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Phylogenetic analysis of peste des petits ruminants virus (PPRV) isolated in Iran based on partial sequence data from the fusion (F) protein gene

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Abstract: In order to gain some insight into the origin of the peste des petits ruminants virus (PPRV) isolated in Iran, a selected region of the PPRV genome in a clinical sample collected from a sheep was amplified using RT-PCR, and the resulting amplicon was sequenced for phylogenetic analysis. The partial nucleotide and predicted amino acid sequence of the Iranian isolate was aligned with the corresponding sequences of 16 previously published F genes. Sequence analysis of the strains showed that the Iranian isolate had the highest degree of homology with the majority of the strains, with the exception of Nigerian isolates and ICV89. In general, a higher degree of amino acid sequence conservation was observed among the various strains of field PPRV. Phylogenetic comparison of the Iranian isolate, along with some published exotic sequences, indicated that the virus has been circulating for years in Iran's neighboring countries, including Turkey and Pakistan. Overall analysis of the amino acid and nucleotide substitutions showed that ICV89 from Ivory Coast was more prone to sequence alterations than the others were. The phylogenetic tree created for F protein nucleotide data was divided into 4 separate lineages. All strains were conserved within the cleavage site of the amino acid sequence, except for Mdn96. Analysis of the sequence data showed that PPRV circulation has been homologous in Asian countries. Determination of the nucleotide sequence of the partial F protein of the Iranian isolate would help clarify the origin of the disease.

Key words: PPRV, sequence, phylogenetic analysis, isolate

Füzyon (F) protein geninin kısmi dizi verilerine dayanarak İran'da izole edilen koyun keçi vebası virüsü (PPRV)'nün filogenetik analizi

Özet: İran'da izole edilmiş koyun keçi vebası virüsü (PPRV)'nün kökeni hakkında biraz fikir edinmek için, koyundan toplanan bir klinik örnekdeki PPRV genomunun seçilmiş bir bölgesi RT-PCR kullanarak çoğaltılmıştır, ve çıkan amplikon filogenetik analiz için dizilenmiştir. İran'a ait izolatın kısmi nükleotit ve tahmin edilen amino asit dizisi daha önce yayımlanan 16 F geninin ilgili dizileri ile hizalanmıştır. Suşların dizi analizi, Nijerya izolatları ve ICV89 hariç tutulursa İran'a ait izolatın, suşların çoğunluğu ile en yüksek seviyede homolojiye sahip olduğunu göstermiştir. Genel olarak, çayır PPRV'nün çeşitli suşları arasında amino asit dizisinin daha yüksek seviyede korunduğu gözlenmiştir. Bazı yayımlanmış ekzotik diziler ile birlikte İran'a ait izolatın filogenetik kıyaslaması, virüsün Türkiye ve Pakistan'ı içeren İran'nın komşu ülkelerinde yıllardır dolaştığını göstermiştir. Amino asit ve nükleotit yer değiştirmelerinin bütün analizleri, Fildişi Kıyısından ICV89'un dizi değişikliklerine, diğerlerinin olduğundan daha eğimli olduğunu göstermiştir. F proteini nükleotit verisi ile yapılan filogenetik ağaç 4 ayrı soya bölünmüştür. Bütün suşların, Mdn96 hariç amino asit dizisinin ayrılma yeri korunmuştur. Dizi veri analizi PPRV sirkülasyonunun Asya ülkelerinde birbirine benzer olduğunu göstermiştir. İran'a ait izolatın kısmi F proteini nükleotit dizisinin belirlenmesi hastalığın kökeninin açıklığa kavuşmasına yardımcı olacaktır.

Anahtar sözcükler: PPRV, dizi, filogenetik analiz, izolat

Introduction

Peste des petits ruminants (PPR) is a highly contagious disease of small domestic and wild ruminants. Goats are severely affected, whereas sheep undergo a mild form of the disease (1,2). The etiological agent of the disease, peste des petits ruminants virus (PPRV), is classified within the genus *Morbillivirus* as a member of the family *Paramyxoviridae* (3). The disease is characterized by high fever, ocular and nasal discharge, pneumonia, necrosis, and ulceration of the mucous membranes and inflammation of the gastro-intestinal tract, followed by severe diarrhea. It causes 50%-80% mortality and about 90% morbidity (4-6). The disease is endemic in the Arabian Peninsula, the Middle East, and in the Indian Subcontinent. PPR has been reported in various parts of Asia and Africa (2,7). PPR is one of the major notifiable diseases of the World Organization for Animal Health (OIE) and existence of PPR can have a catastrophic impact on a region's farming development (8).

In Iran PPR outbreaks have been increasingly reported during the past few years (9). The virus contains a single stranded negative sense RNA ~16 kb in length, encoding 6 structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H), and large polymerase protein (L), and non-structural proteins V and C (10,11). PPRV, like other viruses in the family *Paramyxoviridae*, is an enveloped RNA virus with 2 external glycoproteins (F and H) associated with the envelope. The F protein is responsible for important biological activities, and enables the virus to penetrate cell membranes and enter cytoplasm by effecting fusion of the virus and host cell membranes. This protein is critical for the induction of an effective protective immune response (12,13).

The present study aimed to determine the phylogenic relationship of the PPRV circulating in Iran with strains from around the world. The second purpose of the study was to perform molecular diagnosis of PPRV based on nucleotide sequencing of the partial F protein in clinical samples collected in Iran.

Materials and methods

Viruses

Clinical samples of virus that caused symptoms suggesting PPRV infection were collected from sheep and transferred immediately to sterile containers. Virus specimens were stored at –70 °C in the laboratory of Razi Vaccine and Serum Research Institute. Laboratory and serological tests confirmed the diagnosis of PPRV in the infected samples. Viruses were then passaged on Vero cell monolayer cultures in order to isolate the viral RNA. Isolation of PPRV in cell culture was performed after 5 passages for the extraction of viral RNA. In total, 15 samples (lymph node and cell culture samples) were received from the virology department. Positive results were obtained with 4 of the 15 tested samples.

RNA extraction, cDNA synthesis, and polymerase chain reaction

An isolate of PPRV adapted to Vero cells cultivated in suspension was used for RNA extraction. Infected cell culture supernatants were used for the process of RNA isolation. Total RNA was extracted using a Roche Total RNA Isolation Kit (Roche, Germany), according to the manufacturer's instructions. The cDNA of the F protein-coding sequence was synthesized from total RNA and reverse transcription (RT) carried out using AMV reverse transcriptase. In vitro amplification was run on a programmable thermocycler using Taq DNA polymerase and gene-specific primers.

The PCR-specific primers (forward and reverse) amplified a 322-bp region between the positions of the 254 and 575 nucleotides of the PPRV F gene. Sequences of the 2 primers flanking the target region were as follows: forward: 5'-gagactgagtttgtgacctacaagc-3'; reverse: 5'-atcacagtgtta aagcctgtagagg-3'. The following conditions were observed to be optimal for producing sufficient PCR products: after an initial denaturation at 93 °C for 1 min PCR was performed for 35 cycles, each consisting of 1-min denaturation at 93 °C, 1-min annealing at 51 °C, and 1-min extension at 72 °C, followed by a final extension of 5 min at 72 °C.

Cloning of the PCR products and sequencing

The amplified PCR fragments ~362 bp in size were

identified using 1% agarose gel electrophoresis and ethidium bromide staining (14). Purification of PCR-amplified DNA products was performed in order to prepare for the transformation process using a gel extraction kit (Roche, Germany), according to the recommendations of the supplier. The purified DNA fragment of the F protein gene was ligated into a PNTZ57T vector (Fermentas, Germany). Essential components of the ligation reaction were then used to transform an appropriate strain of *E. coli*. The cloned PCR products were purified using a plasmid purification kit (Roche Diagnostic, Germany) and a sequencing reaction was run with the T7 promoter primer (MWG Co., Germany). Purified fragments were sequenced from both directions.

Data analysis

Multiple nucleotide sequences of the F protein gene region were assembled and aligned using the CLUSTAL V algorithm in MegAlign v.5. Nucleotide and amino acid sequence homology/divergence was calculated using the DNASTAR package program (DNASTAR, Inc.) and the included weighted sequence distances matrix. A multiple amino acid alignment report was generated based on partial sequence data. The following sequences were taken from GenBank and were contained in the analysis (individual accession numbers are given in parentheses): Turkey (AF384687); Sung 96 (AF464883); Avik99 (AF464886); Uri99 (AF464890); Turkey 2000 (AJ849636); FSD/PK07 (AM945963); SAH/PK07 (AM946407); Sungri/96 (AY560591); Ind-TN-2002-G (AY602982); Pakistan (AY823544); PPR-TRabol (EF547922); PPR-TRabo2 (EF547923); ICV89 (EU267273); Ng76/1(EU267274); vaccine strain-Nigeria 75.1 (X74443); Mdn96 (AF464892). The Iranian sequence was submitted to NCBI with the accession number AY948429.

Results and discussion

Fusion (F) and hemagglutinin (H) proteins, both of which are external glycoproteins, provide protection against the PPR in animals, and are thus considered promising candidates for subunit vaccines (1,15). The overall objective of the present study was to estimate the genetic relationship between the Iranian isolate and 16 other PPRV sequences obtained from GenBank by comparing the nucleotide sequences of the PCR product amplified from the partial F gene.

Evolution of RNA viruses is considered a complex process in nature that involves the fast creation of mutants throughout the RNA replication process (16). To assess the genetic similarity and divergence among the strains, multiple data analyses were performed. Sequence data obtained from the Iranian isolate was aligned with that of 16 other strains of PPRV from various geographic areas, including India, Pakistan, Turkey, Ivory Coast, and Nigeria, based on the partial nucleotide and amino acid sequences of the gene encoding the F protein of PPRV. As shown in the Table, the partial (F) protein nucleotide sequence of the Iranian PPRV isolate exhibited the highest degree of homology with most of the strains. None of the field strains had homology less than 97% with the Iranian isolate, with the exception of Nigerian isolates and ICV89 from Ivory Coast. ICV89 showed the lowest degree of homology with the 16 strains studied, in both the amino acid and nucleotide sequences.

At the amino acids level, the Iranian isolate of PPRV exhibited a sequence homology of 100% with 9 strains and of 96%-99.1% with the remaining strains. The alignment of the amino acid sequences of the F protein showed less variation than did the nucleotide sequence alignment. By using phylogeny on diverse sequences, including 17 PPRV strains, the present study's results strengthen the phylogenetic relationship between the Iranian isolate and the other strains used for comparison, and provide information on the relatedness of these strains. The phylogenetic tree created for the nucleotide data was divided into 4 distinct groups. As shown in Figure 2, all the Asian strains were grouped in lineage A. It is clearly observed that the Nigerian isolates and ICV89 clustered into separate lineages (B and C).

The genetic relationships among PPRVs were investigated by comparing the nucleotide sequence of the PCR product amplified from the F gene with the corresponding sequences published in the National Center for Biotechnology Information (NCBI) GenBank (17). PPR is one of the most important animal diseases in Iran, has been identified throughout the country, and has led to the loss of sheep and goats totaling US$1.5 million (18). To date,

Table. Nucleotide sequence variation for PPRV variants isolated from different geographic regions.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1		97.5	97.5	89.4	99.1	94.1	99.1	99.1	98.1	97.8	99.4	98.4	988	99.4	98.8	93.2	98.8	**1**	Iran
2	2.5		97.5	89.1	97.8	93.8	97.2	97.2	96.9	97.8	98.1	97.2	96.9	98.1	96.9	92.9	97.5	**2**	Avik-99
3	2.5	2.5		89.9	97.8	94.1	97.2	97.2	96.9	99.7	98.1	97.2	96.9	98.1	96.9	93.2	97.5	**3**	FSD.PK07
4	11.6	11.7	11.3		89.9	91.9	88.8	88.8	88.8	89.4	89.8	90.1	89.1	89.8	88.5	90.4	88.5	**4**	ICV89
5	0.9	2.2	2.2	11.3		94.7	98.8	98.8	98.4	98.1	99.7	98.8	98.4	99.7	98.4	93.8	99.1	**5**	Ind-TN-2002-G
6	6.2	6.6	6.2	8.7	5.5		94.4	93.8	94.1	94.4	94.7	94.4	93.5	94.7	93.5	97.8	93.8	**6**	Ng76-1
7	0.9	2.9	2.9	12.5	1.3	5.9		99.4	97.8	97.5	99.1	98.1	98.4	99.1	98.4	93.5	98.4	7	PPR-TRabo1
8	0.9	2.9	2.9	12.4	1.3	6.6	0.6		97.8	97.5	99.1	98.1	98.4	99.1	98.4	92.9	98.4	**8**	PPR-TRabo2
9	1.9	3.2	3.2	12.0	1.6	6.2	2.2	2.2		97.2	98.8	97.8	97.5	98.8	97.5	92.5	98.1	**9**	Pakistan
10	2.2	2.2	0.3	11.6	1.9	5.9	2.5	2.5	2.9		98.4	97.5	97.2	98.4	97.2	93.5	97.8	**10**	SAH-PK07
11	0.6	1.9	1.9	11.3	0.3	5.5	0.9	0.9	1.3	1.6		99.1	98.8	100.0	98.8	93.8	99.4	**11**	Sung96
12	1.6	2.9	2.9	10.9	1.3	5.9	1.9	1.9	2.2	2.5	0.9		97.8	99.1	97.8	94.7	98.4	**12**	Sungri96
13	1.3	3.2	3.2	12.1	1.6	6.9	1.6	1.6	2.5	2.9	1.3	2.2		98.8	98.1	32.5	98.1	**13**	Turkey 2000
14	0.6	1.9	1.9	11.3	0.3	5.5	0.9	0.9	1.3	1.6	0.0	0.9	1.3		98.8	93.8	99.4	**14**	Uri-99
15	1.3	3.2	3.2	12.8	1.6	6.9	1.6	1.6	2.5	2.9	1.3	2.2	1.9	1.3		92.5	98.1	**15**	Turkey
16	7.3	7.7	7.3	10.6	6.6	2.2	6.9	7.6	8.0	6.9	6.6	5.5	8.0	6.6	8.0		92.9	**16**	vaccine Strain-Nigeria75.1
17	1.3	2.5	2.5	12.1	0.9	6.2	1.6	1.6	1.9	2.2	0.6	1.6	1.9	0.6	1.9	7.3		**17**	Mdn96
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**		

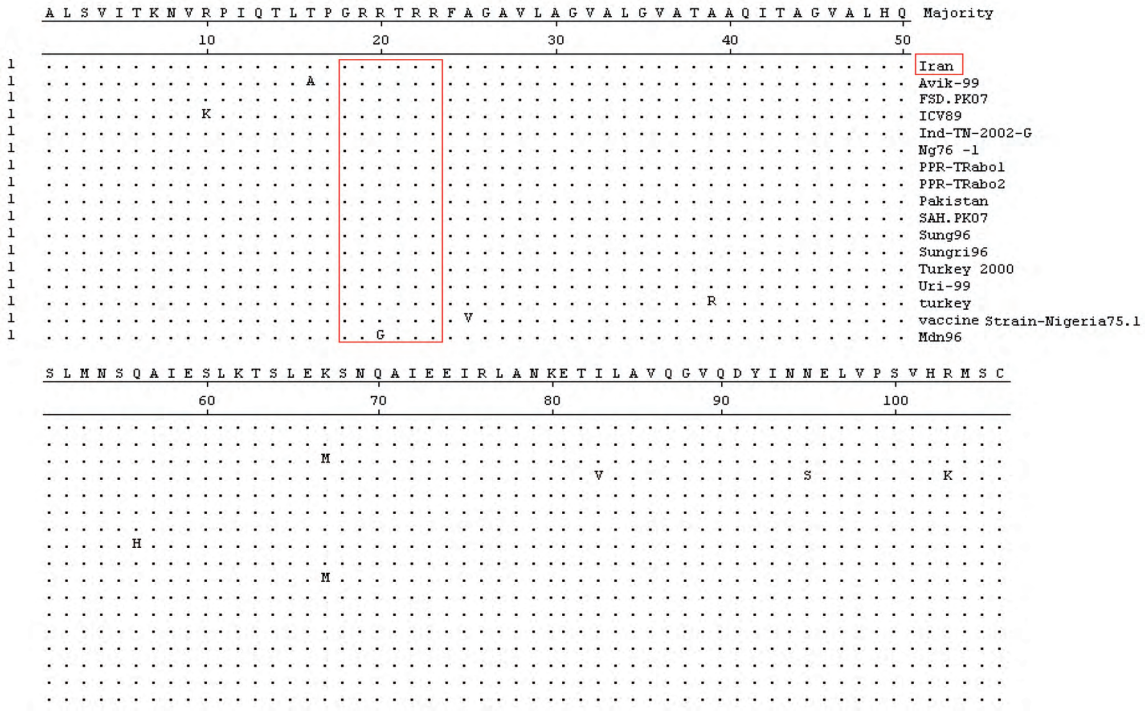


Figure 1. Alignment of the deduced amino acid sequences of partial F protein. The alignment was performed by Jotun Hein method in MegAlign software. The differences at amino acid positions among the strains are shown by a single letter code. Red box exhibits the cleavage site within F protein region.

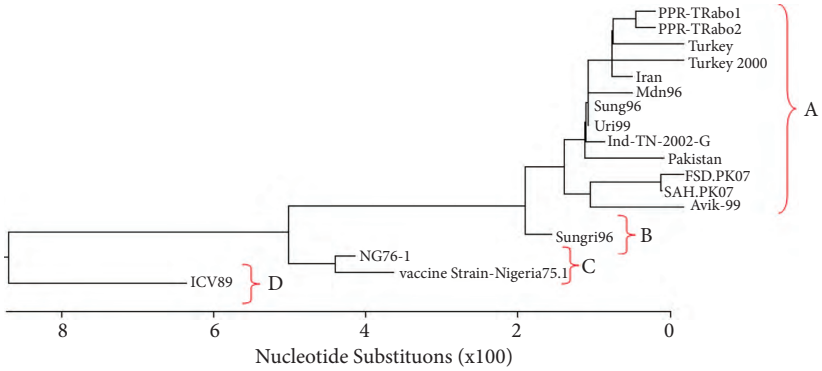


Figure 2. Phylogenetic tree showing the relationships among the PPRV sequences. The dendrogram was constructed using CLUSTAL V algorithm as described, from the partial F protein gene sequences of the referred strains. The 4 divergent clusters are shown on the tree.

no studies have reported the genetic sequence of the F protein region from PPRV isolated in Iran. The observed similarities between the Iranian isolate and strains from Pakistan, Turkey, and India suggest that disease outbreaks have been caused by the same strains. Iran is bordered to the east by Pakistan and to the west by Turkey. In addition, India is a South Asian country bordered by Pakistan to the west. PPRV infection has been reported in the Van and Malatya regions of southeastern Turkey, near the Iranian border.

The border relationships between these countries are probably the cause of PPRV transmission (19). Preliminary data suggest that the virus has been circulating for years in Iran's neighboring countries. Our data allow us to conclude that PPRV circulation has been homologous across the aforementioned regions, with the exception of Nigeria and Ivory Coast. With the advent of molecular biological techniques, such as PCR, rapid and specific diagnosis of PPR has become possible (17). Bailey (20) reported that the F protein and the cleavage site were conserved (20). As the F protein region is highly conserved in sequence, it can hopefully improve early differential diagnosis of PPRV in suspected outbreaks of PPR among flocks of sheep in Iran by employing F gene-based primers, and will lead to improved vaccination and prevention protocols. To further analyze the evolutionary relationships**,** more sequence data would obviously be required. As can be seen in Figure 1, all the strains were conserved within the cleavage site of PPRV, except for Mdn96. The location of the site has been highlighted on the predicted amino acid sequences. The fusion protein cleavage site (FPCS) sequence of PPRV is G-R-R-T-R-R and shows considerable sequence conservation (21).

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