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Genetic analysis of sheep pox virus targeting host immunity evasive genes

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Abstract

The Capripoxvirus genus includes sheep pox virus (SPV), a contagious viral infection of small ruminants primarily affecting sheep. It is characterized by severe skin lesions, fever, diarrhea, difficulty breathing, pulmonary involvement, and death. This leads to enormous economic losses for sheep farmers. Recently, numerous cases of individual and multiple cases of SPV have been reported with failure of treatment and control strategies. This suggests that SPV may undergo genetic mutations affecting host immunity by blocking or inhibiting host immunity proteins more efficiently. Therefore, in this study, three host-immunity evasive viral genes, including soluble interferon-gamma receptor-like protein (IFN-γ), G-protein coupled chemokine receptor (GPCR), and inhibitory Major Histocompatibility Complex (MHC class 2) were sequenced. To do this, 125 suspected cases were examined and then subjected to PCR technique, which revealed 40 SPV positive 32%. The sequence results of most local strains revealed high similarity to the local vaccine strain, and other strains originated from neighboring countries such as Saudi Arabia and Turkey. However, some strains show genetic diversity. Notably, the designed primers efficiently diagnose SPV and could be useful in viral quantification for future studies. This may also explain the efficiency of the vaccination protocol in Iraq. This may help to improve our understanding of SPV infection and its control.

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Introduction

SPV is a contagious viral infection in sheep that, in endemic regions, commonly manifests in skin lesions with marked fever (1,2). However, it also characterized severe losses, particularly in young animals (3), and import and export restrictions on their byproducts (4,5). SPV is an encapsulated DNA virus with two strands belonging to the family Poxviridae (6). Its linear DNA genome is 130-280 kbp. This vast genome encodes every gene needed for its unique intracellular replication (7). Clinically, the illness is marked by high temperatures of 40-42ºC, conjunctivitis, and rhinitis with enhanced widespread numerous focal necrosis in cutaneous and inner tissues such as the lungs, digestive system, liver, and lymphadenopathy (8,9). Other signs include a decline in milk yield and weight loss, high rates of abortion, increased susceptibility to pneumonia, and high mortality. The geographical distribution of sheep pox has been reported worldwide, and it is endemic in many countries, including China, Nepal, Bangladesh, Iraq, Iran, Turkey, Pakistan, India, and Afghanistan. Individual epidemics are occurring in Southern Europe and other areas of the world due to considerable trade with other international countries (10,11). Poxviruses express host genes through genetic recombination and can evade the host's immune system (12). One pathway includes that IFN-γ regulates the immunological response of its host cell (13). G-protein coupled chemokine receptor GPCRs or Guanine nucleotide-binding proteins are a group of proteins that have a pivotal role in the cells. Furthermore, MHC class II-restricted antigen presentation is needed for CD^{4+} T cell-dependent immunological responses, which are required to

form an effective and specific immune response (14). There are few studies on sequence analysis of SPV in Iraq (15).

This study aims to detect the SPV by molecular approach targeting multiple genes, including IFN-γ, GPCR, and MHC class II, and compare them with other global sequences, including the vaccine strain. This could help understand SPV strategies for tackling host immunity.

Materials and methods

Ethical approve

The primary studies under which the samples were collected received ethical clearance from Veterinary Medical Ethics Committee of University of Al-Qadisiya with approval number 375/2023.

Clinical samples

This is the study that has been carried out. In Diwaniyah, Iraq (Al Sannih, Daghara, and Al Badir) from September 2023 to January 2024, a total of 600 Awassi and Al Nuaimi sheep of different ages were examined, and 125 of them were found to be suspected of being infected with SPV. Suspected sheep showed high temperature and skin lesions characterized by 0.25-3 cm of hyperemia and multiple nodular skin lesions widely distributed over the body, especially under the tail, head, perineum, axillae, and udder. In some areas, these spots developed into papules without vesicles that form scabby tissues. Samples were collected aseptically, transported to the laboratory using the cold chain protocol, and maintained at -20ºC until utilized for molecular technique.

Genomic DNA extraction

Skin lesions were put into a Sterilized Petri plate. Minced into smaller pieces with sterile scissors, Transfer to a sterile 1.5ml microcentrifuge tube, homogenize using tissue lysis buffer (AddBio, South Korea), and purified using a silica-based column according to the manufacturer's instructions (ADDBio, South Korea). Eluted DNA was kept at -20 °C until further analysis.

Polymerase chain reaction (PCR)

Amplification of the targeted genes using primers designed in this study (Table 1), specific to SPV. The reaction consists of PCR master mix (ADDBio, South Korea) including 0.4 µl of Taq polymerase, two µl of dNTPs, 6.6 µl of 10X PCR buffer with two µl of each primer (for IFN-γ, GPCR, and MHC class 2) and two µl of template viral DNA, and seven µl of PCR water. PCR thermocycler conditions were done using a conventional PCR thermocycler system, as shown in (Table 2). PCR products were electrophoresed (at 100 volts and 80 AM for 1 hour) by agarose gel 1.6%, stained with ethidium bromide, and illuminated by a gel documentation system (Syngene, Taiwan).

Table 1: Three sets of primers were designed in this study targeting the IFN-γ, GPCR, and MHC class 2 genes of sheep pox virus

Target gene		Sequence '5-------3'	Size	Accession number	Start	End
IFN-γ	Forward	CCGAATTCGCCGTTTAAGCA	563 bp	MN072630	106	125
	Reverse	GCGGAGAATGGAAACAAGCA			668	649
GPCR	Forward	ATCCCACACTGTGATGATGGT	889 bp	KT438551	217	237
	Reverse	CAACACTACTGGTGCTACGC			1105	1086
MHC class 2	Forward	ACTTCATTTTCAACAAAGACGAGGA	541 bp	MN072626	11	35
	Reverse	TCCACAATACACCACCCACT			551	532

Table 2: PCR thermocycler conditions

PCR step	Temp.	Time	Cycle
Initial Denaturation	95°C	3min	1
Denaturation	95°C	35sec	
Annealing	55°C	35sec	39
Extension	72°C	35sec	
Final extension	72°C	5min	1

DNA Sequencing method

Ten samples from each gene were chosen from the positive PCR samples for DNA Sanger sequencing via bidirectional sequence by Macrogen (South Korea). They were slightly trimmed from noise signals and then analyzed for phylogeny using SNAP Gene software. These sequences were submitted to NCBI to obtain accession numbers.

Phylogenetic analysis

A phylogenetic tree and Multiple sequence alignments were constructed using partial sequences of the three genes of local SPV strains using (Mega X software) by maximum likelihood (Tamura-Nei model) with 1000 bootstrap.

Statistical analysis

Chi-square (x^2) and t-tests were used to demonstrate the significant differences between infected and non-infected sheep at a statistical level of $P<0.05$ (16).

Results

Infection percentage

The PCR findings were detected in 40 samples based on 125 sheep of both gender genders and ages selected. The result revealed that 32%. At the same time, 85 samples (68%) were found negative for sheeppox viral DNA (Table 3). As shown in (Table 4); the positively infected sheep were classified into four age categories; according to their gender, out of 43 males, 15 sheep were positive for SPV infection by PCR, as depicted in (Table 5). Statistically, there was a significant difference between Positive and negative cases.

Table 3: Percentages of positive sheep pox cases by PCR

Values	Sheep n (%)
Infected sheep	40 (32%)
Non-infected sheep	85 (68%)
Total	125 (100%)
Chi-square value	32.4
P-value	<0.0001 (HS)

HS: Highly significant difference at P<0.01.

Table 4: Positive cases in sheep according to sheep age

Age (month)	Total number	Sheep n (%)
<1-6	20	6 (30%)
7-12	54	22 (37.03%)
13-18	26	8 (30.76%)
19-24	25	4 (16%)
Total	125	40 (32%)
x^2	4.89	
P value	0.180 (NS)	

Table 5: Positive cases according to sheep gender

Age (month)	Total number	Sheep n (%)
Male	43	15 (34.88%)
Female	82	25 (30.48%)
Total	125	40 (100%)
x^2	0.251	
P value	0.617 (NS)	

Detection of sheep pox virus genes by conventional PCR Assay

The PCR results were detectable in 40 samples out of 125 suspected sheep. Ten samples from the positive PCR technique were sequenced for IFN-γ, GPCR, and MHC class 2 genes. After electrophoresis, the results out of 125 skin lesions, 40 samples produced bands with anticipated sizes of 536 bp (Figure 1), 889 bp (Figure 2), and 541 bp (Figure 3) that corresponded to the universal ladder at 200-1500 bp. In comparison, 85 samples of skin lesions 68% tested negative for sheep pox virus DNA by PCR. The detection of SPV viral DNA in animals suffering from skin symptoms was statistically significant compared to animals with suspected infection.

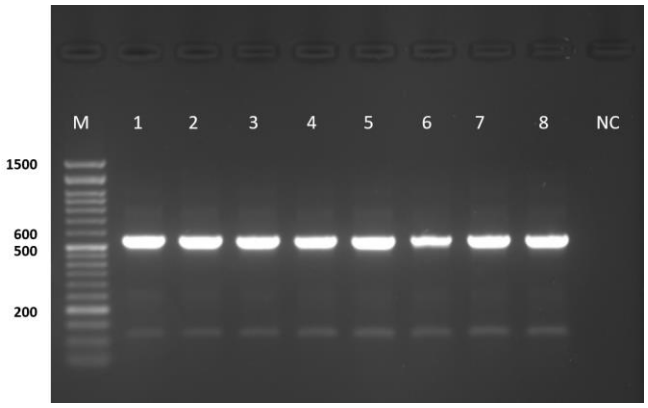

Figure 1: An agarose gel electrophoresis image (1.6 % agarose) shows positive amplicons (1-8) of sheep pox virus targeting a partial region of the Interferon-gamma receptor gene (size = 563 bp). NC is a negative control in which similar PCR conditions were used, except H2O was added instead of DNA.

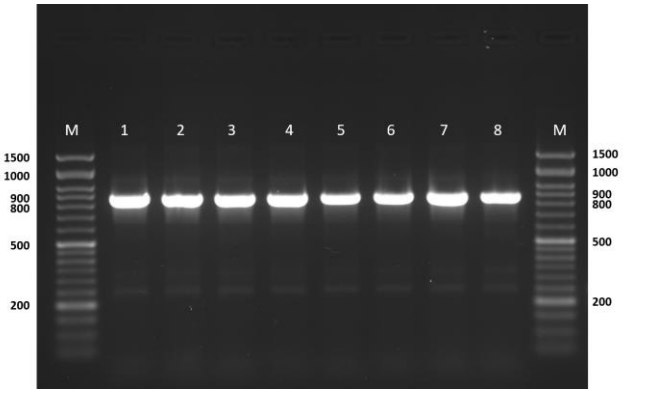

Figure 2: An Agarose gel electrophoresis image (1.6 % agarose) showing positive amplicons (1-8) of the pox virus targeting a partial region of the G-coupled protein receptor gene (size = 889 bp). M is a molecular marker from Genedirx (South Korea).

Gene sequence and phylogenetic analysis

In the IFN-γ gene sequence, ten local strains were submitted in the NCBI database, including OR542752, OR542753, OR542754, OR542755, OR542756, OR542757, OR542758, OR542759, OR542760, and OR542761 (Table 6). These were analyzed and compared to NCBI Gen Bank, which showed some genetic variances between the identified strains and retrieved sequences from NCBI. Phylogenetic tree analysis showed that there was a similarity between locally detected strain and global stain (99.52-100%) (Figure

4). Eight sequences (accession no. OR542752, OR542753, OR542754, OR542755, OR542757, OR542758, OR542759, OR542760) have the same identity 100% in comparison with homologs global sequence in China, Abu Charb, Saudi Arabia, and Turkey, and the sequence OR542756 and OR542761 have identity at 99.52% in China and Abu Charib.

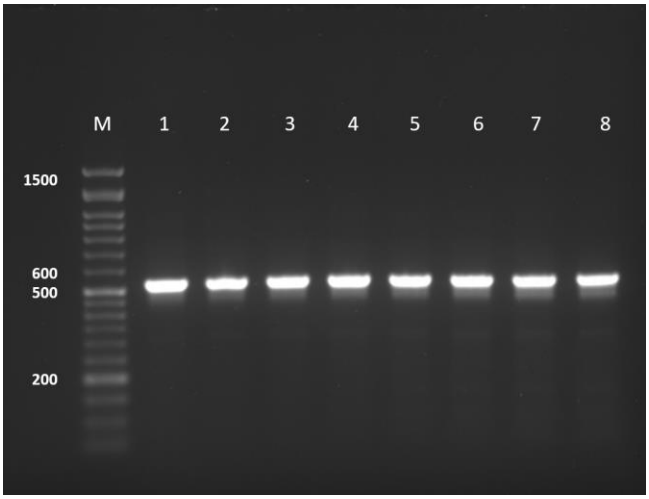

Figure 3: An agarose gel electrophoresis image (1.6 % agarose) shows positive amplicons (1-8) of sheep pox virus targeting a partial region of the MHC Class 2 Presentation Inhibitor gene (size = 541 bp). NC is a negative control in which similar PCR conditions were used, except H2O was added instead of DNA.

In GPCR phylogenetic analysis, the local SPV strains with accession numbers (OR542742, OR542743, OR542744, OR542745, and OR542746) are 100% similar to Turkey's global sequence, while the sequence strains (OR542747, OR542748, OR542749, OR542750, and OR542751) are 99.68% homology with Turkey's global sequence, Abu Gharib, Saudi Arabia (Figure 5). Ten SPV local strains (OR542762, OR542763, OR542764, OR542765, OR542766, OR542767, OR542768, OR542769, OR542770, and OR542771) were compared to NCBI Gen Bank SPV strain found in the MHC class 2 gene. The results revealed variance between the local strains and retrieved sequence from NCBI as nine sequences with (accession no. OR542762, OR542763, OR542764, OR542765, OR542767, OR542768, OR542769, OR54270, OR54271) share 99.79% identity with homology with China, Abu Charb, Saudi Arabia, Turkey, and Nigeria, with the sequence OR542766 sharing 100% identity with Abu Charib strain (Figure 6).

Multiple sequence alignment

As shown in table 7, in the alignment of INF-gamma receptor, GPCR, and MHC Class2 presentation inhibitor genes, there were some genetic replacement mutations. According to the NCBI-Blast that explained the relationship of Iraqi strains with global strains, Interferon gamma receptor, in which a high similarity between the sites nucleated number 54-60, 22-26, and 4-8, conserved for specific changes in particular nucleotides in distinct sites of the gene. In sheep pox -OR542761-sequence 10, A (adenine) replaces T (Thymine) in other strains in the alignment. At site 376 in sheep pox -OR542756-sequence 5, A (adenine) was nucleated instead of T (Thymine) while aligning the G-coupled protein receptor gene, there is a high degree of similarity between the nucleated sites 5-9 except for certain changes in specific nucleotides in distinct locations of the gene sequence. Different nucleotides, C (Cytosine) instead of T in three sequences (OR542748, OR542749, OR542750), were examined by Mega X. The alignment of the MHC Class 2 Presentation Inhibitor gene sequence of sheep pox revealed considerable similarity between the nucleated number 15-24 and 302-309, conserved for specific changes in particular nucleotides at distinct gene locations. Sequence no. 332 is different in (OR542762, OR542763, OR542764, OR542765, OR542766, and OR542771) in which C instead of A in other strains in the alignment. The phylogenetic tree study used partial sequencing of IFN-γ, GPCR, and MHC class 2 genes from local SPV strains for genetic validation.

Table 6: Local SPV strains with their accession numbers

No	Local strains	Accession number		
		IFN-γ gene	GPCR gene	MHC CLASS II gene
1	Sheep pox virus	OR542752	OR542742	OR542762
2	Sheep pox virus	OR542753	OR542743	OR542763
3	Sheep pox virus	OR542754	OR542744	OR542764
4	Sheep pox virus	OR542755	OR542745	OR542765
5	Sheep pox virus	OR542756	OR542746	OR542766
6	Sheep pox virus	OR542757	OR542747	OR542767
7	Sheep pox virus	OR542758	OR542748	OR542768
8	Sheep pox virus	OR542759	OR542749	OR542769
9	Sheep pox virus	OR542760	OR542750	OR542770
10	Sheep pox virus	OR542761	OR542751	OR542771

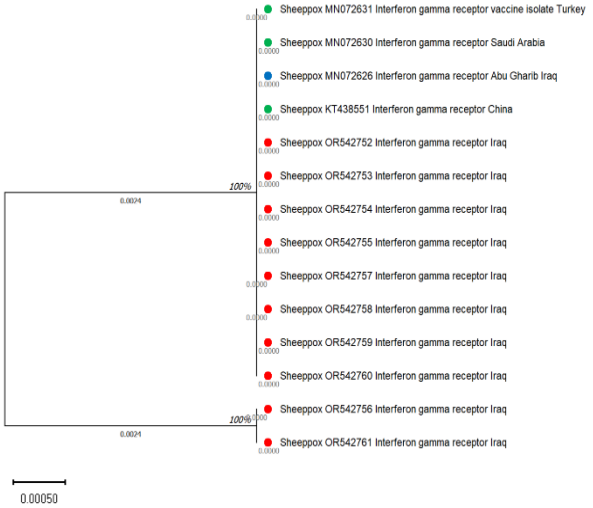

Figure 4: Evolutionary analysis by the Maximum Likelihood method of sheep pox virus (Interferon gamma receptor gene). This was inferred by the Tamura-Nei model and drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches). This analysis involved 14 nucleotide sequences. The final dataset had 414 positions. Evolutionary analyses were conducted in MEGA11.

Figure 5: Evolutionary analysis by the Maximum Likelihood method of sheep pox virus (G-coupled protein receptor gene). This was inferred by the Tamura-Nei model and drawn to scale with branch lengths measured in the number of substitutions per site (below the branches). This analysis involved 13 nucleotide sequences. The final dataset had a total of 312 positions. Evolutionary analyses were conducted in MEGA11.

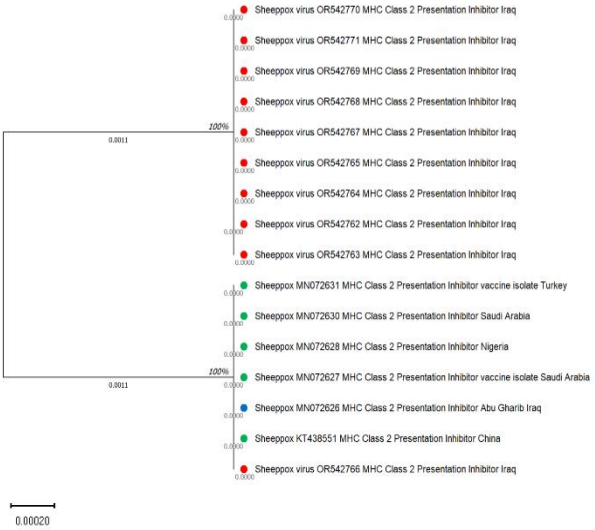

Figure 6: Evolutionary analysis by the Maximum Likelihood method of sheep pox virus (MHC Class 2 Presentation Inhibitor gene). This was inferred by the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches). This analysis involved 16 nucleotide sequences. The final dataset had 471 positions. Evolutionary analyses were conducted in MEGA11.

Discussion

This study showed that some local strains have point mutations in their nucleotides. However, others are highly similar. PCR results demonstrated that positive samples within different areas of Al-Diwaniya reached 32%. This result agrees with another studies Chala (17) that recorded 31.3%. A similar finding was observed by Muhaidi (18), which recorded a morbidity rate of 36% in different ages from Al-Diwaniyah (19). In Duhok Governorate, the morbidity rate was 30% in lambs aged 2-4 months.

The results showed that SPV infection reached 32 %. This means that the studied area is endemic to SPV infection, which agrees with other researchers Muhaidi and Zangana (18,19) who found that the viral dissemination in the environment, the management system of the herd, and the host's immune status are the main reasons for SPV endemicity. Moreover, pox viruses have developed several pathways to overcome innate and acquired host immunity.

This occurs by encoding numerous immunomodulatory proteins, including interferons, chemokines, and cytokines (20). The sequence results showed a high level of similarity, showing that the sequences were identical and offering a greater degree of trust. Special primers were used to achieve the objectives of this study. A partial area of the SPV IFN-γ, GPCR, and MHC class 2 genes was used to suspect clinical cases with numerous skin lesions. These findings are

consistent with earlier studies, as the GPCR gene had been used to diagnose LSDV disease in Thailand (21), Egypt (22), and Uganda (23), as well as other African and Asian countries (24). Characterization of the MHC class 2 encoded gene was well-studied in India and Jordan to demonstrate its pivotal role in host immunity against infections (25,26).

Previous studies in China focused on molecular diagnostics of the IFN-γ gene in ducks, geese, and sheep (27,28). An efficient evading strategy was demonstrated in many viruses, such as herpesviruses and poxviruses (29). Finally, PCR is an ideal technique to detect a wide range of viral infections and demonstrate their pathogenesis (30-40).

Table 7: The site of mutation in the multiple sequence alignment analysis

Gene	Obtained Accession number	Site of mutation
Interferon-gamma receptor	OR542761-sequence 10 OR542756-sequence 5	A (adenine) replaces T (thymine)
G-coupled protein receptor	OR542748 OR542749 OR542750	C (Cytosine) instead of T
MHC class 2 presentation inhibitor	OR542762 OR542763 OR542764 OR542765 OR542766 OR542771	C instead of A (adenine)

Conclusion

This study concludes that SPV strains in Iraq exhibited a substantial similarity with strains worldwide and in neighboring countries, with some genetic development to evade host immunity. Sequencing of these host-evading viral genes could help researchers further analyze SPV regarding viral quantification using the currently designed primers. It would also provide efficient information for epidemiologists and veterinarians.

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Conflict of interests

The authors have no conflict of interest about the findings of this study.

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التحليل الجيني لفيروس جدري الاغنام للجينات المتعلقة بتجنب للجهاز المناعي

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الخلاصة

تعد فيروسات جدري الاغنام من الامراض الفيروسية المعدية الخطرة التي تصيب المجترات الصغيرة ومنها الاغنام حيث يتميز المرض بافات جلدية وحمى واسهال واعراض تنفسية واخيرا هلاك الحيوان وبذلك تسبب خسائر اقتصادية كبيرة. ظهرت موخرا عدد من الحلات المرضية للفيروس مع فشل اساليب العلاج او اللقاح المتبع مما يشير الى وجود طفرات جينية للمسبب المرضي تؤدي الى تثبيط عمل الجهاز المناعي بشكل افضل ولذلك استهدفت هذه الدراسة تحليل ثلاثة جينات فيروسية تتعلق بتجنب الجهاز المناعي ومنها البروتين شبية الانترفيرون كاما، المستقبل المناعي المقترن بالبروتين نوع ج، المعقد المناعي النسيجي للخلايا التائية. تم ذلك من خلال دراسة عدد من الحالات المشتبة باصابتها ١٢٥ حالة والتي فحصت بتقنية تفاعل السلسلة المتبلمر حيث ظهرت عدد الحلات الموجبة ٤٠ حالة اي بنسبة اصابة بلغت ٣٢%. اظهرت نتائج التحليل الجيني لبعض العتر المحلية تقارب جيني لعتر اللقاحات المستعملة بالعراق والدول المجاورة (السعودية وتركيا) بينما اظهرت عتر اخرى اختلاف جيني. كانت البوادئ الجينية المصممة ذات كفاءة عالية بتشخيص الفيروس وبالتالي ممكن استخدامها في الدراسات المستقبلية لتقييم مستوى الاصابة الفيروسية كما بينت نتائج هذه الدراسة كفاءة برامج التلقيح المتبعه في العراق ضد المرض وبالتالي تساعد المهتمين في السيطرة على المرض.