



Molecular characterization of circulating strains of the peste-des-petitis-ruminants virus in Sulaimani province, Iraq

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Abstract

Peste des petits ruminants (PPR) are caused by a *Morbillivirus* that belongs to the family *Paramyxoviridae*. Peste des petits ruminants are an acute, highly contagious, and deadly disease that mainly affects goats and sheep, with subclinical infection occurring in cattle. Peste des petits ruminants occur in many countries, with morbidity and fatality rates as high as 90 percent. Peste des petits ruminants' outbreaks occurred annually in Sulaymaniyah; this study aims to determine genetic variation and the global relationship of circulating strains in the area. RT-PCR was primarily used to identify the virus from mouth epithelial samples. The direct sequencing and subsequent analysis of amplified PCR products for the (N and F) genes revealed the presence of PPRV lineage IV in the study areas. Moreover, phylogenetic analysis revealed N gene (OL702851/PPR/H/SUL/sheep, OL702852/PPR/H/SUL2/Goat) cluster with (china-KP319027.1) strain with identity 98%, while the F gene (PPR/H/SUL3/Sheep, PPR/H/SUL4/Goat) has a cluster with (Turkey-MG744218 and Saudi Arabia-MK922470.1) strains with identities 97 and 98% respectively. Furthermore, phylogenetic tree field virus isolates heterologous with the current vaccine strain (Nigeria 75/1) were used for the mass vaccination program, and the divergence rate was 10%-13%. These variations between vaccines and field isolates could compromise the antibody responses induced by the vaccines in the hosts.

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Introduction

Peste des petits ruminants (PPR) are a highly contagious viral disease of wild and domestic small ruminants (1). Although cattle, swine, camels, and buffaloes can be infected with the PPR virus (2-5). Since it was first identified in Ivory Coast (West Africa) in 1942 (6), its geographic distribution has been expanding within Senegal (7), China (8), India (9,10), Pakistan (11), East Africa (12), Egypt (13), Turkey (14,15), Iran (16), Ethiopia (17,18) Bangladesh (19), Nepal (20), Saudi Arabia (21) and Iraq (22). Peste des petits ruminant's virus (PPRV), an RNA virus from the genus *Morbillivirus* in the *Paramyxoviridae* family, causes the disease (23-25). The genome of PPRV consists of 15,948 nucleotides (1) which encode two non-structural proteins, C and V, and six structural proteins arranged in the order

nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin (H) and viral RNA-dependent RNA polymerase (L) (26). Developing essential molecular diagnostic techniques for PPRV was also aided by sequencing the N and F genes (27). PPRV strains have been classified into four genetically distinct lineages (I, II, III, and IV) based on partial sequences of the N and F genes (28). The acute form of PPR is marked by erosive mouth (erosive stomatitis) with frothy salivation (29), sudden depression, high fever, pneumonia, oculo-nasal discharges, oral necrotic lesions, diarrhea, and dark red spots (congestion) were discovered in several lobes of the lungs, as well as the small and large intestines, during the postmortem examination (30,31). The disease often shows high rates of infertility, and production of underweighted-lambs in young animal, but in mature animal exhibit abortion, stillbirth and lambs born

weak, uncharacteristic body shapes and under-sized (32). The virus is predominantly transmitted by oral, nasal, and conjunctiva secretions and stool of the infected animals, which contain high concentrations of virus. The virus is highly infectious and quickly spreads through direct contact between infected animals' secretions and/or excretions and healthy animals nearby (33). Infectious viral infections are hard to control due to a lack of epidemiological data and different management techniques in the herds where the problems occur (34), but the availability of live attenuated PPRV vaccinations that protect animals from recurrent infection is helping to eradicate the PPRV (e.g., Nigeria/75) (35).

The current study's objective was to genetically describe the PPRV isolate in Sulaimani, Iraq, and to compare its sequence to that of the global PPRV and the existing commercial vaccine strain.

Materials and methods

Ethical approve

Research proposal was accepted and registered under (No. 11 on 27/ 6/ 2021) by the ethics committee of Veterinary Medicine College/ University of Sulaymaniyah.

Sample collection

Throughout March to July 2021, fifty sick sheep and goats with fever, profuse oculo-nasal discharge, moderate ulcerative stomatitis, dyspnea, coughing, diarrhea, and inappetence had their oral swabs and mouth lesions collected from (Halabja and Penjwen) region in Sulaymaniyah province for RT-PCR detection of PPRV RNA. This study selected (Halabja and Penjwen) districts (Figure 1). Since the area is mountainous and has a cold environment, especially in the summer, people who own sheep and goats migrate there to raise their animals. These two locations were chosen for sampling for two reasons: first, there is a greater possibility of a disease outbreak because animals from different areas migrate there; second, it borders Iran.

Extraction of RNA

All samples were extracted by using an RNA extraction tissue kit (Genet Bio, Daejeon, South Korea). The extraction had done according to the manufacturer's protocol.

Oligonucleotides primer

In this study, two primer sets were used. First, the F gene primer set (F1: ATCACAGTGTT AAAGCCTGTA GAGG /F2: GAGACTGAGT TTGTGACCTAC AAGC) was designed for fusion gene of the virus that amplifies 372bp (36). The second primer set N gene: Forward primers N1 (5'-GATGGTCAGA AGATCTGCA 3') and reverse primer N2 (5'-CTTGTCTGTTGT AGACCTGA-3) was amplified partial nucleocapsid gene in PPRV with amplicon size 463bp. The primers were synthesized by (Macrogen Co., Seoul, South Korea) (37).

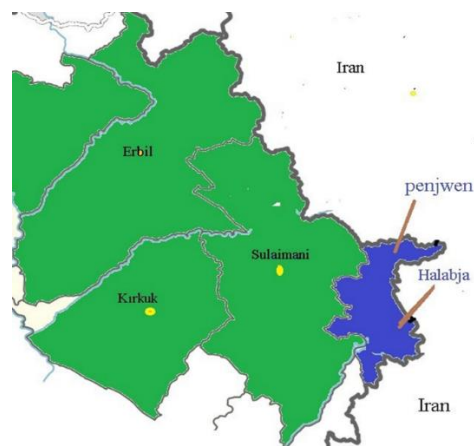


Figure 1: Halabja and Penjwen districts were selected in the current study for the geographic area (Blue zone) where samples were collected.

Complementary DNA (cDNA) and PCR

A partial sequence of the (N, and F) gene was amplified by using one-step RT-PCR Premix (2X) (GeNet Bio). The reactions were carried out in a 0.20 mL PCR tube based on the following specifications: 10.00 μ L RT-PCR premix, 5.00 μ L RNA, 1.00 μ L forward (10.00 pmol), 1.00 μ L reverse primers (10.00 pmol), and 3.00 μ L molecular grade H₂O to make up a final volume of 20.00 μ L. Partial sequence of F and N genes were amplified separately by using a conventional PCR machine (Hercuvan, Carlsbad, USA) that was programmed as follows: cDNA synthesis 50.00 $^{\circ}$ C for 30 min, initial denaturation at 95.00 $^{\circ}$ C for 10 min followed by 40 cycles of 95.00 $^{\circ}$ C for 45-sec annealing at 58.00 $^{\circ}$ C for 45 sec, and extension at 72.00 $^{\circ}$ C for 1 min and a final extension at 72.00 $^{\circ}$ C for 10 min. Both primers set was the same PCR programmed except in annealing step 60 $^{\circ}$ C for N gene protein. The PCR products were analyzed by loading 7.00 μ L on standard 1.00% (w/v) agarose gel (Gendirex, Daejeon, South Korea) in 1X Tris/Borate/EDTA (TBE) buffer. The gel was stained with 4.00 μ L safe dye (EURx, Banino, Poland). Electrophoresis was done on 100v for 1 hr. The PCR product was visualized under a UV trans-illuminator (Uvitec, Cambridge, UK).

Phylogenetic analysis

The PPRV sequences obtained in this study, the sequences of Nigeria/75 vaccine strains available in Iraq, and the sequences retrieved from GenBank were aligned. The evolutionary history was inferred by employing the maximum-likelihood method based on the Tamura Nei parameter model implemented in the MEGA X program (38). The branch lengths were measured regarding the number of substitutions per site. A bootstrap test (1,000 replicates) was done to evaluate the confidence level of branching in the phylogenetic tree.

Results

Result of RT-PCR

Twenty (40%) of fifty samples were positive by agarose gel electrophoretic identification demonstrating an expected amplicon of about 372 bp and 464 bp for F and N genes, respectively (Figure 2). The results were confirmed by sequencing of PCR product, and the sequences were submitted to NCBI Genebank and released under these accession numbers (OL702851-OL702854).

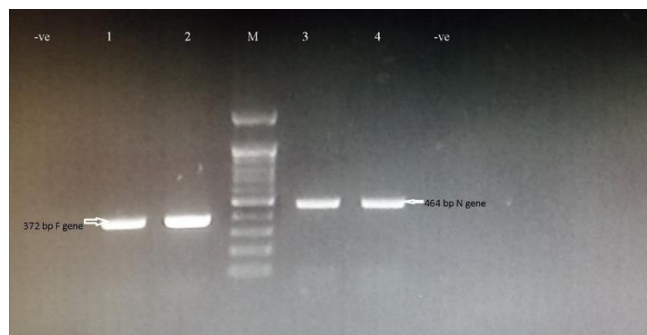


Figure 2: Agarose gel electrophoresis of PCR products using specific primers for N and F gene, M: DNA marker, -ve: negative control, lane 1, 2: positive samples 372 bp for F gene, lanes 3 and 4: positive samples 464 bp for N gene.

Phylogenetic tree and sequences analysis

Identities of 99.30 and 98.80% were observed among the N and F genes of Sulaymaniyah PPRVs, respectively, when analyzing the nucleotide sequences. The analysis of our two isolates for the N gene, which was published in NCBI GenBank, indicated that the 463 bp sequences of two Sulaymaniyah isolates (PPR/H/SUL3/Sheep and PPR/H/SUL4/Goat) have 97% homology with China strain (China/Shaanxi/2014) (Table 1). While 372 bp sequences of our two isolates for the F gene (PPR/H/SUL/Sheep and PPR/H/SUL2/Goat) have 98% homology with the Turkish strain (TR/MANISA/2011), and Saudi strain (Saudi Arabia/93/2017) (Table 2). According to phylogenetic tree construction based on the sequence of both N and F gene alignment of PPRV sequences retrieved from NCBI GenBank, all four PPRV isolates belong to lineage IV (Figures 3 and 4). A nucleotide sequence comparison between the vaccines strain (Nigeria/75) currently used for vaccination programs in Iraq and field strains revealed low identities ranging 87-92% for F and N genes, respectively. The phylogenetic tree revealed that PPRV Field strains belong to lineage IV while vaccine strains belong to lineage II. Compared to other sequences, including vaccination strains, multiple sequence alignment field viral sequences for both genes revealed numerous nucleotide substitutions (Figure 5). As a result, there were few identities and much variance among them.

Table 1: N gene sequence identity of Suleimani isolate with others from Gene-bank

NO.	Accession No.	Countries	Strain/Isolate	Identity %	Lineage
1	KF992797.1	Suleimani/Iraq	PPR/3/2013	95.69-95.01	IV
2	KP319027.1	China	China/Shaanxi/2014	97.86-97.14	IV
3	JQ519935.1	Turkey	TR/MANISA/2011	93.42-93.65	IV
4	GQ122186.1	India	Jabalpur 08	94.69-94.92	IV
5	MG748710.1	India	OD/Gan/2016	93.42-93.20	IV
6	DQ840168.1	Oman	Ibri/83	80.78-80.39	III
7	KY628761.1	Nigeria	75/1	87.30-87.53	II
8	KF483659.1	Mauritania	Mauritania5_2012	89.02-88.63	II
9	HQ131963.1	Senegal	Senegal10	89.41-89.02	II
10	DQ840172.1	Burkina Faso	88	84.71-84.31	I
11	DQ840170	Guinea	88	83.92-83.53	I

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

NO.	Accession No.	Countries	Strain/Isolate	Identity %	Lineage
1	KC252611.1	Suleimani/Iraq	PPR/2012	98.82- 98.76	IV
2	MG744218.1	Turkey	TR/Lamb/616	98.47- 98.14	IV
3	MK922470.1	Saudi Arabia	Saudi Arabia/93/2017	98.82 - 98.53	IV
4	FR667644.1	Kuwait	Kuwait_1999	98.82- 97.81	IV
5	KF648287.1	Erbil/Iraq	Erbil_2011	96.60 - 95.75	IV
6	MK242036.1	Uganda	Uga.2018_Looro014	87.61- 87.02	III
7	KJ124805.1	Benin: IAEA	Benin	92.07- 91.46	II
8	HQ19775.1	Nigeria	75/1	92.33- 91.15	II
9	KY628761.1	Nigeria	75/1	90.00- 89.39	II
10	EU267273.1	Coted'Ivoire	ICV89	88.79- 87.61	I

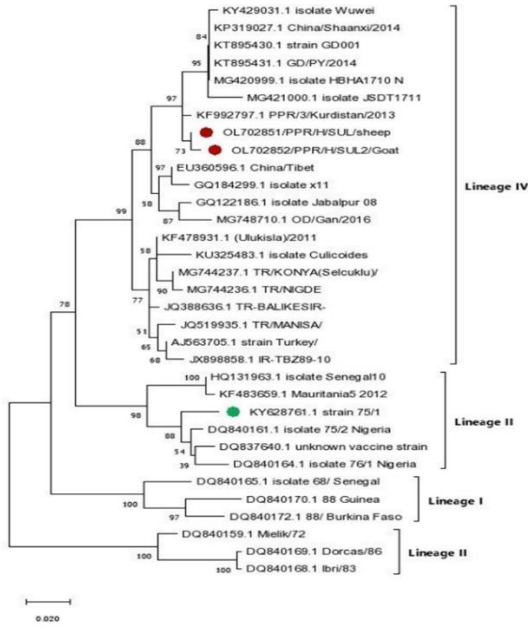


Figure 3: Neighbor-Joining method phylogenetic tree relationship among PPR isolates. N genes are obtained from sheep, goats sequenced in this study are shown with solid circles (Red), and the vaccine strains are shown with solid rectangles (Green).

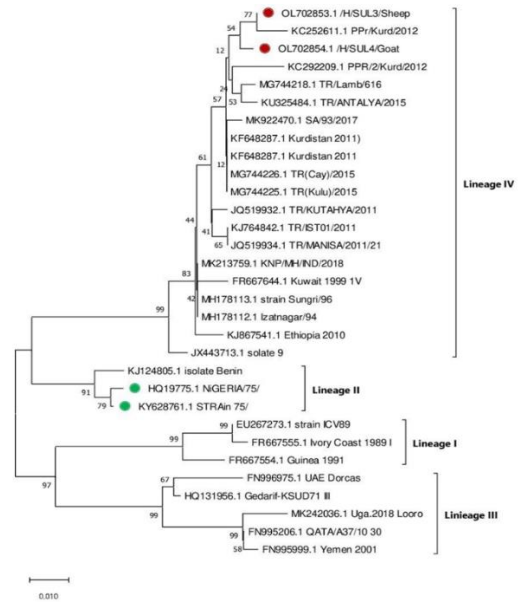


Figure 4: Neighbor-Joining method phylogenetic tree relationship among PPR isolates. F genes are obtained from sheep and goats sequenced in this study are shown with solid circles (Red) and the vaccine strains are shown with solid rectangle (Green).

Discussion

The field data and laboratory results implicate PPR in the Sulaymaniyah province. Based on the RT-PCR which is the most sensitive assay for confirming PPR diagnosis (39). The ability to distinguish between distinct PPR lineages, more precisely identify the origin of outbreaks, and better comprehend the epidemiology of the PPR virus are all made possible by nucleotide sequencing of the PCR product (40). According to sequencing results, the most likely cause is a PPRV field strain that has been circulating in the region and Sulaymaniyah province for over 10 years. There is a 95-98% similarity between our isolates and the Sulaymaniyah isolates of 2012 (41). Iraq has borders with countries where several economically critical infectious diseases are endemic, particularly Turkey, making it difficult to control the introduction of infectious diseases into the country. As a result, the importance of PPRV as a threat to livestock, and other economically significant illnesses, should be evaluated, and actions should be taken to prevent illegal trade with neighboring countries and the entry of animals without quarantine (42). Our use of molecular epidemiologic techniques provided data suggesting cross-border transmission into Sulaymaniyah governorate, northern Iraq, of PPRV infection circulating in neighboring countries. The viruses we isolated are PPRV lineage 4, which includes viruses whose origins are in the Middle East, Saudi Arabia, and south Asia.

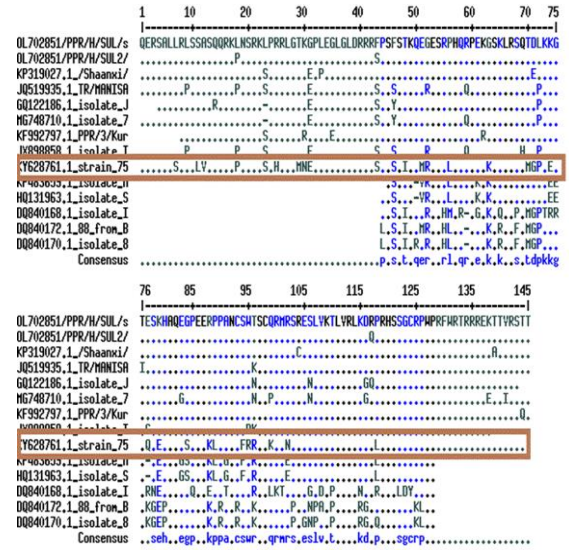


Figure 5: Multiple sequence alignment of the amino acid residue of partial N gene of two fields isolates PPRV with currently available vaccine strain Nigeria/75, (high light in box) and other reference strain in different countries.

For the control of PPR, attenuated live cell culture vaccines are available commercially. Live attenuating PPRV Nigeria 75/1 isolate vaccine, which belongs to Lineage I (43), is currently used in Iraq. However, current and recent studies in Iraq revealed that Iraqi field strains belonged to

Lineage IV, necessitating the development of a specific vaccine by attenuating field isolates of PPRV, and proper administration technique, proper handling, and storage should be followed. The small ruminant populations in the Iraq have not higher protection because of the use of an exotic strain for vaccination in the PPR program (44). Vaccinating ewes during pregnancy with the PPR vaccine is very effective in protecting newborn lambs from the infection (45).

Similar studies are constantly needed in Iraq's disease-endemic regions to improve disease prevention and control methods and prevent the establishment of new outbreaks of certain virus strains. In order to provide appropriate control measures, national effort must be undertaken to develop an alternative vaccination strain in place of Nigeria/75 that has less variability and better antigenic matching with the lineage IV field strain.

Conclusion

This study provided the valuable information to describe the PPR virus strains that exist in Iraq and provided an explanation for why the virus continues to spread throughout the countryside despite the use of regular vaccination in small ruminant populations; this may be due to the field virus isolate heterologous with the current vaccine strain used for the vaccination program, and the divergence rate is between 8 and 13 percent. The outcome of this study allowed us to create an effective vaccination against the virus strain currently infecting fields, allowing us to control and eradicate the infection.

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Conflict of interest

The author declares that there are no conflicts of interest regarding the publication of this manuscript.

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التوصيف الجزيئي لسلاسلات فيروس طاعون المجترات المنتشرة في محافظة السليمانية/العراق

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الخلاصة

يعد فايروس موربي المسبب لطاعون المجترات الصغيرة حيث يصنف الفيروس ضمن عائلة فايروسات بارميكسو. إن طاعون المجترات الصغيرة هو مرض حاد شديد العدوى ومميت يصيب الماعز والأغنام بشكل رئيسي، فيما تكون العدوى غير واضحة في الماشية. يتواجد مرض طاعون المجترات الصغيرة في العديد من البلدان حيث تصل معدلات الإصابة بالمرض والوفيات إلى ٩٠ في المائة. وتعتبر مدينة السليمانية من المدن التي يتفشى فيها المرض سنويا. ولهذا جاءت هذه الدراسة التي هدفت الى تحديد التباين الجيني للسلاسلات المنتشرة في المنطقة ودراسة مدى الترابط الجيني مع العتر أو السلاسلات المنتشرة في العالم. تم استخدام RT-PCR للكشف عن وجود الفيروس في العينات المأخوذة من البطانة الظهارية للفم. وتم تحديد تتابع سلسلة الدنا الناتجة عن تضخيم الجينين N و F بواسطة تقنية PCR حيث أظهرت النتائج وجود سلالة طاعون المجترات الصغيرة الرابع في مناطق الدراسة. علاوة على ذلك، كشف التحليل الوراثي للتطور عن وجود ترابط وراثي بين الجين السليمانية N مع سلالة الصينية بنسبة تطابق تصل الى ٩٨٪، بينما وجد الترابط الوراثي لجين السليمانية F، مع السلاسلتين السعودية و التركية وبنسبة تطابق ٩٧% و ٩٨٪ على التوالي. بالإضافة إلى ذلك، أظهر تحليل شجرة النشوء والتطور لعزلات الفيروس الميدانية، على أنها غير متجانسة مع سلالة اللقاح الحالية (نيجيريا ١/٧٥) لبرنامج التطعيم الشامل، وكان معدل الاختلاف ١٠٪ - ١٣٪. أن هذه الاختلافات الجينية بين العزلات الميدانية والسلاسلات المستخدمة للتطعيم قد تضعف استجابات الأجسام المضادة المنتجة من اللقاحات في المضيف.