 0.05 were considered significant.

Results: The anti-rotavirus IgM and IgG positivity rates among children were 71% (P = 0.001) and 81% (P = 0.0001), respectively. Additionally, the rotavirus stool antigen was positive in 75% of the children (P = 0.001). Among 61 stool samples submitted for conventional PCR technique, the rotavirus RNA was detected in 25 (40.9%). The sequencing and genotyping investigation of 10 PCR positive products revealed that all investigated viral samples belonged to G1P[8] rotavirus A genotype.

Conclusion: The current strains analyzed belonged to the G1P[8] rotavirus A genotypes, indicating high coverage of current rotavirus vaccines and affirming the importance of continuous characterization of circulating rotavirus strains and monitoring vaccine efficacy.

Keywords: Rotavirus; Viral Gastroenteritis; VP7 Genotyping; Diyala Province.

1. Introduction

Diarrhea is the eighth leading cause of annual death among all ages (responsible for more than 1.6 million deaths annually) and the fifth leading cause of death among children younger than 5 years worldwide [1]. According to the WHO (World Health Organization), gastroenteritis affects generally 3 to 5 million children each year, of which nearly 12% are children younger than 5 years (in 2015, responsible for nearly 446,000 deaths) [2]. The morbidity and mortality rate of gastroenteritis is increasing in developing countries. Acute gastroenteritis is one of the main public health problems caused by various well known pathogens, including; bacteria, protozoa and viral pathogens [3].

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It has been shown that enteric viruses, especially rotavirus, are the most important etiological agents for diarrhea mortality among children younger than 5 years with more than 128,000 deaths occur annually in the world [4]. The high percentage of rotavirus-related deaths (> 80%) is found in developing countries [5, 6]. These viruses are transmitted through fecal-oral route, and they are known to be contagious from person to person through person-to-person contact [7]. Rotaviruses, as members of Reoviridae family, are icosahedral, nonenveloped, and composed of three concentric layers of proteins with a core consisting of segmented double-stranded RNA that codes six structural (VP1-VP4, VP6 and VP7) and six nonstructural (NSP1-NSP6) proteins [8].

Serologically, human rotaviruses are divided into ten serogroups (A–J) and each group is based on genetic properties of major structural protein VP6. Group A rotaviruses are the major causes of acute diarrhea in infants and young children worldwide. Based on genetic diversity of two outer proteins, VP7 (glycosylated, G-type) and VP4 (protease sensitive, P-type), rotaviruses are classified into multiple serotypes, including 32 different G and 47 different P genotypes [9]. Of all the possible combinations, 6 genotypes (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8]) contribute to an estimated 80–90% of the global RVA disease burden [10, 11].

Molecular and genotyping studies on rotavirus in Iraq are limited. A study was conducted in three different cities in Iraq among children with acute gastroenteritis during the 2008 pre-vaccination era and it found that the PCR detection rate of rotavirus RNA was 40.0%. The most prevalent genotype was G2 (40%) most often associated with P[6]. G1 was the second most common genotype (16 %) mainly associated with P[8] [12]. In another study, 8 different P and G genotype combinations were detected, and the most common combinations were P[8]G1 (33%), P[8]G4 (21%), P[4]G2 (11%), P[6]G1 (11%), and P[8]G9 (11%) [13]. Different studies from neighboring as well as Eastern Mediterranean countries had yielded different prevalence rates depending on the vaccination status, but G1P[8] was the most prevalent genotype [14, 15]. In Turkey, G1P[8] was the dominant genotype combination (42.2%), followed by G9P[8] (21.1%) and G12P[6] (11.0%) [16]. In another study, RT-PCR (reverse transcription PCR) found rotavirus RNA in 78.2% of the samples tested. G9P[8] was the most common G/P combination (found in 40.5% of the strains), followed by G1P[8] (21.6%), G2P[8] (9.3%), G2P[4] (6.5%), G3P[8] (3.5%), and finally, G4P[8] (3.4%) [17]. Among 20 African countries, the G1P[8] combination (22.64%) was the most encountered followed by G2P[4] (8.29%), G9P[8] (6.95%) and G2P[6] (5.00%) [18]. The most predominant genotype distribution of rotavirus changed from G1P[8] and G2P[4] into the rare and unusual genotypes G3P[8], G8P[8], and G9P[8] in Southeast Asia [19].

2. Material and methods

This is a cross sectional study conducted in Diyala province-Iraq during the period of 2019-2020. One hundred children up to 5 years old with gastroenteritis were enrolled. The mean age ± SD was 24.3 ± 18.5 months with a range of 1-60 months. 54% were males and 46% were females. They were attending hospitals or primary healthcare centers. All were vaccinated with rotavirus vaccine (pentavalent RotaTek, Merck). Blood and fecal samples were collected from each patient. Serum anti-rotavirus IgM and IgG plus fecal rotavirus Ag were tested for using ELISA techniques (Foresight / China). Stool samples positive for rotavirus Ag were submitted for viral RNA according to the instructions of QIAmp viral RNA Mini kit (Qiagen, USA) and then submitted for reverse-transcription conventional PCR for G and P genes using highly specific primers, as shown in table 1.

Table 1 Primers used for VP7 genotyping of human rotavirus strains.

Primer Name	Sequence	Annealing temp. (°C)	Product size (bp)
Beg 9	5`GGCTTTAAAAGAGAGAATTTCCGTCTGG-3`	42	1062
End 9	5`-GGTCACATCATACAATTCTAATCTAAG-3`	42	1062

Primers used for VP7 genotyping of human rotavirus strains [20].

For sequencing, PCR products were sent for Sanger sequencing using ABI3730XL, automated CDNA sequences, by Macrogen Corporation - Korea. The results were received by email then analyzed using Genious software.

The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome. The amino acid sequences of the targeted VP7 protein were retrieved online from the protein data bank (http://www.ncbi.nlm.nih.gov). The observed variants in the

coding portions were translated into a reading frame corresponding to the referring amino acid residues using the Expasy online program (http://web.expasy.org/translate/). Multiple amino acid sequences alignments were conducted between the referring amino acid sequences and their observed mutated counterpart using the "align" script of the BioEdit server.

A specific comprehensive tree was constructed according to the neighbor-joining protocol described by Hussein et al. [21]. The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server [22]. Then, a full inclusive tree, including the observed variant, was built by the neighbor-joining method and visualized as an unrooted tree using iTOL suit. The sequences of each classified phylogenetic species-group in the comprehensive tree were colored appropriately.

Statistical analysis was done using SPSS version 25 and P values ≤ 0.05 were considered significant.

This study was approved by the ethical committee in College of Medicine - Diyala University.

3. Results

A total of 100 children up to 5 years of age were enrolled, 54% were males and 46% were females. 35% were less than 1 year of age and 9% were 5 years old with a mean ± SD of 24.3 ± 18.5 months and a range of 1-60 months. Furthermore, 61% of children were living in rural areas and 39% in urban areas. Clinically, they were complaining from fever, vomiting and diarrhea. The results of ELISA technique found that the anti-rotavirus IgM positivity rate was 71% with a statistically significant difference (P = 0.001). Furthermore, 81% were anti-rotavirus IgG positive with a statistically significant difference (P = 0.0001). The rotavirus stool antigen was positive in 75% with statistically significant difference (P = 0.001), as shown in table 2.

Table 2 Serum anti-rotavirus IgM, IgG and fecal rotavirus Ag among study group.

Immunological results		No.	%	P value
Anti-rotavirus IgM	Positive	71	71.0	0.001*
	Negative	29	29.0	
Anti-rotavirus IgG	Positive	81	81.0	0.0001*
	Negative	19	19.0	
Stool rotavirus Ag	Positive	75	75.0	0.001*
	Negative	25	25.0	

*Significant difference between proportions by Pearson Chi-square test at 0.05 level**.**

A total of 61 stool samples were submitted for PCR detection, 25 (40.9%) were positive and 36 (59.0%) were negative with insignificant difference (P = 0.421), as demonstrated in table 3.

Table 3 Fecal rotavirus RNA detection rate by PCR technique.

PCR detection	No.	%	P value
Positive	25	40.9	0.421*
Negative	36	59.0	

*Insignificant difference between proportions by Pearson Chi-square test at 0.05 level.

Results presented in table 4 found that neither stool rotavirus antigen nor PCR outcome had an association with children age groups (P = 0.413 and 0.789, respectively).

Table 4 Association of stool Ag and PCR outcome with age groups.

Age (Years)	Stool antigen				PCR outcome			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
Less than 1	24	68.6	11	31.4	10	50.0	10	50.0
1	11	84.6	2	15.4	3	33.3	6	66.7
2	9	60.0	6	40.0	4	40.0	6	60.0
3	14	87.5	2	12.5	4	30.8	9	69.2
4	10	83.3	2	16.7	2	33.3	4	66.7
5	7	77.8	2	22.2	2	66.7	1	33.3
P value	0.413*				0.789*			

*Insignificant difference between proportions by Pearson Chi-square test at 0.05 level.

The children gender showed no association with stool rotavirus antigen (P = 0.247) and with PCR outcome (P = 0.714), as shown in table 5.

Table 5 Association of stool antigen and PCR outcome with gender.

Gender	Stool antigen				PCR outcome			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
Male	43	79.6	11	20.4	12	38.7	19	61.3
Female	32	69.6	14	30.4	13	43.3	17	56.7
P value	0.247*				0.714*			

*Insignificant difference between proportions by Pearson Chi-square test at 0.05 level.

Table 6 revealed that both stool rotavirus antigen and PCR results had no association with the residence of children (P = 0.554 and 0.495, respectively).

Table 6 Association of stool antigen and PCR outcome with residence.

Residence	Stool antigen				PCR outcome			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
Urban	28	71.8	11	28.2	9	47.4	10	52.6
Rural	47	77.0	14	23.0	16	38.1	26	61.9
P value	0.554*				0.495*			

*Insignificant difference between proportions by Pearson Chi-square test at 0.05 level.

The stool rotavirus antigen positivity rate was insignificantly higher among those children who were negative for anti-rotavirus IgG (89.5%) (P = 0.105). Similarly, there was no association between PCR outcome and the anti-rotavirus IgG (P = 0.401), as shown in table 7.

Table 7 Association of stool antigen and PCR outcome with IgG positivity.

IgG positivity	Stool antigen				PCR outcome			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
Positive	58	71.6	23	28.4	25	41.7	35	58.3
Negative	17	89.5	2	10.5	-	-	1	100
P value	0.105*				0.401*			

*Insignificant difference between proportions by Pearson Chi-square test at 0.05 level.

The stool rotavirus Ag was higher among children who were positive for anti-rotavirus IgM (83.1%) with a statistically significant difference (P = 0.003). Whereas, the PCR outcome had insignificant association with anti-rotavirus IgM (P = 0.793), as seen in table 8.

Table 8 Association of stool antigen and PCR outcome with IgM positivity.

IgM positivity	Stool antigen				PCR outcome			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
Positive	59	83.1	12	16.9	18	40.0	27	60.0
Negative	16	55.2	13	44.8	7	43.8	9	56.3
P value	0.003*				0.793			

*Significant difference between proportions by Pearson Chi-square test at 0.05 level.

Data presented in table 9 showed that none of the clinical features was significantly associated with rotavirus stool Ag or PCR results.

Table 9 Association of stool antigen and PCR outcome with clinical features.

Clinical feature		Stool antigen				PCR outcome			
		Positive		Negative		Positive		Negative	
		No.	%	No.	%	No.	%	No.	%
Fever	Present	42	75.0	14	25.0	15	38.5	24	61.5
	Absent	33	75.0	11	25.0	10	45.5	12	54.5
P value		--				0.594*			
Vomiting	Present	33	70.2	14	29.8	10	40.0	15	60.0
	Absent	42	79.2	11	20.8	15	41.7	21	58.3
P value		0.298*				0.896*			
Diarrhea	Present	43	69.4	19	30.6	15	40.5	22	59.5
	Absent	32	84.2	6	15.8	10	41.7	14	58.3
P value		0.096*				0.930*			

*Insignificant difference between proportions by Pearson Chi-square test at 0.05 level.
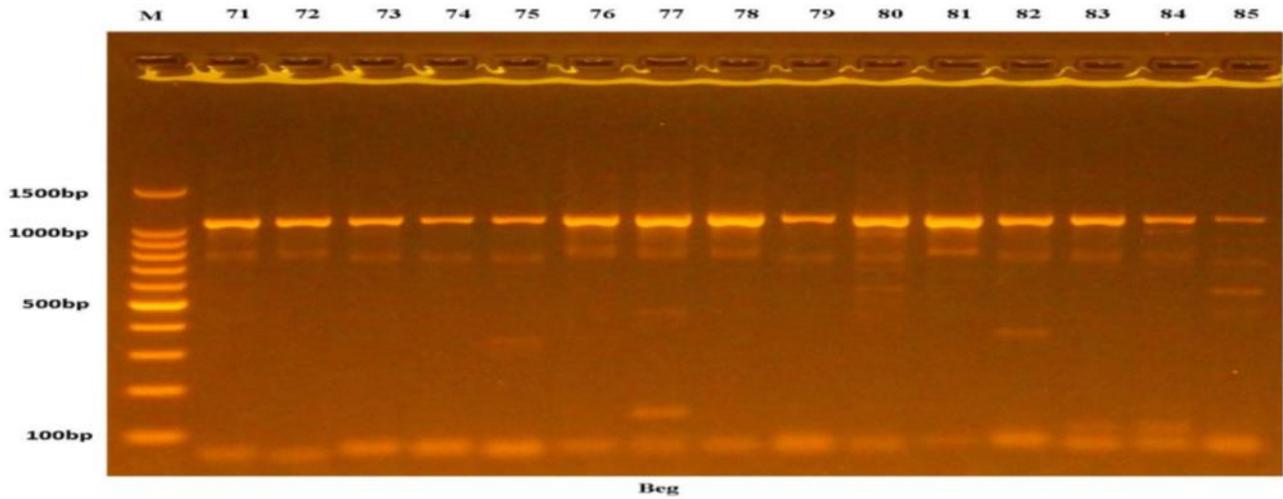
Figure 1 Results of the amplification of rotavirus gene of stool samples were fractionated on 2% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 71-85 resemble 1062bp PCR products.

For the sequencing, 10 PCR positive products were included. These were screened to amplify VP7 gene sequences of rotavirus. The outer capsid protein is one of the structural proteins encoded by rotaviral VP7 gene. Thus, the variation of VP7 gene can be used for rotavirus genotyping due to its ability to adapt remarkable genetic diversity among different viral sequences [23]. The sequencing reactions indicated the exact identity after performing NCBI BLASTn for these PCR amplicons [22]. Concerning the 1062 bp amplicons, the NCBI BLASTn engine has shown about 98% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed nucleic acid sequences of these investigated samples with the retrieved nucleic acid sequences (GenBank acc. GQ452920.3), the accurate positions and other details of the retrieved PCR fragments were identified. The total length of the targeted locus was localized in the NCBI server, and the positions of the start and end of the targeted locus were also confirmed (as shown in figure 2).
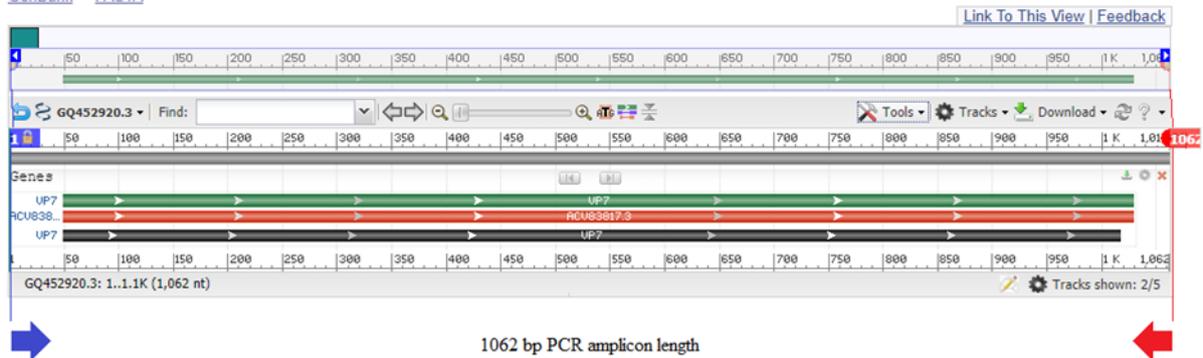
Figure 2 The exact position of the retrieved 1062 bp amplicon that partially covered a portion of the VP7 gene within rotavirus genomic sequences (GenBank acc no. GQ452920.3). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 1062 bp amplicons' sequences within the genomic sequences of rotavirus, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 1062 bp amplified amplicons, as shown in table 10.

Table 10 The position and length of the 1062 bp PCR amplicons that were used to amplify a portion of the VP7 gene within rotavirus genomic sequences (GenBank acc. no. GQ452920.3). The gray-colored sequences refer to the position of the reverse and forward primers, respectively.

Amplicon	Reference locus sequences (5′ - 3′)	length
Nucleic acid sequences within the viral VP7 genetic locus	*GGCTTTAAAAGAGAGAATTTCCGTCTGGCTAACGGTTAGCTCCTTTTGATGTATGGTA TTGAATATACCACAATTCTAATCTTTCTGATATCAATCATTCTATTCAACTATATATTA AAATCAGTGACTCAAATGATGGACTACCTTATATATAGATCTTTGTTAATTTCTGTAGC ATTATTTGCCTTGACAAGAGCTCAGAATTATGGGATTAACTTACCAATAACAGGATCAA TGGACGCCGCATACGCTAACTCTACTCAAGAAGGAATATTTCTAACATCCACATTATGT CTGTATTATCCGACAGAAGCAAGTACTCAAATTAATGATGGTGAATGGAAAGACTCATT ATCACAAATGTTTCTCACAAAAGGTTGGCCAACAGGATCAGTCTATTTTAAAGAGTATT CAAGTATTGTTGATTTTTCTGTTGATCCACAATTATATTGTGATTATAACTTAGTACTA ATGAAATATGATCAAAATCTTGAATTAGATATGTCAGAGTTAGCTGATTTAATATTGAA TGAATGGTTATGTAATCCAATGGATATAACATTATATTATTATCAACAATCAGGAGAAT CAAACAAGTGGATATCAATGGGATCATCATGTACTGTGAAAGTGTGTCCACTGAATACG CAAACGTTAGGAATAGGCTGTCAAACAACAAATGTAGACTCGTTTGAAATGGTTGCTGA AAATGAGAAATTAGCTATAGTGGATGTCGTTGATGGGATAAATCATAAAATAAATTTG ACAACTACGACATGTACTATTCGAAATTGTAAGAAGTTAGGTCCAAGAGAGAATGTAGC TGTAATACAGGTTGGTGGCTCTAATGTATTAGACATAACAGCAGATCCAACGACTAATC CACAAACTGAGAGAATGATGAGAGTGAATTGGAAAAAATGGTGGCAAGTATTTTATAC TATAGTAGATTATATTAACCAGATTGTACAGGTAATGTCCAAAAGATCAAGATCATTAA ATTCTGCAGCTTTTTATTATAGAGTATAGATATACCTTAGATTAGAATTGTATGATGTG ACC**	1062 bp

* Refers to the reverse primer sequences (placed in a forward direction).
** Refers to the forward primer sequences (placed in a reverse complement direction).

To summarize all the results obtained from the sequenced 1062 bp fragments, the precise positions and annotations of the observed nucleic acid substitution mutation is described in the NCBI reference sequences as shown in table 11.

Table 11 The pattern of the observed SNPs in the 1062 bp amplicons of the VP7 gene in comparison with the NCBI referring sequences (GenBank acc. no. GQ452920.3). The symbol "S" followed by a number refers to the numbers of the investigated viral sample.

Sample No.	Native	Allele	Position in the PCR fragment	Position in the reference genome	Amino acid position	Type of mutation	Mutation summary
All	A	G	177	177	Ala43	Silent (p.Ala43=)	g.177 A>G
All	A	G	180	180	Leu44	Silent (p.Leu44=)	g.180 A>G
S1-S9	A	G	192	192	Thr48	Silent (p.Thr48=)	g.192 A>G
All	T	C	300	300	Tyr84	Silent (p.Tyr84=)	g.300 T>C
S7	T	A	427	427	Phe127	Missense (p.Phe127Ile)	g.427 T>A
All	C	T	594	594	Asn182	Silent (p.Asn182=)	g.594 C>T
All	C	T	666	666	Gly206	Silent (p.Gly206=)	g.666 C>T
All	C	T	751	751	His235	Missense (p.His235Tyr)	g.751 C>T
All	T	C	846	846	Ser266	Silent (p.Ser266=)	g.846 T>C
All	C	T	890	890	Thr281	Missense (p.Thr281Ile)	g.890 C>T
All	G	A	1009	1009	Ala321	Missense (p.Ala321Thr)	g.1009 G>A

A comprehensive phylogenetic tree was generated according to the amplified 1062 bp of the VP7 amplicons. This phylogenetic tree contained S1 to S10 samples alongside other relative nucleic acid sequences of rotavirus sequences. The total number of the aligned nucleic acid sequences in this comprehensive tree was 111. The rotavirus sequences

are the only incorporated organism within the tree. Noteworthy, the investigated samples were clustered into several adjacent clades within the rotavirus sequences, as shown in figure 3.

The genetic variations of the coding regions of the VP7 gene of rotavirus were further investigated using a neighbor joining - based phylogenetic analyses based on the versatile role of the protein encoded by the VP7 gene [24].
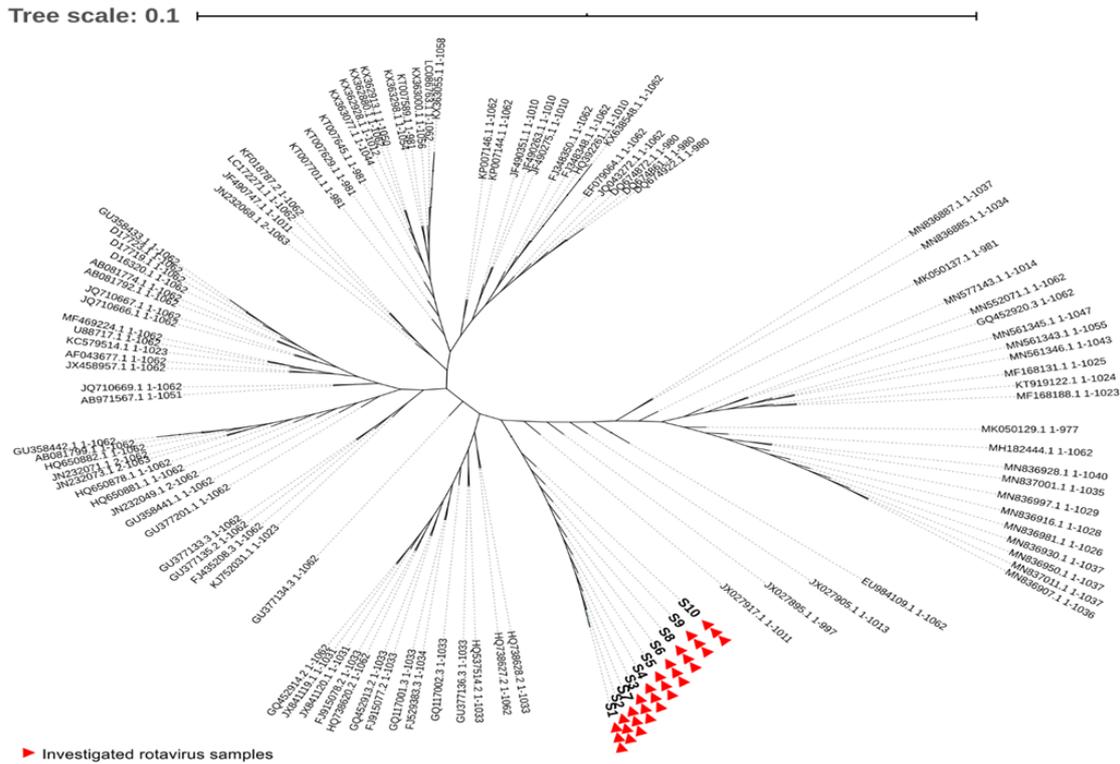
Figure 3 The comprehensive unrooted phylogenetic tree of genetic variants of the VP7 fragment of ten rotavirus local samples. The red triangles refer to the analyzed viral variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The letter "S#" refers to the code of the investigated samples.

The VP7 gene polymorphism is one of the main key factors in the determination of the rotavirus genotype. One genetic fragment covering the entire coding region of the VP7 gene was amplified in this study. Subsequently, a direct sequencing strategy was performed for the observed PCR amplicons to assess the pattern of genetic polymorphism in the screened specimens. Then, a specific comprehensive tree was built to assess the phylogenetic positions of the observed variants. The current results indicated the presence of eleven nucleic acid variations distributed in almost all the investigated samples. All investigated viral samples belonged to G1P[8] genotype. In conclusion, the utilization of VP7 gene polymorphism has precisely given uniform phylogenetic distances amongst all investigated rotavirus samples. These data may suggest no noticeable assortment events between human and animal rotavirus strains in the investigated area.

4. Discussion

In the present study, the seropositivity rate of anti-rotavirus IgM and anti-rotavirus IgG plus the fecal rotavirus Ag detection rate were significantly higher compared to their negative counterparts. The first impression these results had given was the high infection rate of rotavirus in Diyala community. These results are consistent with other local studies [25] as well as with those on the country level [26, 27]. However, the current study's rate was higher than the 40% detection rate of rotavirus stool Ag reported by another Iraqi study which covered three Iraqi cities [12]. Furthermore, these results are also consistent with other studies particularly those from developing countries [6, 28, 29]. Therefore, these results are not surprising in Diyala and probably in Iraq, as one of the developing countries for several reasons; first of these is the low standards of living, inferior standards of community hygiene and inadequate water and food sanitation besides the neglected and deteriorated Iraqi health care as a result of political, financial, and security

instability [30,31]. Another factor that may had contributed to increased rate of rotavirus infection in the community is the in-house breeding of productive domesticated animals since the zoonotic transmission of rotavirus has been well documented [32, 33]. Furthermore, human species of RVA strains that have a high degree of genetic homology and direct animal-to-human transmission can occur, particularly in low-income countries [34]. The situation is further aggravated by the presence of large numbers of refugees, and internally displaced people in Iraq who are suffering from even poorer general health conditions [35, 36].

In the present post-vaccination study, the children rotavirus RNA detection rate by PCR technique was 40.91%. In another study in Iraq which covered three cities during 2008 (pre-vaccination), a 70% detection rate of rotavirus RNA out of 98 stool samples submitted for multiplex PCR was recorded [12]. So, it is logical that the detection rate of rotavirus RNA among children less than 5 years old dropped down from 70% to 41%. It is really encouraging news, clearly ensuring the efficiency of rotavirus vaccine implementation. In spite of that, the protection rate of rotavirus vaccine was only 25.9% [37]. In other Eastern Mediterranean countries, studies found that the highest detection rate in the pre-vaccination era was reported from Iran (59.1%) [38], followed by that in Yemen (57.4%) [39] and then by that in Pakistan (57.3%) [40]. While the highest rate during the post-vaccination era was reported from Morocco (26.6% and 24%) [41, 42] followed by that in Yemen (18.5% and 10.54%) [43, 44] and Saudi Arabia (9.2%) [45]. Upon focusing on the nearest countries to Iraq, In Jordon the rotavirus detection rate was 49.5% among children below 5 years pre-vaccination [46]. In Saudi Arabia the pre-vaccination detection rate was 31.6% which thereafter dropped down to 9.2% post-vaccination [45, 47]. In Kuwait, the pre-vaccination rotavirus detection rate was 43.6% among children less than 5 years [48]. In Turkey, out of 329 stool samples analyzed, the rotavirus detection rate was 33.1% [16]. Accordingly, it is easy to interpret that the post-vaccination detection rate in Iraq is relatively higher compared to neighboring countries. This can be attributed to the unfavorable conditions during the last two decades represented by intermittent armed conflicts with partial or sometimes complete destruction of infrastructures, all that lead to deterioration of municipal, health and humanitarian services [30, 31, 35, 49].

Worldwide studies had obtained variable results, for instance, in post-vaccination cross sectional study in Nairobi, Kenya, the rotavirus RNA was detected in 60.1% by PCR technique [50]. In Spain, among children less than 5 years of age, 30.1% had symptomatic rotavirus infection despite the fact that they were vaccinated, while among unvaccinated children the rate was 69.9% [51]. In Côte d'Ivoire prior to rotavirus vaccine introduction, the stool Ag positivity rate by EIA was 34%, while using multiplex PCR yielded a positivity rate of 60.8% [52]. In India, four-year data on rotavirus gastroenteritis in hospitalized children < 5 years of age prior to vaccine introduction revealed that 35.5% were positive for rotavirus by EIA, and of these 97.1% were positive for rotavirus genotyping by RT-PCR [53]. It is clearly evident that the geographical location, standards of living and hygiene, food and water sanitation, the dominant circulating rotavirus genotypes plus vaccine implementation are playing a major in determining the prevalence of rotavirus infection [1, 54, 55].

In the present study, the PCR technique found that 40.9% of post-vaccination children were positive for rotavirus RNA. In Iraq, limited studies were conducted on genotyping of rotavirus. The present results are partially in agreement with a study that found that the PCR detection rate of rotavirus RNA was 40.0%. The most prevalent genotype was G2 (40%), most often associated with P[6]. G1 was the second most common genotype (16 %), mainly associated with P[8]. Furthermore, 5 G8P[6], and 7 G12 RVA strains in combination with P[6] and P[8] were detected for the first time in Iraq [12]. The current findings are also consistent with the main outcome of another study in Iraqi Kurdistan in 2005, that was conducted among children with acute diarrhea. The molecular detection showed that 38% were G1, 17% were G2, 20% were G4, and 11% were G9. A total of 11% were P[4], 15% were P[6], and 68% were P[8]. One showed mixed P[4] and P[8] genotypes and 3 (5%) were P non-typeable. Of 8 different P and G genotype combinations that were detected, the most common combinations were G1P[8] (33%), G4P[8] (21%), G2P[4] (11%), G1 P[6] (11%), and G9P[8] (11%) [13]. Based on the previous and present results, we can conclude that the main rotavirus genotypes circulating in Iraq showed minor or no change between pre- and post-vaccination periods.

The current results are totally concordant with those reported from the 13 Middle East countries that concluded that the G1P[8] was the most common circulating strain pre-vaccination [14]. The same findings were reported at the period immediately after vaccination, since G1P[8] was the most common strain in the 3 countries; Yemen [44], Morocco [41], and Saudi Arabia [45]. In Saudi Arabia, it has been found that G1P[4] was the most common (88%), followed by G1P[8] (61.9%) during pre-vaccination period [47]. Similar results were obtained by another study that found that G1P[8] was the dominant strain (44%) in KSA [56]. Additionally, the current results are consistent with the results of the one available study conducted after vaccination in Saudi Arabia that found that G1P[8] was the predominant strain [45].

Similarly, the rotavirus genotype obtained in this study is identical to that of 4 studies conducted in Morocco, 2 were pre-vaccination, one study was post-vaccination, and one compared the situation before and after vaccination. During

the pre-vaccination period, G1P[8] was the most common strain (57%,33%, and 55%) [57, 58] , and the same findings were found after introduction of vaccine (51.7% and 40.5%) [41,42]. In Morocco, the pre-vaccination rate of G1P[8] was the highest followed by G2P[4], G9P[8], G2P[6], G4P[8], and G3P[8]. After vaccination, G1P[8] continued to be the most frequent strain followed by G2P[4] and G4P[8]. One year later, G2P[4] strains were the most common, followed by G1P[8], and G9P[8]. Three years after vaccination, a huge change was reported in rotavirus strains. G1P[8] was not found, but G9P[8] and G2P[4] were found at 67% and 33%, respectively [41, 42]. Similar genotype distribution was reported from Yemen since 4 studies were conducted, 2 were before vaccination and 2 pre- and post-vaccination. The findings of pre-vaccination studied showed that G1P[8] was the most prevalent with a rate of 55% [43, 59] and 12% [39]. Another study showed that G2P[4] was the most prevalent (76.6%) followed by G1P[8] (45.5%), and then post-vaccination, it was reported that G1P[4] was the most prevalent (87.5%) followed by G9P[8] (57%) and G1P[8] (18.5%) [44]. Therefore, in Yemen, G2P[4] strain was the predominant type (55%), followed by G1P[8] (15%) post-vaccination. Furthermore, G1P[8] strain was the prevalent strain (31%), followed by G9P[8] (27.5%) [43]. Therefore, after introduction of rotavirus vaccination program, it was reported that the rates of strain combinations G1P[8], G2P[4], G9P[8], G12P[8], and G1P[8] dropped down from 51% to 37.1%, while G2P[4] increased from 21.6% to 33.3%18 [14].

The current study is totally consistent with the systematic review that included 27 studies in 20 African countries during the period of 2006-2016 among children less than 5 years of age, that revealed that the G1P[8] combination (22.64%) was the most encountered followed by G2P[4] (8.29%), G9P[8] (6.95%) and G2P[6] (5.00%), and that 65.70% of the circulating rotavirus strains prevalent in North Africa had the P[8] genotype [18].

The present molecular findings are also partially consistent with those reported by a study conducted in Spain that found that 30.1% of infants had symptomatic rotavirus infection despite the fact that they were previously vaccinated and the G9P[8], G12P[8] and G1P[8] were the most prevalent genotypes [51]. However, the current molecular outcome is inconsistent with the results from Kenya that found that the strains among the vaccinated were G3P[4], G12P[6], G3P[6], G9P[4], G mixed G9/3P[4] and G1/3P[4] indicating the diversity in circulating genotypes with emergence of new genotypes [50].

The present results are also consistent with those from India, that revealed that rotavirus genotypes G1P[8] (56.3%), G2P[4] (9.1%), G9P[4] (7.6%), G9P[8] (4.2%), and G12P[6] (3.7%) were the common genotypes in southern India and G1P[8] (36%), G9P[4] (11.4%), G2P[4] (11.2%), G12P[6] (8.4%), and G3P[8] (5.9%) in northern India, affirming the diversity of rotavirus genotypes across different geographical regions, signifying the importance of pre-vaccine surveillance to evaluate the potential change in circulating rotavirus genotypes after vaccine introduction [53].

In Turkey, G1P[8] was the dominant genotype combination (42.2%), followed by G9P[8] (21.1%) and G12P[6] (11.0%) [16]. In another study, G9P[8] was the most common G/P combination found in 40.5% of the strains followed by G1P[8] (21.6%), G2P[8] (9.3%), G2P[4] (6.5%), G3P[8] (3.5%), and finally, G4P[8] (3.4%). The rate of uncommon genotypes was 14%, suggesting high coverage of current rotavirus vaccines [17]. A study conducted in Iran found that 60.8% of fecal specimens contained only one rotavirus strain, 30.4% were mixed infections and 8.8% were non-typeable. The predominant single G/P combination was G1P[8] (57.82%), followed by, G2P[8] (16.98%), G2P[4] (1.72%), G3P[8] (4.69%), G4P[8] (3.13%) G8P[8] (3.13%), and G9P[8] (6.26%) [60]. Another molecular epidemiological survey revealed that G1P[8] is the predominant genotype in Iran, although other genotypes were also reported [61]. Other studies affirmed the predominance of G1P[8] genotype in Iran, with a rate of 59.2% [62], and 53.4% [63].

The current study suggests that a special focus should be directed towards the rotavirus genotypic changes that occurred in Turkey, Iran and Syria (unfortunately no data available online), since the Tigris and Euphrates arise and/or flow across their mainland and as it is well known that Iraqi people are largely dependent on these two rivers for irrigation and municipal supply. Of note, it was widely documented that human or animal sewage contaminated water is the main vehicle for rotavirus transmission [64, 65]. Additionally, during the last few years, active trading of different fresh or processed foods from these countries became much more common [66]. The current study suggests that collectively these sources may impact and alter the rotavirus epidemiology in Iraq.

Since this study is a part of a larger study including molecular detection of rotavirus among calves, another fascinating result was that the common rotavirus genotype G1P[8] was detected among both children as well as calves. Similar results were obtained from a Nigerian study which reported a human rotavirus in diarrheic calves aged 29-56 days, that was explained by the close association between the herdsmen and their animals and the sharing of a common source of drinking water in the predominantly livestock-producing communities [67]. Furthermore, it was reported that interspecies transmission between livestock, domestic animals and humans is commonly observed [68].

In Diyala community, the first impression of this result points to the common source of transmission of rotavirus infection in both human and calves. In this regard the most incriminated route is contaminated water. It is well documented that the drinking water is the primary source of rotavirus infection and outbreaks among human and animals particularly in low-income communities [64, 65]. It is important to remember that viral shedding with the stool in clinical or asymptomatic rotavirus infection continues for up to 57 days post exposure [7, 28, 69]. What is aggravating the situation in communities like that in Diyala is that the human and animal excreta return to the rivers successively as sewage treatment stations are absent and that the municipal water repumping stations are located on the same rivers. So, the epidemiological trajectory cycle of rotavirus passes through three stages; human or animals, excreta, water, and human or animals again [70, 71]. Additionally, Diyala province is an agricultural one and peoples are used to breeding domesticated animals in or near their houses. Therefore, the direct contact with these animals may hasten virus interspecies transmission [33, 72]. Furthermore, human and animal excreta are still being used as a fertilizer for vegetables and of course the most dangerous of these are those eaten fresh without cooking as a source of rotavirus infection [66, 73]. Eventually, even pet animals were proved to be able to transmit rotavirus to human [68, 74]. Moreover, in this regard, a study reported the detection of two rotavirus genotypes; G3P[9] and G6P[9] in cats, stressing that the first report of feline G6P[9] questions the previous belief that G6P[9] in people is of bovine origin [75]. In connection with interspecies transmission of rotavirus, it has been reported that genetic reassortants containing segments from a number of animal species and/or humans (interspecies transmission by reassorted animal rotaviruses) and viruses in which all segments were from the same animal species (interspecies transmission by non-reassorted animal rotaviruses). Molecular analysis has documented many more examples of interspecies transmission of genetic reassortants. Among the earliest documented reassortants were two G3P[3] human strains that were shown to be rotaviruses of canine/feline origin [74]. G6 and G10 are the predominant rotavirus serotypes in cattle in most but not all areas of the world. Bovine G6 strains were reported in many countries to be associated with P[1], P[5], and P[11] [76, 77]. Two G8P[1] strains were described as evidence of direct transmission of bovine rotaviruses to humans [78, 79]. Accordingly, the present study pointed out the importance of continuous surveillance studies to follow up any changes in local rotavirus genotype.

Another important result of sequence genotyping of the current G1P[8] rotavirus is the absence of reassortment. This result may be related to non-employment of bovine rotavirus vaccine for calves and recent introduction of human rotavirus vaccines (since 2014) in Iraq. Several molecular studies had reported genotypic changes of rotavirus as a result of vaccine pressure [50, 55, 80]. These rotavirus vaccines were designed to protect against disease caused by the most prevalent strain types; globally, G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12 in combination with P[6] or P[8] account for over 90% of the genotypes that infect humans [81]. Accordingly and based on the current rotavirus genotype G1P[8] detected in Diyala province, the current study is speculating that the rotavirus vaccine under use in Iraq (RV5; RotaTeq, Merck) is still protective for human vaccinees, as the currently used vaccine was designed to provide protection against the most common rotavirus serotypes (G1, G2, G3, G4, G9) and be able to decrease disease severity, reduce hospitalizations, and decrease disease-related costs [82].

5. Conclusion

All the investigated strains in this study belong to G1P[8] genotype, with no noticeable assortment events between human and animal rotavirus strains in Diyala province. Since the Tigris and Euphrates arise and/or flow across the mainland of Turkey, Iran and Syria, and Iraqi people are largely dependent on these two rivers for irrigation and municipal supply, special focus should be directed towards the rotavirus genotypic changes in these countries. Moreover, continuous follow-up of changes in rotavirus genotypes through molecular surveillance and vigilant system for persistent clarification of epidemiological changes and for monitoring rotavirus vaccine employment, should be exercised in Iraq.

Compliance with ethical standards

Disclosure of conflict of interest

Ammar Talib Nasser declares that there are no relevant financial or non-financial competing interests to report.

Abdulrazak Shafiq Hasan declares that there are no relevant financial or non-financial competing interests to report.

Amer Khazaal Saleh declares that there are no relevant financial or non-financial competing interests to report.

Mohammad Kassem Saleh declares that there are no relevant financial or non-financial competing interests to report.

Statement of ethical approval

This study was approved by the ethical committee in College of Medicine - Diyala University. Informed consent was obtained from all individual participants included in the study.

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