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Molecular epidemiology and phylogenetic analysis of bovine picobirnaviruses causing calf diarrhea, in Iran

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Abstract

Picobirnavirus (PBV) is an enteropathogen virus causing diarrhea as an opportunistic virus in its vertebrate host. There is no information about human or animal PBVs in Iran. The aim of the present study was the investigation of the epidemiology of bovine PBV in the broad geographical area of Iran. Four hundred and eighty-five stool samples of up to 1 month old diarrheic calves were collected from 14 provinces and were tested with polyacrylamide gel electrophoresis (PAGE), and reverse transcription polymerase chain reaction (RT-PCR). Five samples were positive in PAGE assay (1.00%) and all of them were amplified using GI specific primers in RT-PCR. Phylogenetic analysis of one of the amplicons (strain Nazaktabar-14) revealed a low relationship to bovine PBV sequences and more identity to PBV isolates from other hosts. The structural alignment of the deduced amino acids of the partially sequenced RdRp gene of the Nazaktabar-14 strain showed high conservation. Sequences obtained from other amplicons showed a high mutation rate and further analysis of one of them showed that, despite the potential of forming deleterious mutations, most of the point mutations occurred in the RdRp gene of PBVs may be a silent mutation. There is little information about the molecular epidemiology of bovine PBVs. This study was the first report on the occurrence of PBVs in Iran and the first study on the molecular epidemiology of bovine PBV in the Middle East, revealing its low frequency as a diarrhea causative agent.

Introduction

Picobirnavirus (PBV) is a none-enveloped small RNA-virus (30-40 nm in diameter), with double-stranded and bi-segmented genomic RNA. Based on biochemistry of the genomic features of the virus, and the size of viral particles, the International Committee of Taxonomy of Viruses (ICTV) nominated and classified them as a new family, *Picobirnaviridae,* which contains one genus named *Picobirnavirus*. To date, three species named human, equine, and Beihai picobirnavirus are classified in this genus.[1]

The genomic RNA can be easily distinguished by silver-stained polyacrylamide gel electrophoresis (ss-PAGE) that visualizes two distinct bands of the viral bi-segmented dsRNA. Two long and short migration patterns of genomic dsRNA segments are seen in the PAGE. The size of segments in large migration patterns is 2.70 kbp and 1.90 kbp for segments 1 and 2, respectively. The size of segments 1 and 2 in the small migration pattern is 2.20 kbp and 1.20 kbp, respectively.[2,3] Segment 1 has two open reading frames (ORF), ORF1 encodes a presumptive hydrophobic protein. The start codon of ORF 2 begins at -1 nucleotide backsliding at the stop codon of ORF 1 and encodes the viral capsomere with 552 amino acids in its structure. Segment 2 encodes a protein with 534 amino acids. This protein is the viral RNA dependent RNA polymerase of PBVs.[4]

The PBVs are divided into three genogroups of GI and GII and most recently GIII based on their genetic properties of segment 2. [1] In most cases, this genogrouping is based on sequencing of PCR products obtained from the primer sets suggested by Rosen *et al*.[5] Most of strains were detected and sequences submitted in GenBank® belonged to GI.[3]

Since the first detection of PBV in humans and rats in 1988,[6,7] many studies have been conducted on its epidemiology suggesting that this virus is present in several countries and has been detected in various species

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including both wild and domestic mammalian, reptile, and birds such as foals, calves, buffalo calves, dogs, rabbits, giant anteaters, dogs, cats, monkeys, orangutans, armadillo, snakes and different species of birds.[8-12] Some studies have also shown the presence of this virus in sewage and surface water.[13] Interestingly, it has been suggested that PBVs may be prokaryote virus which is present in vertebrate alimentary tracts without infecting eukaryotic cells.[14]

Although PBV infects a wide range of different hosts, its pathogenicity as a primary cause of diarrhea is debatable, Currently, it has been determined that the virus after the development of persistent infection in the early days of life remained as an opportunistic agent to cause diarrhea and it may get activated following the onset of immuno-compromising conditions.[15] A longitudinal study conducted on 44 hematopoietic stem cell transplantation (HSCT) recipients revealed that the PBVs of human gut virome could act as a risk factor in developing enteric graft-versus-host disease (GVHD).[16] In the past years, some authors have reported the detection of picobirnaviruses from the human and porcine respiratory tract and it made more controversy on its pathogenicity and biology.[2]

At the time of writing this article there are only seven papers on the epidemiology of bovine picobirnavirus (BPV) in countries including Brazil, India, and Russia, Belgium and the United Kingdom[2,17-19] in which molecular studies have been conducted only in three countries China, Brazil, and India,[17] Therefore, there are few sequences of bovine PBV deposited in GenBank®. The purpose of this study was to investigate the molecular epidemiology and phylogenetic analysis of the virus in Iran. In the present study, 485 stool samples of calves were collected from 14 provinces of Iran which covered a broad area and the molecular epidemiology of bovine PBV was investigated for the first time in both Iran and the Middle East.

Materials and Methods

Fecal samples. 485 stool samples were collected from up to one month old diarrheic calves. Samples were collected from both industrial and rural farms located in 14 provinces of Iran named Tehran, Alborz, Qazvin, Zanjan, East Azarbaijan, Markazi, Isfahan, Fars, Qom, Mazandaran, Golestan, Sistan and Baluchestan, Razavi Khorasan and, South Khorasan. All samples were collected immediately after excretion and were frozen before transferring to our laboratory and then kept frozen at –40.00 ˚C or lower temperatures until the time of analysis.

Extraction of dsRNA. To extract the viral dsRNA of stool samples firstly, aliquots of 200 µL from fecal suspension (20.00% v/v in phosphate-buffered saline) were prepared, and then, the extraction process was carried out using Viral Gene-spin™ DNA/RNA extraction kit (Intron Biotechnology Co., Seongnam-si, South Korea) according to the manufacturer's recommendations.

Detection of BPV by polyacrylamide gel electrophoresis. All extracted RNAs were investigated by PAGE according to Laemmli system to detect the bi-segmented dsRNA of BPV.[20] Briefly, 25.00 µL of extracted RNAs were mixed with 7.00 µL of sample buffer and applied to 10.00% polyacrylamide gel, 10.00 cm×10.00 cm×1.20 mm thick (Paya-Pajoohesh-Pars, Tehran, Iran) at 50.00 mA/plate constant current for 4 hr. The nucleic acid bands were visualized using silver staining described by other authors.[21]

RT-PCR. All positive samples in ss-PAGE were submitted to RT-PCR assay. The RT-PCR reaction was performed by specific primers PicoB25 (5' TGGTGTGGATG TTTC 3') and PicoB43 (5' ARTGYTGGTCGAACTT 3') as forward and reverse primers, respectively to detect the genogroup I of PBVs which described by Rosen *et al*.[5] To denature the viral dsRNA, 2.00 µL of extracted RNA along with 1.00 µL of dimethyl sulfoxide (DMSO) were solved in 13.00 µL of nuclease-free water. The mixture was incubated at 98.00 ˚C for 5 min and then immediately chilled on ice for 2.00 min. The denatured dsRNA mixture was added to 9.00 µL of reaction mixture containing 5.00 µL of 5x Qiagen One-step RT-PCR kit PCR buffer, 1.00 µL mixed dNTP, 1.00 µL (10.00 pmol μL^{-1}) of each forward and reverse primers, 1.00 µL of RT-PCR enzyme mix (Qiagen One-step RT-PCR kit, Qiagen Sciences LLC, Germantown, MD, USA) to a final volume of 25.00 µL. The reverse transcription reaction was performed at 50.00 ˚C for 30 min and followed by 95.00 ˚C for 15 min to destroy the RT enzyme. The PCR reaction was followed by 35 cycles of 94.00 ˚C for 45 sec, 52.00 ˚C for 60 sec, and 72.00 ˚C for 60 sec. Final elongation was carried out at 72.00 ˚C for 10 min. PCR products were run in 1.50% agar gel electrophoresis and visualized by ethidium bromide.

Sequence analysis. Three RT-PCR products from positive samples were selected and submitted to direct sequencing reaction (BigDye terminator kit on ABI 3730XL DNA Analyzer sequencer; Bioneer, Daejeon, Korea). The sequencing of each sample was done in both directions using forward and reverse primers. One of three sequenced RT-PCR products has been submitted in GenBank® as the strain named as GI/PBV/bovine/IRN/Nazaktabar_14/2016 (accession number MG646008). Obtained sequences were edited by BIOEDITH software and the quality of them were analyzed using Phred software (http://asparagin.cenargen.embrapa.br/phph/). The identification of the sequences was verified by BLAST. Subsequently, the following practices were performed using Mega software (version 10.0; BioDesign Institute, Tempe, USA).[22] Alignment of obtained sequences was performed along with some of the selected nucleotide sequences of PBV from GenBank® using the Clustal W method and computing the best evolutionary model, and then construction of the phylogenetic tree was done based on the neighbor-joining method.[23] The validity of the phylogenetic tree was evaluated by bootstrapping with

500 replicates. Translation of nucleotide sequences to amino acid sequences was carried out using the translation tool available in the ExPASy site (http://web.expasy.org/translate/). Structural alignment of amino acid sequences was carried out using the Expresso tool prepared by the web-based software T-Coffee server (http://tcoffee.crg.cat/apps/tcoffee/do:expresso).

Results

PAGE and RT-PCR assay. Five of 485 stool samples were positive (1.00%) and disclosed the two special bands of the bi-segmented genome of PBV in the ss-PAGE assay (Fig. 1). Positive samples were collected from Tehran (two samples), Qazvin (one sample), Razavi Khorasan (one sample) and Markazi (one sample) provinces. All positive samples were tested by RT-PCR assay with Rosen primers specific for GI genogroup and produced 200 bp bands in agar gel electrophoresis. Products of three samples belonged to Tehran, Qazvin and Razavi Khorasan provinces were selected for direct sequencing. In this study, only one migration pattern was found in the PAGE exam and all positive samples in the PAGE were reacted with the specific primers of genogroup GI. No pattern of other gel migration similar to the GII genotype was observed.

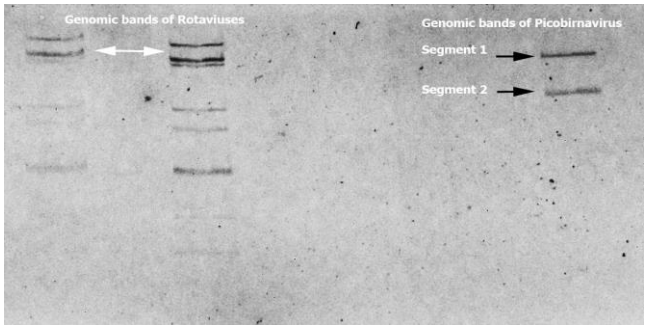

Fig. 1. Polyacrylamide gel electrophoresis of the bi-segmented bovine PBV genome. On the right, two bands of the genomic fragments of PBV are displayed. On the left, typical dsRNA bands of two samples infected with rotavirus A can be observed.

Sequence Analysis. One of three sequenced amplicons (from Tehran province) was suitable for phylogenetic analysis and submitted to GenBank® (accession number MG646008). In two other sequences, a noisy chromatogram was observed. In the chromatogram of one of the co-infected sequences (from Qazvin province), well-formed and distinctively clarified single peaks were seen, however, there were overlapping peaks in some residues which could be representative of point mutations. Because of the high quality of the chromatogram, this sequence was submitted for further analysis to investigate the potential effect of point mutations on its deduced amino acids sequence. First, the sequence was translated according to the original sequence proposed by the factory using the ExPASy services and then nucleotides of unread peaks were considered and their potential corresponding amino acid(s) were speculated. Forty-three nucleotides from 153 residues were mutated and showed overlapping peaks in the chromatogram (28.00%) from which 25 point mutations were observed in codons encoding 23 amino acids with no changes in their deduced amino acid residues compared to the primary sequence, therefore, they were considered as silent mutations. The nucleotides encoding 19 amino acid residues were not mutated (including sequences encoding of D--S-D residues of motif A), however, mutations that occurred in the nucleotides of the codons encoding nine amino acid residues according to probability rules might have led to change in their deduced amino acid residues while hydropathy features of most of them were not changed. In one residue (Y_{239}), three coding letters were mutated (because its chromatogram showed three overlapping peaks) as a result of potentially forming the TAG stop codon and a possible deleterious mutation was considered as. Figure 2 shows 56 nucleotides of this sequence. In this Figure, unread residues from overlapping peaks have been shown and possible amino acids that could be translated regarding these residues.

Supposed Nt sequence	CTG	GTT	CCT	GCT	TGG	GTT	AGC	ATG	GAA	T A T	GTG	GAT	CGA	CGC	ATT	ACT	AAT	ATG	TTC
Overlapping unread peaks (point mutations)	- - A	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	G C G	- - T	- - -	- - G	T - T	- - C	- - A	CGA	- - -	- - -
Possible codons and (their corresponding amino acids)	CTG (L) CTA (L)	(V)	(P)	(A)	(W)	(V)	(S)	(M)	(E)	TAT (Y) TCT (S) TCG (S) GAT (D) GCT (A) GCG (A) GAG (E) TAG Stop	GTG (V) GTT (V)	(D)	CGA (R) CGG (R)	CGT (R) CGC (R) TGT (C) TGC (C)	ATT (I) ATC (I)	ACT (T) ACA (T)	AAT (N) AAA (K) CAT (H) CAA (Q) CGA (R) GGT (R) AGA (R) AGT (S)	(M)	(F)

Fig. 2. Analysis of 56 nucleotides from the sequence obtained from Qazvin province and its deduced amino acids are shown. In the first row, the nucleotides supposed by the sequencer company are displayed. In the second row, the nucleotides that were seen in the chromatogram as overlapping peaks are shown. Nucleotides without overlapping peaks (unmutated) are shown as hyphen <->. In the third row, the amino acids for each triplet codons are displayed in IUPAC code in parentheses. If there was overlapping peaks, each of possible codons and their deduced amino acids are written in front of them. Non-mutated and silent-mutated sites are displayed in pale gray and sites where mutations led to the amino acid change are shown in light gray. The sites where the mutation could possibly lead to the formation of a stop codon are shown in dark gray.

The structural alignment of the deduced amino acids sequence of the obtained partial sequence of RdRp gene of the strain Nazaktabar-14 along with some homologous sequences from GenBank® using the Expresso tool determined that contrary to nucleotide sequences, the sequence of amino acids of this gene was highly conserved.

Phylogenetic Analysis. In the phylogenetic study, the nucleotide sequence of strain nazaktabar-14 was stayed far from the bovine PBV sequences and placed in the same cluster together with strains of humans and monkeys isolated from India and China, respectively. Furthermore, this sequence showed an evolutionary relationship with some strains obtained from dromedary, horse, and wastewater which were detected from Dubai and China (Fig. 3). The identity with bovine strains such as the prototype bovine PBV RUBV-P detected in India (GenBank® GQ221268) and PBV18_PR detected in Brazil (GenBank® KP 843617) were 61.00% and 72.00%, respectively.

Discussion

The PBVs possess a high variable genome causing persistent infection milieu in the gut of various vertebrate hosts. Based on the phylogenetic results, it seems the virus has no limit on the selection of its vertebrate hosts and infects them non-specifically.[3] In addition, PBVs can survive in the environment and pollute surface water, therefore, it simply finds its new host, human beings, to create a persistent infection.[13]

In this study, the presence of PBVs was investigated for the first time in calf population from a broad area in Iran, in many of which the dairy industry was very important and there were many crowded dairy farms located there, such as the provinces of Tehran, Qazvin, Isfahan, Fars, Zanjan, Markazi and Razavi Khorasan. The results showed that the frequency of bovine PBV causing calf diarrhea in Iran was low and this finding was consistent with the results of Buzinaro *et al.* as they detected PBV in 4 samples among 567 stool samples using PAGE in Brazil.[18]

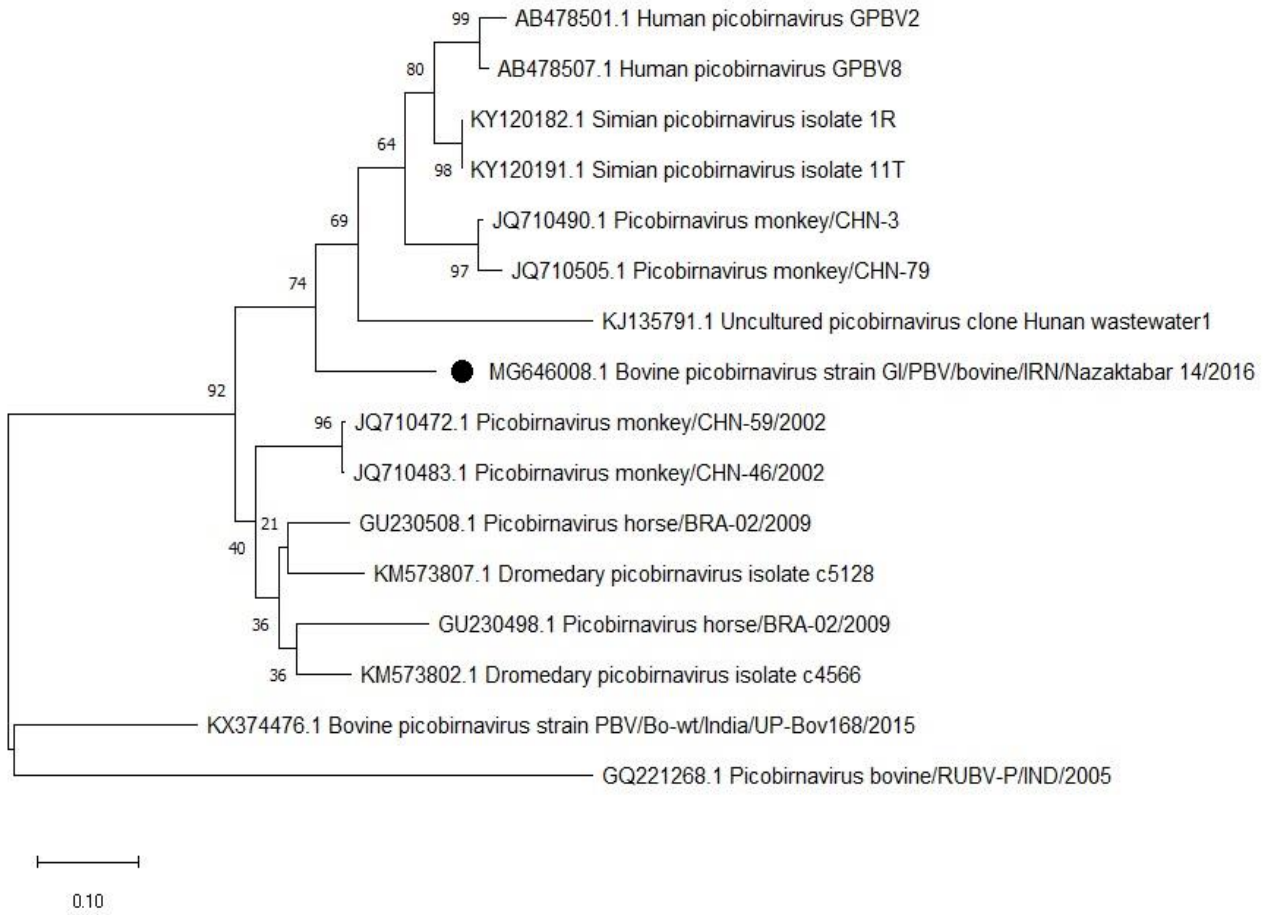

Fig. 3. Phylogenetic analysis by the neighbor-joining method of partial sequences of the RdRp gene of genogroup I PBV strains based on nucleotide sequences. The sequence of this study is indicated by ●. The percentage of bootstrap probabilities (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method and the rate variation among sites was modeled with a gamma distribution. Evolutionary analyses were conducted in MEGA X.

Due to the specific characteristics of the PBVs' genome, PAGE is the most common method to detect PBV in stool samples, however, the sensitivity of PAGE to detect the dsRNA molecules is low and it can detect viral dsRNA molecules only when a large amount of the virus is present in the stool which is only occurred in clinical diarrhea.[24] PAGE is also unable to detect the presence of different strains with the same pattern of migration in one sample. In contrast, the sensitivity of RT-PCR is higher, and it can detect the lower load of the virus in fecal specimens, especially in subclinical infections in which the shedding of the virus is low and PAGE is not sensitive enough to detect it. Studies have also shown that the rate of detection of the virus may be much higher in epidemiological studies if the RT-PCR is used in place of the PAGE;[10,11,15] However, since there are different pathogens causing diarrhea, using sensitive methods (such as RT-PCR) to detect the PBV in diarrheic stool samples can lead to an increase in the false-positive rate of PBV-positive samples detected as the primary cause of diarrhea. On the other hand, because of the high rate of genetic variability of PBVs, the sensitivity of the RT-PCR test can be affected by the inactivity of primers and, consequently, it may not be able to detect some of PAGE positive samples.[15]

Although the prevalence of the GI genogroup is higher in the world, some reports indicate that both genogroups GI and GII could be isolated from the bovine population.[17,25] Given that PBVs can establish a persistent infection in their host with three distinct periods in which the fecal shedding of the virus is high (concomitant with diarrhea, detectable by PAGE), low (without the clinical disorder, detectable only by RT-PCR), and/or undetectable (inactive infection),[15] very small amounts of subclinical infections of GII may not be recognized by PAGE. Thus, to get a better knowledge of the GII epidemiology, more sensitive techniques such as RT-PCR should be used.

Analysis of the sequences of the RdRp gene showed that these highly mutable viruses can also be present as a quasi-species virus in cattle, Bányai *et al.* proved that the high rate of single point nonsense and deleterious mutations was occurred in the RdRp gene of PBVs and suggested that these viruses replicated in the alimentary tract as a quasi-species virus.[26] Inappropriate sequences for the phylogenetic study were also reported in a molecular study of bovine PBVs in Brazil.[17]

In silico sequence analysis of one inappropriate sequence present study revealed that despite the high rate of mutation in PBVs (28.00% in the present analysis) many of these point mutations are silent and/or conservative mutations which may not structurally alter the hydropathy of the RdRp protein and its function, however, some mutations are likely to be deleterious. This finding was inconsistent with the result of Bányai *et al.* and confirmed the potential existent of PBV as a quasi-species virus in the bovine alimentary tract.[26]

As mentioned in the results section, the structural alignment amino acid sequences of the RdRp gene of PBVs using the Expresso tool revealed high conservation. This finding was inconsistent with the results of Collier *et al.* suggesting this conservation led to similar three-dimensional structures of RdRps of various mammalian and avian PBV strains.[27]

The phylogenetic study showed that the sequence of this study was less similar to that obtained from cattle, and was more similar to other species including a virus isolated from camels in the UAE, a country neighboring Iran. The widely distributed phylogenetic results of PBVs have been reported in all other studies.[28] However, in a recent report by Woo *et al*., the diversity in bovine PBV sequences was more than in others (monkeys).[29]

It should be noted that the number of sequences of PBVs, especially bovine in the GenBank® was very low, and given the high mutability in the nucleotide sequences of these viruses, the results of phylogenetic studies of these viruses were currently low in bootstrap scores. To obtain more valid phylogenetic results from the RdRp gene more sequences of this gene (especially from bovine PBV) are needed to be deposited in the GenBank®. Although, the high rate of the variability of the genome of PBV limited its molecular study with simpler methods such as RT-PCR. Therefore, most of the sequences of the virus deposited in GenBank® were obtained from a pair of primers amplifying a small portion of the RdRp protein-coding genome. More full-length genomic sequences are required to illustrate more details of the molecular biology of these high genetic divergent, opportunistic, and resistant viruses infecting several hosts.

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Conflict of interest

Author declares that there is no conflict of interest regarding the publication of this article.

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