International Journal of
Enteric Pathogens

Int J Enteric Pathog. 2020 May;8(2):66-72

Original Article

http://enterpathog.abzums.ac.ir

doi 10.34172/ijep.2020.14

Phylogenetic Study of VP6 Gene of Bovine Rotavirus A and Molecular Survey of Bovine Rotaviruses B and C, and Human G and P Genotypes of Rotavirus A in Calves in Iran

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Published Online May 28, 2020

Keywords: Rotaviruses A, Rotaviruses B, Rotaviruses C, VP6 gene, Phylogenetic analysis, Genotyping

Abstract

Background: Rotavirus (RV) is one of the most important causes of diarrhea in the calf and human neonates. Rotaviruses are divided into nine different serogroups, of which group A is more important compared to other groups.

Objective: This study was performed because of the lack of information about the importance and prevalence of bovine rotaviruses B (RVB) and C (RVC) and human genotypes of rotavirus A (RVA) in the bovine population in Iran. Phylogenetic analysis of VP6 of bovine RVA was the second aim of the present study.

Materials and Methods: A total of 581 stool specimens were collected from diarrheic calves of 14 provinces and were analyzed by reverse transcription polymerase chain reaction (RT-PCR) and 485 of them were investigated by PAGE electrophoresis to determine the frequency of three rotaviruses A (RVA), B (RVB), and C (RVC). The presence of human G and P genotypes in Iranian bovine population was also evaluated using semi-nested multiplex RT-PCR.

Results: RVA was detected by RT-PCR (VP6 gene detection) in 16.2% (94/581) and by PAGE in 22.16% (108/485) and no positive cases of RVB and RVC were confirmed by polyacrylamide gel electrophoresis (PAGE). This study showed that non-A RV groups (B and C) have little role in calf diarrhea in Iran. The results of the phylogenetic study of VP6 sequences of rotaviruses A identified in this study showed that they all belonged to genotype I2 and were classified into three different branches. Specimen isolated in Zanjan showed the highest difference (maximum identity of 94%) with other sequences and clustered along with the Japanese strain, R22. Human G and P genotypes were not found in the studied samples.

Conclusion: The results showed that non-A rotaviruses and human genotypes of RVA are of little importance in calf rotavirus diarrhea in Iran. Also, there is the first phylogenetic study of rotavirus A VP6 protein in Iran.

Received Janaury 26, 2020; **Revised** May 12, 2020; **Accepted** May 21, 2020

Background

Rotaviruses (RVs) are enteric viruses that propagate in the gastrointestinal tract of many birds and mammals and cause diarrhea in human and animal infants. These viruses are classified in the genus Rotavirus and belong to the Reoviridae family. The genome of RVs consists of 11 segmented dsRNAs that are all encapsulated in a triple-layered non-enveloped capsid. The external layer of the viral capsid is composed of two capsomeres, VP7, and VP4 that carry the major neutralizing antigens of the virus. The middle layer consists of 260 copies of VP6 trimer, the most abundant protein in the RV particle. The inner layer of the virus consists of VP2.[1]

RVs are divided into nine serogroups named from A to I, based on the nucleotide sequence, antigenic characteristics of the VP6 protein, and the migration pattern of their genomic fragments in gel electrophoresis.[2] Rotavirus A (RVA) is the most important serogroup and it is the main cause of diarrhea in newborns of humans and calves. RVs group B (RVB) and C (RVC) are also important in humans and some animals. Other serogroups have rarely been detected in animals.[3] The importance of non-A RVs is less than group A; however, because of the existence of few studies about them, especially RVB and RVC, there is little information about their role in diarrhea.

RVB has been detected in humans, cattle, sheep, swine, ferrets, and rats.[3] In cattle, it has been detected in diarrhea samples of adults and calves. Seroepidemiological studies

indicate that RVB infection is common in cattle.[4,5] RVC has been reported as a diarrheal pathogen in humans, swine, and cattle.[3] It was first detected in diarrheic piglets, and then in humans, it was detected in sporadic cases and epidemic outbreaks of diarrhea in children and adults worldwide.[6-8] In cattle, RVC has been detected in adults suffering from diarrhea with reduced milk production in Japan. There is evidence that adult cattle are natural reservoirs of RVC.[6]

Although some reports indicated that suckling piglets may be infected with RVC alone or co-infected with RVA and RVB,[9,10] there are few reports on the importance of RVC and RVB alone or in co-infection with other viruses, especially RVA, in newborn calves. One of the objectives of the present study was to investigate the importance of the role of RVB and RVC in calf diarrhea in Iran.

RVA is classified into different genotypes based on the sequence characteristics of the viral genomic segments. Hence, based on the genomic characteristics of the outer layer proteins (VP7 and VP4) and the intermediate layer protein (VP6) of the viral capsid, these viruses are classified as G, P, and I genotypes, respectively.[1,11] The main neutralizing antibodies are developed against antigens of the two RVA surface proteins; therefore, G and P genotyping is an important issue in epidemiological studies of RVA. To date, 36 G and 51 P genotypes have been recognized. The most important bovine genotypes include G6, G10, and G8 in combination with P[11], P[5], and P[1] genotypes. Common human genotypes include G1-4 and G9 in combination with P [4], P [6], and P [8].[2]

Since some reports indicate that RVA can act as a potential zoonosis, numerous cases of pathogenicity of RVA in cattle and other animals have been reported that were isolated from diarrheic children[12-14]; As a complementary part of this study, fecal specimens of calves were examined using specific primers of common human G and P genotypes to give a better insight into the epidemiology of RVA in Iran.

The VP6 protein carries subgroup determining antigens and accordingly, RVA is divided into four subgroups SGI, SGII, SGI/II, and non-SGI/II. Previously, VP6 has not been considered to have an important role in inducing classical neutralizing antibodies. However, it now appears that the VP6 specific antibodies neutralize RVA particles during the transcytosis phase.[1] The genotypes derived from the genetic characteristics of the VP6 encoding gene are named as I genotype. To date, the Rotavirus Classification Workgroup (RCWG) has defined 26 different I genotypes.[15] Since there are no studies on the determination and phylogeny of I genotypes of bovine RVA in Iran, it was considered as a purpose of the present study to gather more information about the epidemiology and the origin of circulating RVAs in the bovine population of Iran. Iran is an Asian country with a very diverse climate, which occupies a large area of the Middle East and is surrounded with more than 12 neighboring countries. Therefore, obtaining more information about the epidemiology of RV infection across Iran can provide a good insight into the epidemiology of them in the Middle East and the information obtained from Iran can also be useful to neighboring countries.

Materials and Methods

Sampling

From industrial and non-industrial dairy farms located in 14 provinces of Iran, 581 stool specimens were collected from one-month-old calves suffered from diarrhea. All specimens were kept frozen at -35°C until the test. The samples were collected from Tehran, Alborz, Qazvin, Mazandaran, Golestan, Zanjan, East-Azarbaijan, Qom, Markazi, Isfahan, Fars, Khorasan-Razavi, South Khorasan, and Sistan and Baluchestan provinces.

RNA Extraction

To extract the viral dsRNA genome, 300 µL of 20% fecal suspension in PBS (v/v) was mixed with 700 µL of lysis buffer provided by the extraction Kit (Viral Gene-spin, iNtRON Biotechnology Co., Korea). The extraction process was carried out according to the manufacturer's instructions and finally, the extracted RNAs were eluted with 50 µL of elution buffer.

Polyacrylamide Gel Electrophoresis Assay

Polyacrylamide gel electrophoresis (PAGE) assay was used to detect the dsRNA fragments of RV according to the Laemmli method described previously[16] with some modifications. Briefly, 12 µL of the extracted dsRNA were mixed with bromophenol blue as the loading buffer and loaded in a 0.75 mm mini-gel cassette (Paya Pajouhesh Co., Iran) containing 4% spacer gel and 10% resolving gel and then was run with a constant voltage of 170 µV for 5.5 hours.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Multiplex RT-PCR

Some of RVA positive samples in PAGE assay were used as indicators to set up a multiplex RT-PCR for simultaneous detection of three RVs A, B, and C. For this purpose, a triplex RT-PCR assay using specific primers of all three viruses was performed according to the method described previously by Fukuda et al with some modifications.[17] Briefly, 2 µL of extracted dsRNA was mixed with 1 µL DMSO in 13.5 µL of double distilled water and denatured at 98°C for 5 minutes. Then, it was chilled immediately on ice. Afterwards, 5 µL of 5x buffer (Qiagen one-step RT-PCR kit) along with 1.5 µL of the mixture of primers (containing 4 µmol concentration of each primer) (Table 1), 1 µL dNTP, and 1 µL mixture of reverse transcription enzymes and Taq polymerase (Qiagen one-step RT-PCR kit) were added to the denatured dsRNA. Following the reverse transcription step, the PCR assay consisting of 35

Table 1. Primers Used for Multiplex and Single RT-PCR Assays

RV Group	Target Gene	Primer	Sequence (5'-3')	Size of Product (bp)	Ref
RVA	VP6	GEN-VP6F	GGCTTTWAAACGAAGTCTTC	928	17
		GAR VP6-928R	GGYGTCATATTYGGTGG		
RVB	VP7	9B3	CAGTAACTCTATCCTTTTACC	281	18
		9B4	CGTATCGCAATACAATCCG		
RVC	VP6	ShintokuVP6-370F	ATCGCATTAGCTTCATCAAA	563	17
		ShintokuVP6-933R	CTGTACATACTGGGTCATAGC		

cycles of 94°C for 45 seconds, 52°C for 45 seconds, and 72°C for 60 seconds was performed. Final elongation was performed at 72°C for 10 minutes. All the RT-PCR products in the present study were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining.

RT-PCR to Detect the RVB

Following the multiplex RT-PCR and PAGE, specimens suspected to be RVB positive along with 200 randomly selected samples from Khorasan-Razavi, Sistan and Baluchestan, Mazandaran, Tehran, Qazvin, Alborz, Isfahan, and Fars provinces were tested again by the method discussed previously.[18] Briefly, after reverse transcription reaction which was performed as described above with the same material, the PCR assay was performed using the primers 9B3 and 9B4 (1 µL of each) over 35 cycles of 95°C for 1 minute, 52°C for 1 minute, and 72°C 45 seconds. Final elongation was performed at 72°C for 10 minutes.

RT-PCR to Detect the RVC

Similarly, all specimens suspected of the presence of RVC in multiplex RT-PCR and PAGE together with 200 specimens described above were examined with a single RT-PCR assay to detect RVC. To this end, specific primers of RVC (Table 1) were used. The single RT-PCR assay was the same as the multiplex RT-PCR assay, with a minor modification in which only 2 µL of the specific primers of RVC (ShintokuVP6-370F/ShintokuVP6-933R) was added to the reaction instead of the mixture of the three virus-specific primers.

Sequence Analysis

Among the PCR products obtained from each of the reactions mentioned above, 24 samples that showed appropriate and strong bands in agar gel electrophoresis were selected for direct nucleotide sequencing (Bioneer Co., South Korea) using specific forward and reverse primers. The obtained sequences after identification using BLASTN and edition by BioEdit software were subjected to phylogenic analysis by the neighbor-joining (NJ) method with Tamura 3-parameter method and the rate variation with gamma distribution and 1000 bootstrap replicates using MEGA5 software.[19] I genotypes of bovine RVA were determined using the RotaC 2.0 web-based tool (http://www.regatools.be/rota20/). To determine the occurrence of intramolecular recombination, the obtained sequences were compared with sequences deposited in the GenBank database using RDP4 software.

Results

PAGE Results

In PAGE assay, genomic RNA fragments of RV were detected in 116 samples (23.9%), all of which belonged to RVA. Non-A RVs (RVB and RVC) were not detected (Table 2). All of RVA electrophoretypes belonged to the long pattern. At least four electrophoretypes were identified in terms of the mobility of the four primary and three intermediate bands of RVA genome fragments. No abnormal movement of genome fragments was observed in PAGE. Co-infection with two or more different electrophoretypes was not observed in any sample.

RT-PCR Investigation and Sequencing

Of the 581 samples analyzed by multiplex RT-PCR, 103, 32, and 5 showed the expected band of RVA, RVB, and RVC in agar gel electrophoresis, respectively (Table 2). In the single RT-PCR assay performed to individually detect RVB and RVC using their specific primers, both were negative and no RVB and RVC were detected. The sequencing results of the RVA PCR products showed that they all belonged to the VP6 gene of this virus, but the multiplex RT-PCR products sequenced for RVB and RVC were all negative. In the part of the investigation of human G and P genotypes in diarrheic calves, two specimens showed expected bands of G4 in agar gel electrophoresis. However, as mentioned in the next section, sequencing results showed that they belonged to bovine G10. The rest of the samples showed no bands corresponding to human G and P genotypes.

The sequences obtained in this study were deposited in GenBank with accession numbers MH424424.1, MH424423.1, MH424422.1, MH424421.1, MH424420.1, and MH424419.1 for the VP6 and MH424425.1 and MH424426.1 for the VP7 genes of RVA.

I Genotyping and Phylogenetic Analysis of VP6

The results of the analysis of sequences of the VP6 gene

Table 2. The Frequency of RVA, RVB, and RVC Detected by Multiplex RT-PCR and PAGE in Different Provinces of Iran

	RVA		RVB		RVC	
	M RT-PCR +/All	PAGE +/All	M RT-PCR +/all	PAGE +/All	M RT-PCR +/All	PAGE +/all
Tehran, Alborz, Qazvin	16/108	20/90	10/90	0	1/108	0
Razavi-Khorasan	16/88	17/70	1/88	0	0/88	0
South Khorasan	4/25	4/20	1/25	0	0/25	0
Sistan and Baluchestan	1/29	1/25	0/29	0	1/29	0
East-Azarbaijan	16/57	19/49	4/57	0	0/57	0
Zanjan	3/42	9/35	2/42	0	2/42	0
Markazi	19/51	12/45	7/51	0	0/51	0
Isfahan, Qom	8/59	18/50	3/59	0	0/59	0
Fars	12/62	11/50	4/62	0	1/62	0
Mazandaran	9/43	6/36	0/43	0	0/43	0
Golestan	0/17	0/15	0/17	0	0/17	0
TOTAL	103/581	116/485	32/581	0/485	5/581	0/485

of RVA using the RotaC web-based tool revealed that they all belong to genotype I2. Phylogenetic studies showed that these sequences, although constructed three distinct branches, were all clustered alongside bovine RV strains isolated in Thailand (e.g., 61A and 22R). They also resembled some isolates from Turkey (a neighboring country in northwestern Iran). This phylogenetic similarity with Turkish strains was particularly evident in the isolate that had been collected from East Azarbaijan province (located in northwestern Iran). The isolates from Fars and Tehran provinces were phylogenetically related to each other as well as to isolates from Khorasan-Razavi and East-Azarbaijan provinces. The strain isolated in Zanjan was less phylogenetically related to the other four strains and showed more phylogenetic similarity with the strains isolated in Thailand (e.g., 22R). The sequences isolated in this study showed a little similarity with the major bovine RVA strains (including B223, UK, and NCDV) and did not cluster alongside them (Figure 1).

The phylogenetic tree was constructed using MEGA 5 software based on the Neighbor-Joining method with the bootstrap test (1000 replicates). The evolutionary distances were computed using the Tamura 3-parameter method and the rate variation among sites was modeled with gamma distribution. All positions containing gaps and missing data were eliminated and there were a total of 879 positions in the final dataset. The sequences obtained in the present study are marked by black circles.

Discussion

Diarrhea in newborn calves is one of the major problems in dairy farms. Diarrhea, in addition to accounting for more than 50% of the mortality in suckling calves, indirectly causes economic losses to the farmer.[20-23] Many studies have shown that RVA is one of the major causes of diarrhea around the world with a prevalence ranging from 9% to 93.7%.[12,24] Several studies have been conducted in Iran to determine the prevalence of RVA in calf diarrhea. Mayameei et al reported that the prevalence of RVA in Khorasan-Razavi province was 26.9% using ELISA. Additionally, Madadgar et al in a study reported that the RVA prevalence in 11 provinces of Iran was 28.2% using the same method.[25,26] In another study by Nourmohammadzadeh et al, the prevalence of RVA was reported to be 48% in winter and 16% in summer. In other words, RV was found to be present in 34% of cases using electron microscopy.[27] However, the prevalence of RVA in Iran has also been investigated by RT-PCR, which was estimated to be 49.4% by Pourasgari et al.[28] As can be seen from the above-mentioned results, the prevalence of RVA can vary depending on the season, the geographical area, and the sensitivity of the detection method.

In the present study, the prevalence of bovine RVA in the 581 fecal samples of one-month-old calves suffering from diarrhea was investigated using two methods in 14 different provinces of Iran. The frequency of detected RVA was not the same in all provinces and geographical regions. However, in total, using multiplex RT-PCR assays to simultaneously detect the three RVs A, B, and C, RVA was seen in 103 out of 581 samples (17.72%) and using PAGE in 116 out of 485 samples (23.9%). In other words, the PAGE test was more sensitive than the PCR method used in this study. Since PAGE is economically less expensive than RT-PCR and ELISA, it is still used as an appropriate method to detect RV infections in stool samples.

In East Azarbaijan and Markazi provinces with a cold and relatively humid climate, the samples were all collected from large industrial dairy farms, and the prevalence of RV using PAGE and multiplex RT-PCR was reported to be much higher than the prevalence obtained from all samples in this study.

For example, the prevalence of RVB in stool samples

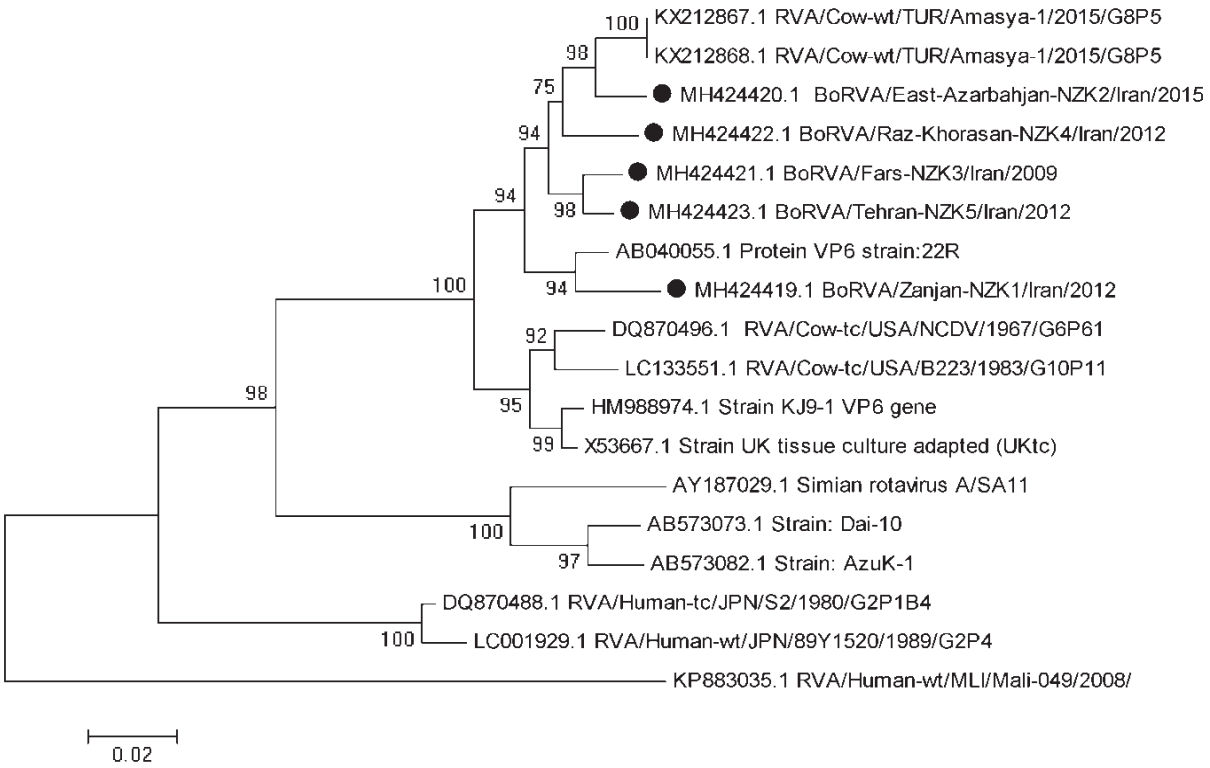

Figure 1. The Evolutionary History of the Nucleotide Sequence of the VP6 Gene of bovine RVA circulating in Iran.

in Germany[10] and the United States[4] in some studies was reported to range from 3 and 5.6%, respectively, while in another study in California State, which used a similar method and primers to our study, the prevalence of RVB in stool samples was reported to be very high (81%).[18] In contrast with the results of the study by Chinsangaram et al[18] in California, the prevalence of RVB in other parts of the world does not appear to be high. The prevalence of RVB in Japan and the United States is reported to be about 10% to 11%.[29] Little is known about the epidemiology of bovine RVC strains. In a study by Otto et al, its presence was reported in 6% of cattle using real-time RT-PCR.[10] However, some studies indicated that RVC can be considered as a potential zoonosis.

Since there is no commercial method to detect RVB and RVC, all samples were first screened for the presence of their genomic dsRNA fragments using PAGE. As mentioned above, it was interesting to detect RVA in 116 samples, while none of the samples were positive for RVB and C. It should be noted that the PAGE assay as a method for detecting RVs is highly specific although its sensitivity is relatively low. Therefore, when the amount of virus (genomic dsRNA) in the sample is very high (10^6 or more copies of the genomic molecule), PAGE assay is able to detect RVs. Moreover, the rate of RVB shedding is even lower than that of RVA in cases of diarrhea so its concentration in the feces is lower.[18] Therefore, to confirm the PAGE results, all of 581 samples were re-examined by RT-PCR to detect RVs B and C and as mentioned above, a multiplex RT-PCR assay including primers of A, B, and C RVs was first performed. RVA was used as a marker and a positive control indicator to set up the reaction and to ensure the accuracy of the PCR results. Besides high-quality material, the primers used in this reaction to detect RVs A, B, and C were derived from well-referenced studies.[17,18]

The sequencing revealed that unlike the results of the multiplex RT-PCR test, none of 32 and 5 positive samples were positive for RVB and RVC, respectively, even with repeated examinations. Since the results showed that the sensitivity of the multiplex RT-PCR method in detection of RVA was lower than that of the PAGE, all positive samples of multiplex RT-PCR assay for RVC and RVB along with randomly selected 200 samples were rechecked separately using single RT-PCR and all samples were negative. Based on the results obtained from PAGE, RT-PCR, and sequencing, it can be concluded that unlike RVA which circulates with high frequency in Iranian cattle, the prevalence of both RVB and RVC is probably very low and these viruses do not cause many health problems for dairy cattle in Iran. To determine the prevalence of RVB and RVC in Iran more precisely, given the low prevalence of them, the use of a larger sample size as well as more sensitive methods is recommended.

Survey of the prevalence of human G and P genotypes of RVA in the bovine population of Iran was another objective of this study, which was performed according to standard methods recommended by WHO[30] and

indicated the absence of these genotypes in the studied samples. The aim of this part of the study was to further clarify the status of RVA infections in Iran and to get more information about its epidemiology. As shown in the results section, two samples in multiplex semi-nested RT-PCR assay produced expected human G4 genotype bands in agar gel electrophoresis. However, sequencing results showed that these PCR products belonged to the G10 genotype of bovine RV. It can be due to the mispriming especially in the second round of semi-nested multiplex PCR in which the annealing temperature was as low as 42°C. Another reason could be the occurrence of mutations or genetic recombination in priming sites of the viral genome.[31,32] However, phylogenetic and recombination analysis rejected the latter reason, as it indicated that these two sequences were completely similar to sequences previously recorded in Tehran and Khorasan-Razavi in G10 genotype (results not shown). Besides, no evidence of molecular recombination was seen in the analysis of the obtained VP7 and VP6 sequences using RDP4 software.

Phylogenetic analysis of VP6 gene of the RVA sequenced in this study showed that all of them belonged to genotype I2. This study is the first report on I genotyping of bovine RVA in Iran. Earlier studies indicated that G6, G10, P [11], and P [5] are the most abundant G and P genotypes of bovine RVA and that there is no evidence for the existence of G8 and P[1] genotypes in Iran.[25,28,33,34]

There are fewer studies about I genotyping compared to the G and P genotypes of bovine RVA in the world. The results of these studies show that I2 is the most common I genotype in bovine RVA and almost all of the major strains of bovine RVA (e.g., UK, NCDV, and B223) possess I2 genotype.[35] In the phylogenetic analysis, I2 genotypes are clustered into different clades. One of these clades contains most of the bovine I2 genotypes, including important bovine RVA strains (NCDV, B223, and UK) which, despite being different in terms of G and P genotypes, are phylogenetically related in their I2 genotype and clustered together. However, East Asian strains such as 22R and A61 showed little similarity with NCDV, B223, and UK and constructed a separate cluster.

Phylogenetic analysis showed that all sequences of this study were clustered alongside the 22R strain despite being isolated in different geographical regions of Iran. The strain isolated in Zanjan province showed the highest similarity with the 22R strain. Sequences of Azarbaijan and Khorasan-Razavi provinces showed high similarity and were placed next to Turkish isolates (access number KX212868, Amasy a-2). Sequences of Tehran and Fars provinces were also closely related.

There is little information about the I genotype of human RVA circulating in Iran but only two sequences of human VP6 have been deposited by Iranian researchers in GenBank with accession No. KF219615 and KF219616 (Unpublished data), and the analysis by RotaC showed that they belong to I2 and unlike the I2 genotypes determined in this study, phylogenetically related to bovine NCDV strain (with 100% identity). No further information on these isolates has been published yet, but observing these bovine genotypes in the human population of Iran indicates the breakdown of host barriers and transmission of bovine RVA to the human or the occurrence of reassortment. As noted earlier, the presence of human genotypes and the reassortment of the human G and P with bovine RVA were not observed in the present study.

Three subjects were studied in this study: (1) the epidemiology and importance of RVB and RVC, two of the most important non-A RVs, (2) the determination of genotype I and phylogenetic analysis of the VP6 gene of bovine RVA, and (3) the presence of G and P genotypes of human RVA in the feces of diarrheic calves. The results showed that bovine RVB and RVC, as well as human RVA genotypes, are of little importance in causing calf diarrhea in Iran. However, the results of the phylogenetic study of the VP6 gene in the present study in comparison with previous results from genotype I2 in the human population show that the epidemiology of RVA infection in Iran is complicated. Therefore, every few years, further studies and examinations on both bovine and human populations are needed to obtain new insights about its epidemiology and biology.

Authors' Contributions

The present study was based on the substantive intellectual contribution of the corresponding author, Dr. Ahmad Nazaktabar.

Ethical Approval

The study was performed in a laboratory and did not interfere with ethical considerations.

Conflict of Interest Disclosures

There are no competing interests to declare.

Financial Support

This study was supported by Research Deputy of Ministry of Science and Technology and Amol University of Special Modern Technologies, Amol, Iran

Acknowledgments

This study was supported by the Research Deputy of Ministry of Science and Technology and Amol University of Special Modern Technologies, Amol, Iran.

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