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Shehbaz HA
Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Al-Jadriya Street, Baghdad, Iraq

Hassan IQ
Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Al-Jadriya Street, Baghdad, Iraq

Correspondence
Shehbaz HA
Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Al-Jadriya Street, Baghdad, Iraq

Phylogenetic analysis of sheepox virus isolates based on P32 gene in Iraq

Shehbaz HA and Hassan IQ

Abstract
The present study was conducted to diagnose sheeppox virus (SPPV) by PCR with phylogenetic analysis of 12 positive local skin isolates of SPPV and vaccinal strains in Al-Diwaniya, Iraq. Result revealed that the expected amplicons size 515 bp of SPPV was detected in 36 samples (36%) of skin lesions. Number of base substitutions per site between sequences was shown. The identity score of *P32* of ten positive local isolates was 98-100% with Pox vac and Iraq Kandi Sheeppox vaccines. However with NCBI-BLAST Sheeppox virus (MF289491.1), Goatpox virus (KJ026556.1) and Lumpy skin virus (KX960778.1), was 95-100%. Phylogenetic tree analysis based on the partial *P32* showed that all samples tested were closely related in sequence alignment with NCBI-BLAST capripoxviruses: sheeppox virus envelope protein *P32* gene (MF289491.1), four sheeppox virus isolates of Sulaimani Governorate, Iraq and two strains of Al-Hassa, Saudi Arabia. Meanwhile, (LSDV (KX960778.1) and Tunisian sheeppox virus (KT964235.1) were out of tree.

Keywords: Sheeppox, Phylogenetic analysis, *Capripoxvirus*, P32, PCR, Sequence alignment

1. Introduction
The etiological agent of sheep pox disease is *Sheeppox virus* (SPPV), belongs to the genus *Capripoxvirus* within the family *Poxviridae* [1]. The genus includes also *Goatpox* virus (GTPV) of goats and lumpy skin disease virus (LSDV) of cattle [2]. Sheep pox is notifiable to the World Organization of Animal Health (OIE) [2,3], and pose serious socioeconomic impact to small ruminant productivity in terms of hide damage, morbidity, mortality, and trade restriction [4]. Clinically, the disease is characterized by pyrexia, rhinitis, conjunctivitis, excessive generalized multifocal necrotic lesions in the skin and internal organs including lungs, gastrointestinal tract, liver, lymphadenopathy and death [5, 6]. Geographical distribution of the sheep pox has been relatively stable. Sheep pox and goat pox are endemic in China, Nepal, Bangladesh, Iraq, Iran, Turkey, Pakistan, India, Afghanistan, and Africa. Sporadic outbreaks occur in Southern Europe and other parts in the world because of extensive trade between other foreign countries [7, 8].
Identification of these viruses needs molecular methods. Sequencing genes, such as (*P32*), RNA polymerase subunit *(RP030)* or G- protein coupled chemokine receptor *(GPCR)* gene have shown differences between these viruses [9, 10].
In Iraq, sheeppox virus was isolated for the first time by Al-Aubaidi [11], but sequence analysis of the virus in the country is very scarce, expect one research conducted by Rashid *et al.* [12]. So, the aim of study was to detect sheep pox from field samples, phylogenetic analysis, sequencing of the partial *P32* gene of the positive local isolates and studying their relationship with world reference strains.

2. Materials and Methods
2.1 Clinical Samples
In this present study 100 scab samples were collected from clinically suspected sheep during the period of September -December 2016 in Al-Diwaniya Governorate. Samples were aseptically taken and transported in a cool bag to the laboratory and stored at -20° until used for molecular diagnosis.

2.2 Genomic DNA Extraction
The scab sample of each case was put in a sterile Petri dish then cut by sterile scissor to a smaller pieces and transferred to sterile 1.5ml microcentrifuge tube and homogenized by using

Tissue Lysis Buffer according to the manufacturer's instruction of AccuPrep®Genomic DNA extraction kit (Bioneer. Korea), and then freezed at -20 °C until used.

2.3 Polymerase Chain Reaction (PCR)

The PCR assay was performed [13] according to the manufacturer's instruction (AccuPower PCR PreMix Kit). The PCR primers were designed from *P32* gene with the following sequence: forward primer CCAATCGTTGGTCGCGAAAT and reverse primer TCATATCCCCCTGTGTACGA to amplify 515 bp. The primers were constructed by Macrogen (Korea). The PCR reaction was performed with 5 µl DNA template, 1.5µl of Forward primer (10pmol), 1.5µl Reverse primer (10pmol) and PCR water 12 µl with final total volume of 20 µl. The thermocycler (Mygene. Bioneer. Korea) parameters were; initial denaturation one cycle at 95 °C for 3 min, 30 cycles denaturation at 95 °C for 30 sec., 30 cycles annealing at 60 °C for 30 sec., 30 cycles extension at 72 °C for 1min and final extension one cycle at 72 °C for 5min.

2.4 PCR Products Analysis

The amplified PCR products of the viral DNA were detected by electrophoresis on 1% agarose gel containing ethidium bromide, which was placed in TBE buffer and run at a constant voltage (100V) and 80 AM for 1hour.and viewed under UV transilluminator and photographic.

2.5 DNA Sequencing Method

The PCR amplicons of *P32* gene for 10 positive sheep pox isolates and two vaccinal strains were purified from agarose gel by using (EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada) as mentioned in the manufacturers protocol. After that, the purified *P32* gene PCR product samples were sent to Macrogen Company in Korea for performing the DNA sequence by the applied biosystem (AB) DNA sequencing system. The coding sequencing of *P32* gene of 12 strains was submitted to GenBank with accession numbers in Table 1.

Table 1: Local SPPV isolates and vaccinal strains with their accession numbers

No.	Local isolates	Accession number
1	SPV-IQ1	MF572285
2	SPV-IQ2	MF572286
3	SPV-IQ3	MF572287
4	SPV-IQ4	MF572288
5	SPV-IQ5	MF572289
6	SPV-IQ6	MF572290
7	SPV-IQ7	MF572291
8	SPV-IQ8	MF572292
9	SPV-IQ9	MF572293
10	SPV-IQ10	MF572294
11	Sheeppox virus vaccine strain (Iraq Kandi) 11	MF572295
12	Sheeppox virus vaccine strain (Pox vac) 12	MF572296

2.6 Phylogenetic Analysis

The number of base substitutions (mutation) per site between sequences was Analysed using the Maximum Composite Likelihood model. The nucleotide sequences of *P32* gene of capripoxviruses strains worldwide were obtained from Genbank. Multiple alignments of these sequences were performed by the neighbor-joining method using MEGA version 6.0 software [14], which it is multiple alignment analysis tool used for building Phylogenetic tree.

Statistical Analysis

The Statistical Analysis System-SAS (2012) program (statistical. Version 9.1 SAS. Inst Inc. Cary. N. C. USA) [15] was used. The Chi-Square test was applied to determine significant differences between percentages in this study.

3. Results

3.1 Detection of Sheep Viral DNA by Conventional PCR Assay

The extracted DNA from scab suspensions, when subjected to PCR using primers specific for *P32* gene yielded expected amplicon of size 515 pb (Fig. 1). The result revealed that the expected amplicons size 515 of SPPV was detected in 36 samples out of 100 skin lesions. Whereas 64 samples (64%) were negative for sheeppox viral DNA (Table 2). Statistically, there was significant difference between positive and negative cases with $P< 0.01$. Ten of the amplified isolates and 2 vaccinal strains were confirmed by sequencing.

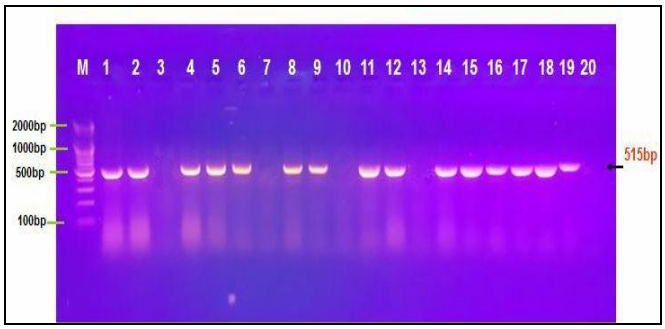

Fig 1: Agarose gel electrophoresis image that showed the PCR product analysis of P32 gene in sheeppox virus. Where lane M: marker (2000-100bp), lane 1,2,4,5,6,8,9,11,12,14,19 were positive and 3,7,10,13,20 were negative skin lesion samples at 515bp PCR product size.

Table 2: Percentages of positive sheep pox cases by PCR

Number of sheep with skin lesions	Number of sheep	Percentage
Infected sheep	36	36%
Non-infected sheep	64	64%
Total	100	100%
Chi-square value	----	9.271 *
P- value	----	0.0003

* ($P< 0.01$)

3.2 Gene sequence and phylogenetic analysis

As shown in Table 3 sheep pox virus isolate IQ1 had no base substitutions (mutation) with the isolates IQ2, IQ4, IQ5, IQ8, IQ10 and with the two vaccinal strains (Iraqi Kandi and Pox Vac), whereas there was little difference(0.002%) in the base substitutions of sheeppox virus isolates IQ3 and IQ9. However, isolates IQ6 and IQ7 had more difference in the base substitutions (0.010%).The identity score of *P32* of the ten local isolates on comparing with the two Iraqi vaccinal sheeppox virus strains (Pox vac and Iraq Kandi Vaccines) was of 98-100%. But on comparing with NCBI-BLAST Sheeppox virus (accession number of MF289491.1), Goatpox virus (KJ026556.1), Lumpy skin virus (KX960778.1), the identity score was of 95-100%.Phylogenetic tree analysis based on the partial *P32* showed that all samples tested including SPPV vaccine strains were closely related in sequence alignment analysis, when were compared with NCBI-BLAST capripoxviruses: sheeppox virus envelope protein *P32* gene

(MF289491.1), four sheeppox virus isolates of Sulaimani Governorate and two strains of Al-Hassa of Saudi Arabia. While GTPV (KJ026556.1) and sheeppox virus Iranian isolate (FJ917518.1) showed less relation. Meanwhile, LSDV (KX960778.1) and Tunisian sheeppox virus (KT964235.1) were out of tree (Fig.2 and 3).

Table 3: Estimation of Evolutionary Divergence between Sequences

	1	2	3	4	5	6	7	8	9	10	11	12
1. Sheeppox virus Diwanyia-IQ1												
2. Sheeppox virus Diwanyia-IQ2	0.000											
3. Sheeppox virus Diwanyia-IQ3	0.002	0.002										
4. Sheeppox virus Diwanyia-IQ4	0.000	0.000	0.002									
5. Sheeppox virus Diwanyia-IQ5	0.000	0.000	0.002	0.000								
6. Sheeppox virus Diwanyia-IQ6	0.010	0.010	0.008	0.010	0.010							
7. Sheeppox virus Diwanyia-IQ7	0.010	0.010	0.008	0.010	0.010	0.002						
8. Sheeppox virus Diwanyia-IQ8	0.000	0.000	0.002	0.000	0.000	0.010	0.010					
9. Sheeppox virus Diwanyia-IQ9	0.002	0.002	0.000	0.002	0.002	0.008	0.008	0.002				
10. Sheeppox virus Diwanyia-IQ10	0.000	0.000	0.002	0.000	0.000	0.010	0.010	0.000	0.002			
11. Sheeppox virus Vaccine strain (Iraq Kandi)	0.000	0.000	0.002	0.000	0.000	0.010	0.010	0.000	0.002	0.000		
12. Sheeppox virus Vaccine strain (Pox vac)	0.000	0.000	0.002	0.000	0.000	0.010	0.010	0.000	0.002	0.000	0.000	

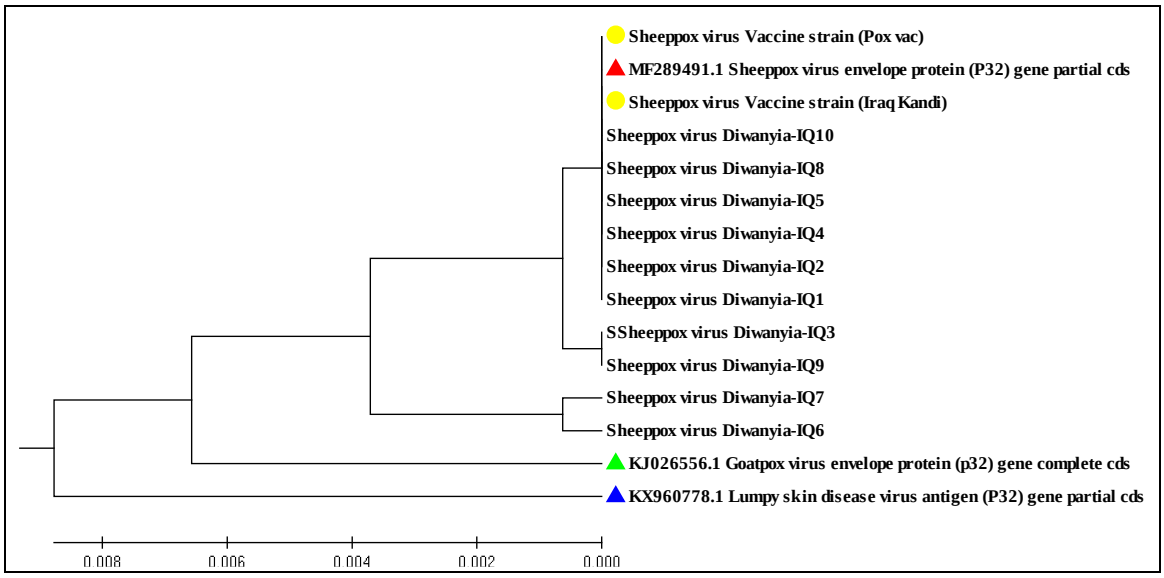

Fig 2: Phylogenetic tree of different capripoxviruses based on the partial nucleotide sequences of *P32* gene.

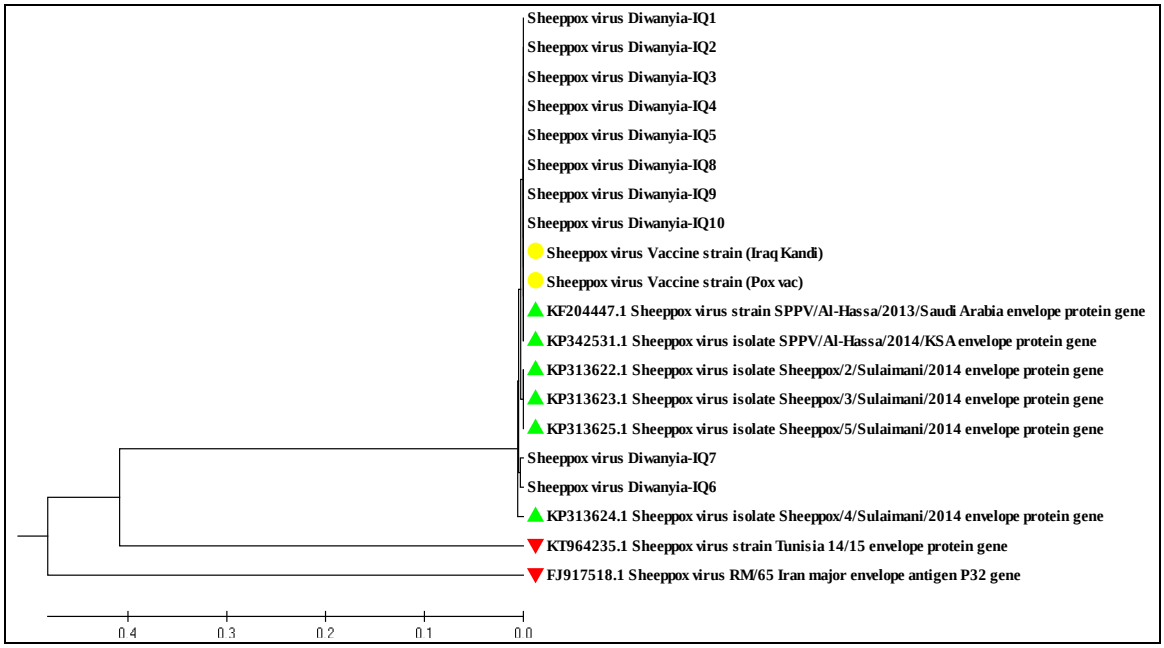

Fig 3: Phylogenetic tree analysis of different sheeppox viruses depending on the partial nucleotide sequence of *P32* gene. Phylogenetic tree was constructed using neighbor joining algorithm in MEGA 6.0 version.

4. Discussion

Subjecting of the extracted DNA from skin lesions to PCR using primers specific for *P32* gene yielded expected amplicon of size 515 bp No amplification was obtain in negative cases which was supported by Parthiban *et al* [16] and Rajamuthu *et al* [17]**,** indicating that the primers were highly specific for detection of sheeppox virus. Percentage of positive sheep cases in different areas of Al-Diwaniya reached to 36% which was higher than the finding of Zangana and Abdullah [18], who recorded morbidity rate of 30% in lambs of 2-4 months old during an outbreak occurred in Duhok Governorate. While, in the study of Musa [19] high percentage of positive sheep pox cases (70%) was recorded in Al-Diwaniya Governorate. The variation in the percentages of the aforementioned studies may be due to several factors: endemicity of the disease in the area, environmental conditions, overcrowding, quality of management, sheep immunity and vaccination program. This opinion was supported by the explanation of Yeruham *et al* [20]; Chanie [21]; Zangana and Abdullah [18].

The classification of *Capripoxviruses* based on animal species from which virus is isolated indicating that these viruses are strictly host-specific [22, 23]. But, there are many reports proved that SPPV could infect goat and vice versa [23, 24]**.** So it needs molecular methods for differential diagnosis. Several molecular markers including *P32* gene, *GPCR* and *RPO30* were used for the differential diagnosis and phylogenetic analysis. They are highly conserved among capripoxviruses. the *P32* gene is one of the structural proteins present in all *Capripoxviruses* and contains major immunogenic determinants. The gene is homologous to the *P35* protein encoded by vaccinia virus *H3L* gene [13]. Because of the size difference of *P32* between *Sheeppoxvirus* and *Goatpoxvirus*, they can be distinguished by sequence comparisons [25, 26].

According to the NCBI-Blast alignment, all the local sheeppox virus strains SPV-IQ (10 isolates) possessed 98-100% homology with sheeppox virus vaccine strain (Pox Vac) and sheeppox virus vaccine strain (Iraq kandi) at the nucleotide level. This means, the local isolates were closely related to both vaccinal strains. Hence nucleotide sequence comparison and antigenic and immunogenic properties of local isolates and vaccine strains are to be analyzed to confirm any change in the selection of vaccine strain [8]. But deletant vaccine may be the better choice for differentiation of local and vaccine strains of sheeppox virus in the countries where vaccination is used regularly [16, 17, 27]. Meanwhile all the local sheeppox virus isolates, the two sheeppox virus vaccine strains and also NCBI- BLAST sheep pox virus (MF289491.1) had high identity (98-100%). but identity with goatpox virus (KJ026556.1) was 95-97% and with LSDV (KX960778.1) was 95-96%. The result of the present study support the hypothesis that GTPV and LSDV were emerged from a common ancestor close to sheeppox viruses as proposed by Hosamani *et al* [9] and Stram *et al* [28]. They carried out their phylogenetic studies on different genome segments but their assumption is contradicted another study, which concluded that small ruminant poxviruses may be emerged from a common LSDV-like ancestor [29].

As shown in the constructed tree of all sheeppox virus isolates and the two vaccinal strains were closely related to the NCBI-Blast Sheeppox virus Sulaimani and Saudi Arabia isolates. Whereas sheeppox virus Iranian isolate (FJ917518.1) showed less relationship. Tunisian sheeppox virus (KT964235.1) found to be in a separated branch and out of the tree. The close relationship between Iraqi isolates strains (including Sulamani isolates) and Saudi Arabia strains, probably associated with illegal trade and unauthorized sheep movement across borders of both countries.

5. Conclusion

The present study recorded the first genetic analysis and confirmation of SPPV isolates circulating in Al-Diwaniya Governorate, Iraq. Addtionally, the phylogenetic analysis of envelope protein *P32* gene is suitable for epidemiological research on Iraqi SPPV isolates because of its available abundant information. There was high degree of similarity between Iraqi local SPPV isolates (including Sulaimani strains, Iraq) and vaccinal strains with Saudi Arabia isolates at the nucleotide level.

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