# Genetic characterization of peste des petitis ruminants virus (PPRV) from Sulaimani/ Iraq by phylogenetic analysis and sequencing of nucleoprotein and fusion protein gene.

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**Abstract**

**An outbreak of peste des petitis ruminants (PPR) in sheep was studied in Sulaimani/ Iraqi Kurdistan Region. The study provided the first molecular characterization of the PPRV lineage associated with fatal PPR infections in small ruminats. RT-PCR was used for diagnosis of PPRV outbreaks during 2012-2013 and the phylogenetic analysis results based on F and N genes. It was observed that three tested samples were positive for PPR, from which the two F genes (ppr/Kurdistan/2012, ppr2/Kurdistan/2012) were genetically close to the (KF478924 & JF274480) strain (turkey and Egypt) of lineage IV, respectively with 99% nucleotide sequence homogeneity. Regarding N gene, it was noticed that one sample (ppr3/Kurdistan/2013) where genetically close to (FJ795511, DQ840197 & DQ840190) strain (Emirate, Saudi Arabia & Israel) %99, %98 and %97 respectively. The current study suggested that there are at least two sources of PPRV in Iraq.**

**Keywords:** PPRV; Sulaimani; peste des petits ruminants; Kurdistan

## I. Introduction :

Peste des petits ruminants (PPR) is classified as economically important and an acute highly contagious transboundary viral disease in small ruminant (1, 2). It is characterized by pyrexia, nasal ocular discharges, erosive stomatitis, mucopurulent, bronchopneumonia, and anorexia and sever diarrhea, followed by recovery or death (3, 4, 5). PPR literally means "plague of small ruminant" cause fatal deaths in sheep and goat, (6, 7). PPR disease is grouped under list A of the Office International des Epizooties (OIE), because of its highly contagious nature and capacity for rapid spread (8, 6).

The causative agent, peste des petits ruminant's virus (PPRV), is grouped in the genus Morbillivirus within family Paramyxoviridae (9). PPRV is pleomorphic, with negative sense single stranded RNA genome containing approximately 16 kb nucleotides (10,11) .The Morbillivirus genome is organized into six contiguous, non-overlapping transcription units separated by short intergenic regions and encoding six structural proteins nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin protein (H), polymerase protein (L). (12, 13, 14). In addition, there are two non-structural proteins (C and V), which

are translated from the P gene open reading frame (ORF) by different mechanisms (15,16).

N protein encapsulates the genomic RNA to form the N-RNA template to facilitate replication and transcription by the L-P polymerase complex. M is a matrix protein around which the envelope is assembled. F and HN are two surface glycoprotein, which enable the entry of the virus into the host cell. H protein recognizes the host cell receptor, whereas F protein mediates the fusion of host and viral envelopes (17, 18). PPRV is genetically grouped intofour distinct lineages (I, II, III and IV) (19,20) based on the F and N genes; despite the fact that only a single serotype of PPRV has been reported (20 ), PPRV belonging to lineages I and II are exclusively isolated from the countries of PPRV origin in West and central Africa(21,22). Lineage III currently present south of the Arabian Peninsula, (Yemen, Qatar and Oman) and East Africa, (22) although some of the viruses that belong to lineage III have also been isolated from southern India. Lineage IV is considered a new lineage comprising newly emerging viruses, and is most Prevalent in Asian countries, Southeast Asia, the Middle East, and more recently northern Africa (16, 19).
Historically, OIE and FAO officially reported PPR in Iraq in 1998 (FAO 2003). Another outbreak occurred in 2000 in which the virus caused high morbidity and low mortality rates among small ruminants (12). Recently, devastating outbreak occurred in Erbil province in Kurdistan region among wild goat (Capra aegagrus) which caused 750 deaths (8).

This study aimed to determine the phylogenic relationship and molecular characterization of PPRV circulating in Sulaimani province, Kurdistan region of Iraq, based on partial sequence of the F and N gene in clinical samples.

## II. Materials and methods :

*A. Sampling.*

Mouth epithelial tissue samples from sick sheep where collected by local veterinary department (Pshder, Bazian, and Sirwan), based on clinical sings. The samples chilled on ice and transported to veterinary directory of Sulaimani laboratory

*B. RNA extraction.*

RNA extraction was performed from epithelial tissues of mouth lesion using RNA extraction tissue kit (Genaid, Korea) according to manufacturer's protocol. the extracted RNA immediately used for cDNA synthesis.

*C. Oligonucleotides Primers*

Two sets of primers were used in this study (Table I). First set primers (F1 & F2) specific for detection of PPR fusion (F) gene (23), second set primers (N1 & N2) specific for detection PPR nucleocapsid (N) gene (24).

*D. Reverse transcription*

The cDNA of the F and N protein-coding sequence was separately synthesized from total extracted RNA using *AccuPower*® RT PreMix (Bioneer, Korea), the reaction carried out in 0.2 ml PCR tube with following condition, 5 µl extracted RNA, 1 µl reverse primer (10 mole) and finally complete to 20 µl by adding 14 µl ultra pure water, this reaction was run in PCR thermocycler (Bio Rad ,U.S) at 70 °C for 5 minute and 4 °C for 5 minute. The mixture was then added to lyophilized pellet of RT Premix which consists of M-MLV Reverse transcriptase, dNTPs, reaction buffer, DTT, RNase inhibitor, and stabilizer. The reaction was run in thermocycler programmed at 42 °C for 60 mints, then stopped at 95 °C for 5 mints.

*E. PCR amplification*

Partial sequence of F & N genes were amplified separately by using AccuPower PCR PreMix (Bioneer, Korea) the reaction carried out in 0.2 ml PCR tube with following

reaction, 5 µl cDNA, 1 µl forward (10 pmole), 1 reverse primers (10 pmole)( Table I), and finally complete to 20 µl by adding 13 µl ultra pure water The PCR thermocycler was programmed as following; an initial denaturation at 94 °C for 2 min , 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s for F gene primers, but the annealing temperature for N gene primers was 50 °C for 30 s, and extension at 72 °C for 40 s and final extension at 72 °C for 5 min. 10 µl of the amplified PCR products was run on a 1 % agarose gel and stained with ethidium bromide. The PCR product visualized under UV transilluminator (UVETIC, U.K).

*F. Sequencing the PCR products*

Twenty ul of PCR product of F and N gene was sequenced from both primer in, (Macrogen sequencing crevice , Korea) and (Bioneer sequencing crevice , Korea). Both sequence were aligned and trimmed by using NCBI (bl2seq) then published in Genbank as ppr/Kurdistan/2012, accession number (KC292209), ppr2/Kurdistan/2012 accession number KC252611 for F gene and ppr3/Kurdistan/2013 accession number (KF992797) for N gene.

*G. Sequence and phylogenic tree analysis*

The partial nucleotide sequences of F and N genes were aligned with corresponding sequences available in GenBank, The sequence homology and multiple sequence alignment at the nucleotide and the amino acid level were performed by CLUSTAL W program (25). Phylogenic tree were constructed among Kurdistan/ppr isolates and isolates from different lineage around the world, based on the neighbor-joining method using Kimura2-parameter model in Mega5.2. The bootstrap values were determined from 1000 replicates of the original data (26).

**Table I: list of primers which used for detecting PPRV by Rt-PCR**

| Name | Sequence Primer | Target Position | Amplicon | Reference |
|---|---|---|---|---|
| F1-f | ATCACAGTGTTAAAGCCTGTAGAGG | F gene 777-801 | 372 bp | ( 23) |
| F2-r | GAGACTGAGTTTGTGACCTACAAGC | F gene 1124-1148 | | |
| N1-f | GATGGTCAGAAGATCTGCA | N gene 1208-1226 | 463 bp | (24) |
| N2-r | CTTGTCGTTGTAGACCTGA | N gene 1670-1652 | | |

## III. Result :

*A. Detection of PPRV by F and N gene based RT-PCR*

All the suspected samples were showed positive result for PPRV, based on agarose gel electrophoresis, which demonstrated expected amplicon about 372 bp and 463 bp for F and N gene, respectively fig(1& 2 ). The results were confirmed by sequencing of PCR product and the sequences were submitted in NCBI Genbank

***B. Sequence Homology and phylogenetic analysis***

The analysis of PPRV of Iraqi isolates which was published in NCBI Genbank indicated that the 372 bp F gene sequences of two Sulaimani isolates (Kurdistan/ppr & Kurdistan/ppr2) accession No. (KC252611 & KC292209) have 99% homology. The analysis of both Sulaimani isolates with recent outbreak in Erbil (Kurdistan/2011) was also showed 99% homology where as similarity with previous outbreak Iraqi isolate Iraq/2002 ranged from 97-98%. The F gene sequence of Sulaimani isolates were also aligned with the PPRV isolates outbreak in the neighbor countries and it showed 92-99% homology (Table II ).

Similarly 436 bp N gene of sulaimani isolate Kurdistan/ppr3 was subjected to multiple sequence alignments with Iraqi and neighbors PPRV isolates the result showed 96% identity with Erbil strain Kurdistan/2011 and 89-99% identity with other seven countries in the region (TableIII).

According to phylogenetic tree construction based on the sequence of F gene alignment of PPRV sequences retrieved from NCBI Genbank, all the PPRV isolates were grouped intofour lineages both Sulaimani isolates were belong to Lineage 4 and cluster with Egypt/2009 & TR/Asparta/2013 isolate. This lineage include all PPRV isolate from Asia including both Erbil strain Kurdistan/2011 and Iraq/2002 of Iraq fig(3)

According to phylogenic tree of N gene sequences, all PPRV isolate also divided to four group, most of Asian isolate were grouped in lineage 4, Iraq ppr3/Kurdistan /2013 was cluster with emirat/2009 isolate within lineage 4, the Erbil isolate Kurdistan/2011 was also grouped in lineage 4 but it originated from different route, so it has different sub lineage from ppr3/Kurdistan /2013. fig (4).

## IV. DISCUSSION

PPR is a serious economic disease. Several outbreaks were occurred in Iraq, two of them were in 2000 and 2001(11, 8) PPR in Iraq most often diagnosed based on clinical symptoms and serological test (27). The present study described an outbreak of PPR in sheep in sulaimani province Kurdistan region during 2012-2013.

To understand the epidemiology of these PPRV outbreaks in Iraq, it is important to perform genetic characterization of these viruses. Therefore classification of PPRV intolineages has broadened our understanding of the molecular epidemiology and worldwide movement of PPR viruses. PPR outbreak in this study was confirmed based on molecular diagnosis by using conventional reverse transcription PCR and sequence analysis of the virus (13).

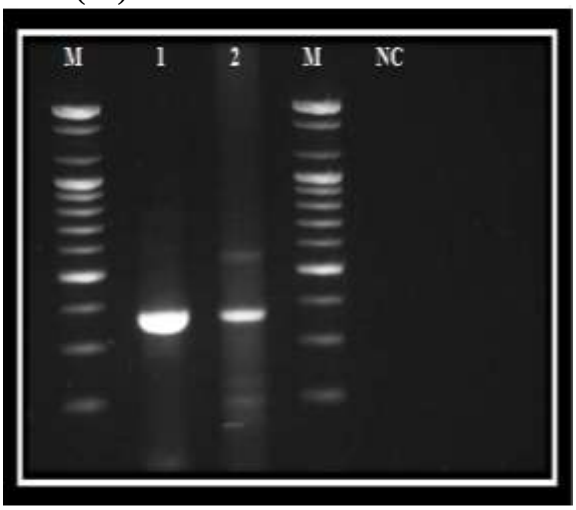


**Fig. 1: agarose gel electrophoresis of amplified PCR product using specific primers for PPRV F gene. M;100bp DNA ladder, Lane 1& 2 showing 372 bp. Lane NC, negative control.**


**Fig. 2: Ethidium bromide stained agarose gel of amplified PCR product using specific primers for PPR virus N gene. M; 100bp DNA ladder, lane NC, negative control and Lane 1: showing 463 bp of clinical sample**

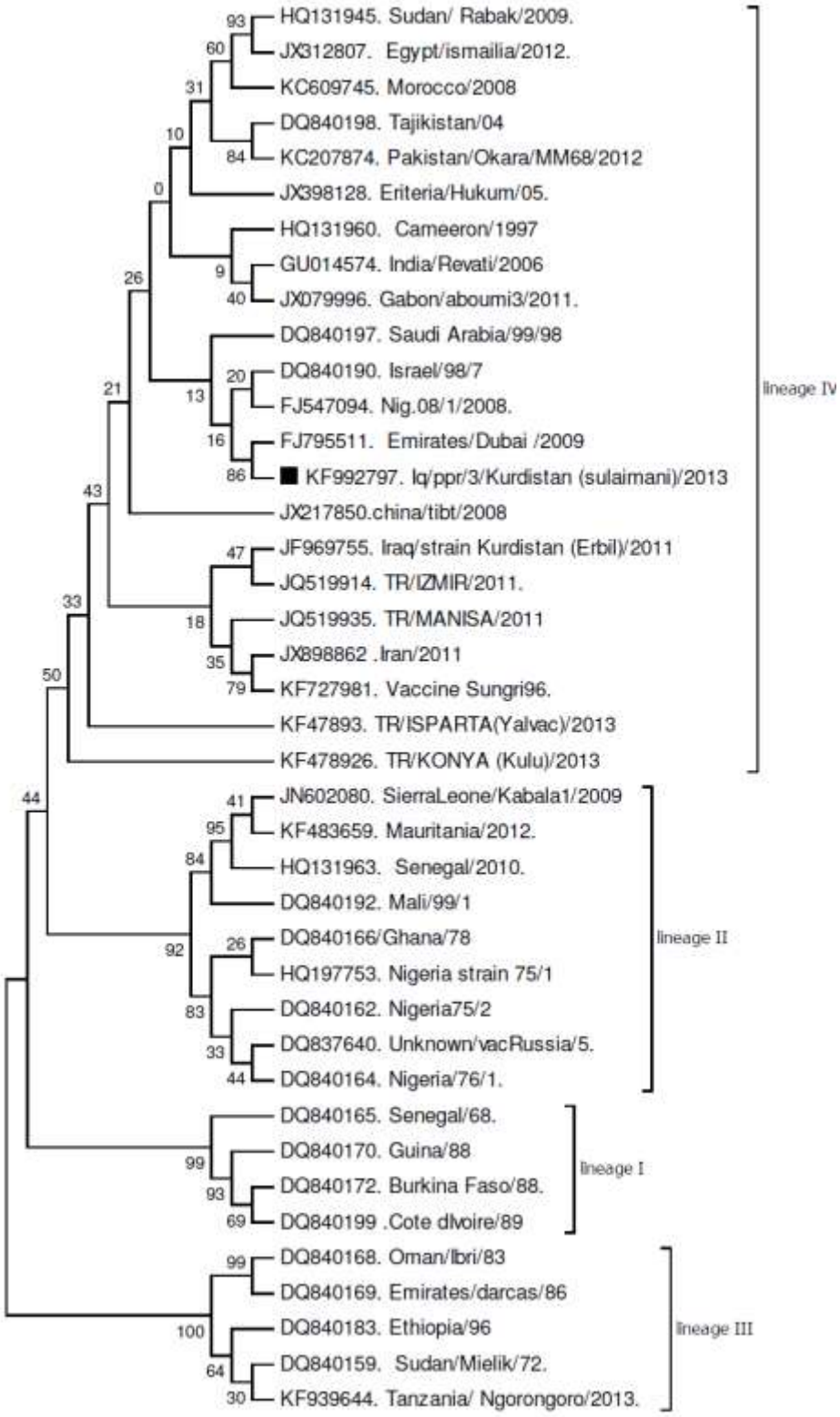


**Fig. 3 : Phylogenetic tree between PPRV detected in Sulaimani in 2013 and PPRV sequences found in GenBank.. the analyses of phylogenetic tree was according to N gene and it was indicated 4 lineage .the black square indicate ppr3/Kurdistan/2013 isolate which was in lineage IV**

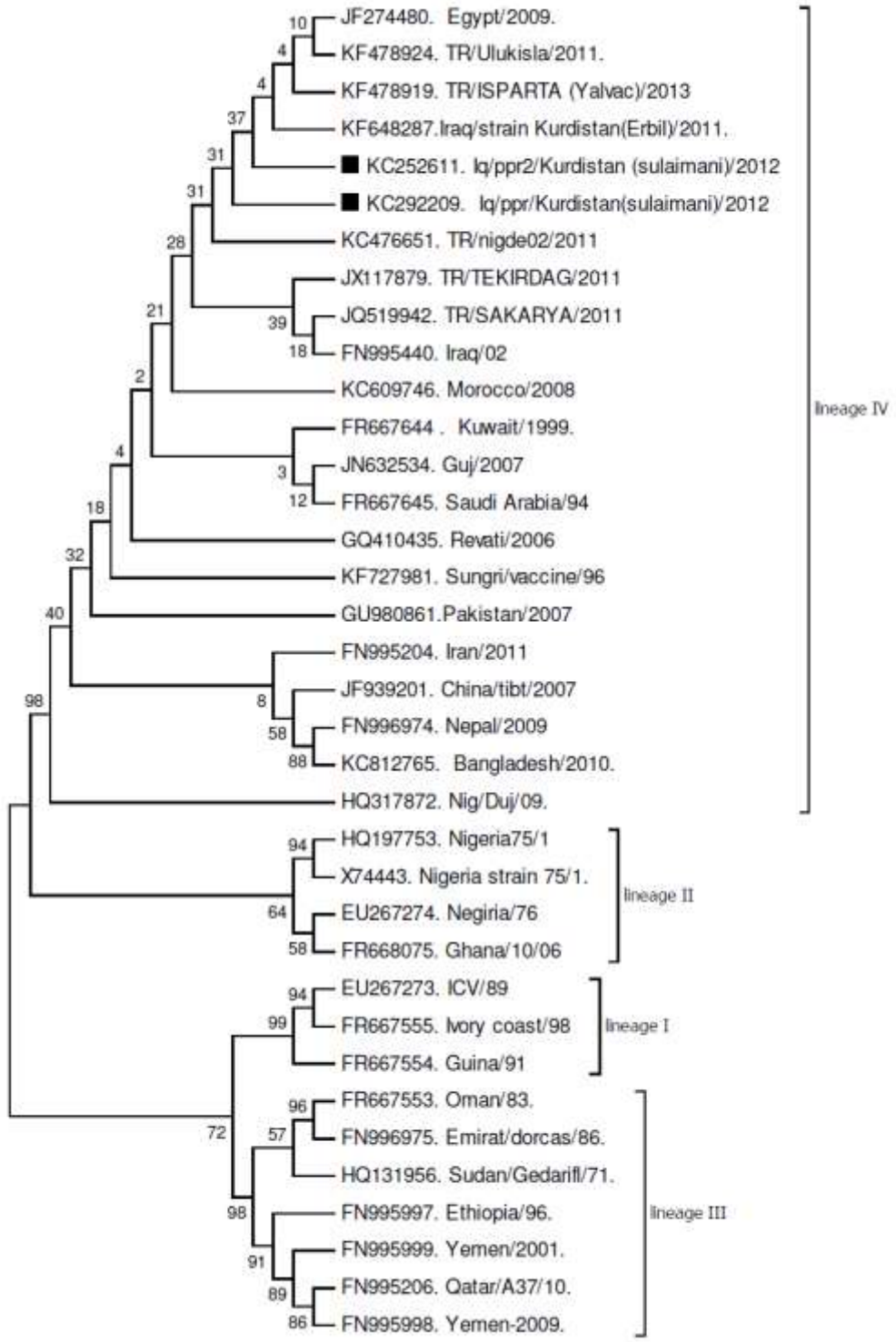


**Fig. 4: Phylogenetic tree showing the relationships among the PPRV sequences. Analyses of phylogenetic tree was based on partial sequence of fusion protein gene and it indicate 4 lineage. The black square indicating ppr/Kurdistan/2012 and ppr2/Kurdistan/2012 isolate which was in lineage IV**

**Table 2: F gene sequence identity of Sulaimani isolate with others from Gene-bank**

| NO. | Accession No. | Country/year | Identity F gene sequence |
|---|---|---|---|
| 1 | KF478924 | Turkey/2011 | 99% |
| 2 | KF648287 | Iraq-Erbil/2011 | 99% |
| 3 | JF274480 | Egypt/2009 | 99% |
| 4 | FN995440 | Iraq/ 2002 | 97--98% |
| 5 | FN995204 | Iran /2010 | 97-96% |
| 6 | KF727981 | Sun-gri/vaccine/96 | 97-98% |
| 7 | HQ197753 | Nigeria 75/1 | 92-93% |

**Table 3 : N gene sequence identity of Sulaimani isolate with others from Gene-bank.**

| No. | Accession No. | Country/year | sequence Identity N gene |
|---|---|---|---|
| 1 | FJ795511 | Emirate/2009 | 99% |
| 2 | KF969755 | Iraq/Erbil/2001 | 96% |
| 3 | DQ840197 | Saudi Arabia/99/98 | 98% |
| 4 | DQ840190 | Israel/98 | 97% |
| 5 | JX898862 | Iran /2011 | 97% |
| 6 | KF478931 | Turkey/2011 | 94% |
| 7 | KF727981 | Sungri-vaccin/96 | 94% |
| 8 | HQ197753 | Nigeria75/1 | 89% |

Close genetic relationship of Iraqi PPRV isolates with those of turkey, Israel Egypt (4), Saudi Arabia and Iran and the previous out break in wild goat in Erbil (8) suggest that the disease outbreaks have been caused by the same strain with different sources of transmittion The border relationships between these countries , animal trading both legal and illegal , transit shipment of animal from different country are probably the cause of transmission of closely related PPRV isolates (4).

In Iraq, vaccination is performed as a part of control action in certain endemic area. However the used PPR vaccine is based on Nig75/1 which is in lineage II, and the circulating field isolates in Iraq according to the phylogenetic tree and sequence analysis are grouped in lineage IV. It may be better to use a domestic strain for vaccination as has been practiced in other country. Therefore, it is crucial to have good genetic characterization of PPRV in Iraq. For this purpose, RT-PCR and sequencing is recommended to be added to regular serological surveillance in all over the country (13, 16).

In conclusion, data from this study have shown that, lineage IV of PPRV is currently circulating in the country. It was demonstrating also population of PPRV in the same lineage which warrants future need to perform such studies at country level to ascertain the complete picture of circulating viruses in the country. Such understating is crucial for devising future control plans.

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