

Original Article

Molecular Characterization and Phylogenetic Analysis of the G6 and G10 Genotypes of Bovine Rotaviruses from Iran

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Abstract

Background and Aims: Rotaviruses are the main cause of gastroenteritis in the young of human and a variety of animals in the world. Rotavirus G6 and G10 are the most prevalent genotypes in cattle worldwide, but are also found in human with possible origination from animals.

Materials and Methods: In this work, we have detected the G6 and G10 genotypes of bovine rotaviruses isolated from diarrheic calves in Tehran province by RT-PCR. The amplified VP7 genes were cloned, sequenced and compared to the sequences deposited in the GenBank database from the world.

Results: The phylogenetic analysis showed that the Iranian G6 and G10 genotypes are most identical to the genotypes from Ireland (>98.6% amino acid homology) and Canada (>97.3% amino acid homology), respectively.

Conclusion: This high similarity between the Iranian sequences and those from the far countries may suggest that the virus can be spread in a wide geographic region possibly by animal transportation.

Keywords: Bovine rotaviruses; VP7 molecular analysis; Iran

Introduction

Group A rotavirus (RVA) is recognized reportedly as the most common viral cause of severe gastroenteritis associated with significant morbidity, mortality, and economic burden among newborn calves worldwide (1). Symptoms such as anorexia, immobility, and eventually severe diarrhea are evident in young calves between 1-4 weeks (1-3). Bovine rotaviruses

are prevalent in both dairy and beef cattle around the world and are major cause of loss of productivity and loss of bulk animal protein sources. According to the studies, the economic damage caused by diarrhea in calves is primarily related to the rotaviruses (4).

Rotaviruses belong to the family of *Reoviridae* and have seven groups based on protein VP6.

Its genome comprises of 11 double-stranded RNA segments that generate six structural proteins (VP1, VP2, VP3, VP4, and VP6 & VP7) and six nonstructural proteins (NSP1-6) that play at the replication cycle. The outer protein coat is made of VP4 and VP7 proteins which elicit neutralizing antibody responses, and form the basis of dual classification system of G (VP7) and P (VP4) types. VP7 is the

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major neutralization antigen and is involved in attachment and the cell entry process (5). Given that vaccination is the best way to prevent rotavirus gastroenteritis, these two proteins produce criteria for making vaccines against rotavirus (6). To date, 16 G types have been described for group A rotaviruses and G6, G8 and G10 are the most common G genotypes identified in cattle worldwide (7, 8). It is important to know the profile of epidemiologic distribution of the genotypes in the area and to detect the possible interspecies transmission. In addition, study of the G and P types of group A rotaviruses is an absolute requirement for the development of a potent vaccine against the virus.

Bovine rotaviruses have been studied in Iran (9, 10) but this is the first report confirming the existence of G6 and G10 serotypes in the country and giving a picture of their divergence with human and animal rotaviruses around the world at both nucleotide and amino acid levels. These data are valuable and prerequisite for better understanding the molecular epidemiology of rotaviruses in Iran and application of the most potent vaccine.

Methods

Samples

The samples belonged to under one-month-old calves with diarrhea from dairy farms around Tehran during 2010-2011. These samples had previously been found to be positive by ELISA and PCR for VP6 gene.

RNA extraction

Viral RNA was extracted from fecal samples using QIAzol solution (Qiagen) according to the manufacturer's protocol. Precipitated RNA was solved in RNase free water. The purified RNA was subjected to reverse transcription (RT).

cDNA synthesis

Total cDNA synthesis was carried out by RevertAid Reverse TranscriptaseTM (Fermentas). The extracted RNA and random hexamer primer were heated at 95 °C for 5 min and cooled on ice, then 5X Reaction buffer (4 µl), RiboLock RNase Inhibitor 1 µl (20 U), 2 µl of 10 mM dNTP Mix, RevertAid Reverse

Transcriptase 1 µl (200 U) were added and the reaction reached to the final volume of 20 µl with DEPC water. The mixture was put in a thermocycler at 25 °C for 5 min, followed by 42 °C for 60 min and 72 °C for 5 min.

PCR amplification of VP7 gene

The PCR mixture contained 2.5 µl of 10 X buffers, 1 µl dNTP (5 mM), 1 µl MgCL₂ (50mM), 1µl cDNA, 1 µl (10 pmol) of each primer, VP7-Forward (5' - ATG TAT GTT ATT GAA TAT ACC AC- 3') at position 51-71 and VP7-Reverse (5' - AAC TTG CCA CCA TTT TTT CC- 3') at position 914-933 and 1 unit (0.2 µl) of Taq DNA polymerase (Amplicon) in a total volume of 25 µl. The PCR cycling program was started with initial denaturation at 95°C for 5 min then followed by 35 cycles of 95°C for 60 seconds, 52°C for 60 seconds, 72°C for 60 seconds and, a final extension at 72°C for 10 minutes. PCR products were run on agarose gel. Samples were identified as positive based on the presence of the expected amplicon (880 bp) with ethidium bromide staining. The PCR products were purified with RBC Bioscience column according to the manufacturer's instruction.

Cloning of VP7 gene

Cloning was performed by InsTAclone PCR Cloning Kit according to the manufacturer's instruction (Thermo Scientific) to construct pTZ57-VP7 plasmids. The constructs were digested with BamHI and HindIII enzyme and the digested products were run on the agarose gel to check their correct bands size

Sequencing and phylogenetic analysis

Both plasmids with VP7 genes insert were subjected to sequencing by M13 forward and reverse primers. The resulted nucleotide sequence data were compared with the corresponding rotavirus sequences from the GenBank database. Multiple sequence alignments were performed using the MEGA.5 and BioEdit 7.0.5 software. Phylogenetic analyses were conducted using the neighbor-joining method and the Kimura-2 parameter using MEGA5 software (11). The reliability of the branching was evaluated by the bootstrap test with 100 replicates (12).

Results

Amplification and cloning of rotavirus VP7 gene

The VP7 genes of isolates were successfully amplified by RT-PCR. The amplified genes showed the expected size about 880 bp (Fig.1). The amplified VP7 cDNA was cloned into the polylinker site of pTZ57R/T cloning vector and the resulted plasmid named as pTZ57-G6 and pTZ57-G10

Sequencing and phylogenetic analyses

Using BLAST, the sequences of the both genotypes (G6 and G10) obtained were confirmed and compared to the sequences available in the GenBank database. The resulted G6 and G10 genotypes sequences were also deposited in the GenBank database with the Accession numbers of KM243015 and KM243016, respectively. The sequences of this study were aligned with the the representative of sequences from human and other animals, and phylogenetic analysis was performed to draw the most possible tree. According to the phylogenetic tree, all the G6 genotypes were grouped into four distinct lineages; the lineage I consists of only bovine isolates in that the Iranian and Irish isolates are the most identical, the lineage II shows host diversity, the lineage III includes only the human isolates and the lineage IV consists of both bovine and human isolates. Also the Iranian G10 genotype exhibits the highest similarity with the sequence submitted from

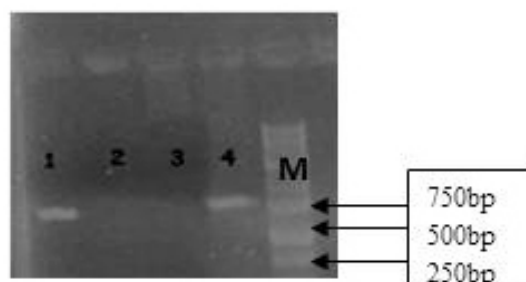
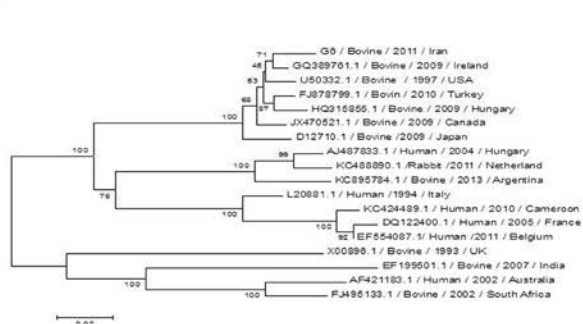


Fig. 1. PCR Product of VP7 gene (883bp) on 1% agarose gel.

Canada, however except the human sequences available from Africa, no specific lineage can be considered as all closely related isolates are bovine and human within the branches (Fig. 2). Additionally, the amino acid sequences of the Iranian G6 and G10 isolates (295 aa) were aligned to the corresponding amino acid sequences using BioEdit software. Comparison of the Iranian G6 amino acids with that of Ireland indicated that they are extremely similar with 98.6% amino acid homology and three amino acid substitutions including M29I, A225V, V268I occurred within the conserved regions. Also, alignment of the Iranian G10 amino acids with the Canadian isolate showed that they are closely related (>97.3 % amino acid homology) with eight amino acid substitutions including I16T, I55V, S66T, T71A, I87T, N147S, A181T and Q189R while two changes identified in antigenic region A (at position 87) and B (position 147), respectively (Fig. 3).

A



B

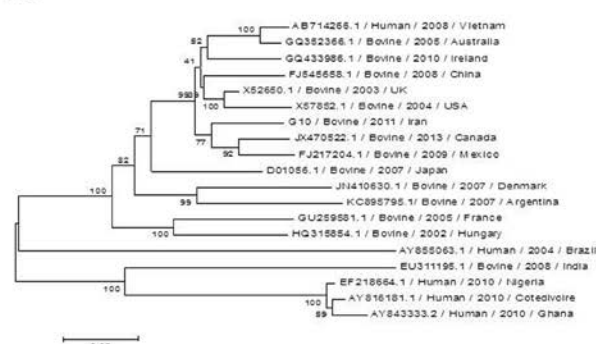


Fig. 2. Phylogenetic tree based on the VP7 genes of the Iranian bovine rotavirus strains and globally distributed strains, G6 (A) and G10 (B). The trees were drawn by nucleotide alignment using neighbor-joining method and Kimura-2 parameter.

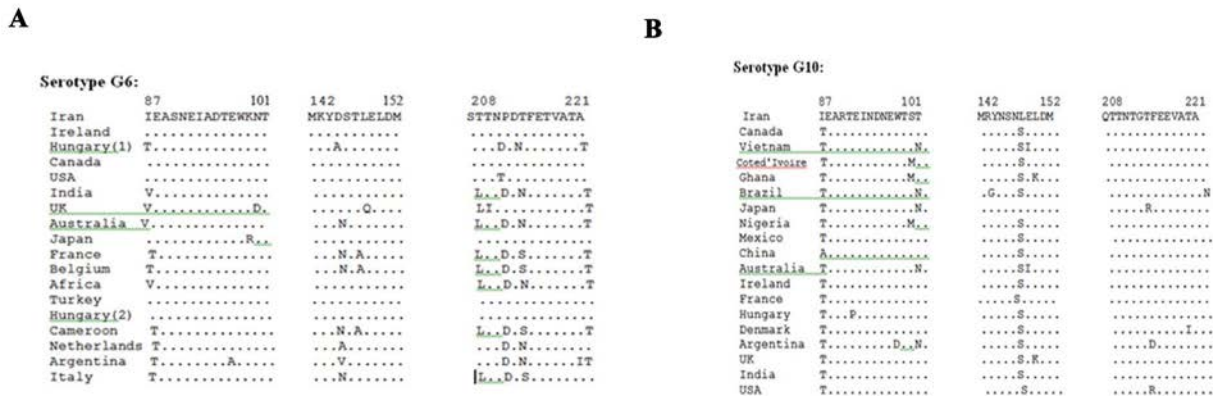


Fig. 3. Alignment of the Iranian bovine G6 (A) and G10 (B) amino acids sequences with those of bovine and human origins from the world. The antigenic regions A (amino acids 87-101), B (aa 142-152) and C (aa 208-221) are indicated (Hoshino et al.,1994).

Discussion

RVA is the major cause of gastroenteritis in cattle, being responsible for economic losses in terms of death, reduction of weight gain and treatment cost (13). There is a great potential of diversity for rotaviruses due to the wide range of hosts and different mechanism of viral evolution including point mutation, genomic reassortment and rearrangement (14). The presence of rotaviruses in livestock has also epidemiological importance in human health as the virus has been found in human with animal origin, interspecies transmission and genomic reassortment are the possible cause of viral transmission between human and animals (7, 15, 16). Epidemiological studies have shown that the group A rotaviruses affecting calves belong to G1, G6, G8, G10 and G15 serotypes as the G6, and G10 serotypes are the most common, being reported in different part of the world as well as Iran (7, 9, 10). However, identification of the amino acid change in antigenic site of the VP7 protein within a serotype have been observed and explained to affect specific neutralizing antibodies (17).

We tried to find the differences of the two sequences of Iranian genotypes (G6 and G10) with the sequences from the world. Phylogenetic analysis was performed on these genotypes which were isolated from diarrheal

calves in industrial dairy cows around Tehran. Phylogenetic analyses of the G6 genotype showed presence of 4 lineages, the Iranian G6 with the highly identical Irish isolates and other bovine isolates are clustered in the lineage I. Except the lineage III which includes only the human isolates, the other two lineages comprise of human and animal isolates. Also, G10 genotype sequence analysis revealed that the Iranian isolate has the maximum identity with the bovine isolate from Canada but except the human sequences registered from Africa, no special geographic distribution can be considered. Finding the highly similar G6 and G10 sequences among different hosts can be resulted from the interspecies transmission or genetic reassortment of rotaviruses as explained previously (7, 14, 15, 18). Additionally, identification of the closely similar sequences from quite far countries may imply the worldwide distribution of these viral genotypes over the world possibly by animal transportation.

Comparison of the amino acid sequences of the G6 genotype indicated that there are three amino acid substitutions between the isolate from Iran and that from Ireland which are not located in the antigenic regions of A, B and C but diversities between the Iranian isolate and those from other parts of are observed. Furthermore, alignment of the G10 amino acid sequences revealed that again differences exist among the sequences from the world and the

two identical Iranian and Canadian isolates have eight substitution as two of them are located in the neutralization domains; amino acids 87(I87T) and 147(N147S). These results, together, with data available from previous studies again emphasize the divergence of a specific rotavirus genotype and their potential to cross host barriers. To obtain the more accurate data about the variation of the segmented rotavirus genome, regular monitoring of the whole rotavirus genome among different host across the continents is necessary (15). This requirement will definitely rise when live vaccines are continuously administered in more countries (7) and novel rotavirus strains will overcome the predominant immunity provided by vaccine (3, 7). The results of the present study provide essential molecular evidence for the most antigenic VP7 protein; however to define the complete molecular epidemiology of the viruses circulating in Iran, monitoring other parts of the country and identification of the whole genome of these viruses, especially VP4, are recommended.

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References

- Holland RE. Some infectious causes of diarrhea in young farm animals. *Clinical Microbiology Reviews*. 1990;3(4):345.
- Pisanelli G, Martella V, Pagnini U, De Martino L, Lorusso E, Iovane G, et al. Distribution of G (VP7) and P (VP4) genotypes in buffalo group A rotaviruses isolated in Southern Italy. *Veterinary microbiology*. 2005;110(1):1-6.
- Rathi R, Kadian S, Khurana B, Grover Y, Gulati B. Evaluation of immune response to bovine rotavirus following oral and intraperitoneal inoculation in mice. *Indian journal of experimental biology*. 2007;45(2):212.
- Dhama K, Chauhan R, Mahendran M, Malik S. Rotavirus diarrhea in bovines and other domestic animals. *Veterinary research communications*. 2009;33(1):1-23.
- Papp H, László B, Jakab F, Ganesh B, De Grazia S, Matthijnssens J, et al. Review of group A rotavirus strains reported in swine and cattle. *Veterinary microbiology*. 2013;165(3):190-9.
- Agrawal D, Singh N, Chauhan R. Colostral antibodies against rotavirus infection in neonatal calves. *Journal of Immunology and Immunopathology*. 2002;4(1and2):107-9.
- Armah GE, Hoshino Y, Santos N, Binka F, Damanka S, Adjei R, et al. The global spread of rotavirus G10 strains: Detection in Ghanaian children hospitalized with diarrhea. *Journal of Infectious Diseases*. 2010;202(Supplement 1):S231-S8.
- Garaicoechea L, Bok K, Jones L, Combessies G, Odeon A, Fernandez F, et al. Molecular characterization of bovine rotavirus circulating in beef and dairy herds in Argentina during a 10-year period (1994–2003). *Veterinary microbiology*. 2006;118(1):1-11.
- Shoja Z, Tagliamonte M, Jalilvand S, Ziaee A-A, Mokhtari-Azad T, Hamkar R, et al. Molecular characterization analysis of the outer protein layer (VP7) from human rotavirus A genotype G1 isolate identified in Iran: Implications for vaccine development. *Microbiologica-Quarterly Journal of Microbiological Sciences*. 2012;35(4):415.
- Mayameii A, Shapouri M, Ghorbanpour M, Hajikolaei M, Keyvanfar H. Molecular G typing of bovine rotaviruses in Iran. *Pakistan journal of biological sciences: PJBS*. 2007;10(19):3466-9.
- Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution*. 1980;16(2):111-20.
- Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 1985;783-91.
- Martella V, Bányai K, Lorusso E, Bellacicco AL, Decaro N, Camero M, et al. Prevalence of group C rotaviruses in weaning and post-weaning pigs with enteritis. *Veterinary microbiology*. 2007;123(1):26-33.
- Tsugawa T, Hoshino Y. Whole genome sequence and phylogenetic analyses reveal human rotavirus G3P [3] strains Ro1845 and HCR3A are examples of direct virion transmission of canine/feline rotaviruses to humans. *Virology*. 2008;380(2):344-53.
- Abe M, Ito N, Morikawa S, Takasu M, Murase T, Kawashima T, et al. Molecular epidemiology of rotaviruses among healthy calves in Japan: isolation of a novel bovine rotavirus bearing new P and G genotypes. *Virus research*. 2009;144(1):250-7.

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16. Gerna G, Steele AD, Hoshino Y, Sereno M, Garcia D, Sarasini A, et al. A comparison of the VP7 gene sequences of human and bovine rotaviruses. *Journal of General Virology*. 1994;75(7):1781-4.

17. Hoshino Y, Nishikawa K, Benfield DA, Gorziglia M. Mapping of antigenic sites involved in serotype-cross-reactive neutralization on group

A rotavirus outer capsid glycoprotein VP7. *Virology*. 1994;199(1):233-7.

18. Cooney M, Gorrell R, Palombo E. Characterisation and phylogenetic analysis of the VP7 proteins of serotype G6 and G8 human rotaviruses. *Journal of medical microbiology*. 2001;50(5):462-7.