



FIRST MOLECULAR DETECTION OF MAEDI-VISNA VIRUS IN AWASSI SHEEP OF MIDDLE IRAQ REGIONS

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Summary

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Respiratory viral infections cause significant economic losses in sheep production. This preliminary molecular study aimed to detect the Maedi-Visna virus infection in Awassi sheep in three governorates in the middle region of Iraq. The presence of one or more of the specific four genes (*gag*, *pol*, *env* and *LTR*) were considered as positive result. A total of 210 blood samples of Awassi sheep were collected for the purpose of the project. The molecular prevalence of Maedi-Visna virus in sheep was 12.85% (27/210). As a result, Maedi-Visna virus was observed in sheep with chronic respiratory system disease with non-significant difference between governorates and between primers percentage ($P > 0.05$). Sequencing studies strongly suggested that Maedi-Visna virus originated in Iraq. This is the first study describing Iraqi Maedi-Visna virus sequences with molecular characterisation of *gag*, *pol*, *env* and *LTR* genes, suggesting that Maedi-Visna virus originated in Iraq.

Key words: Awassi sheep, first molecular detection, Maedi-Visna virus, middle Iraq regions

INTRODUCTION

Maedi-Visna is a chronic and persistent viral infectious disease of sheep and rarely goats, caused by the Maedi-Visna virus (MVV), family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Lentivirus*, which are genetically related to caprine arthritis encephalitis virus (CAEV) (Fauquet *et al.*, 2005). The disease is manifested clinically by two forms: Maedi causing respiratory signs while Visna vi-

rus is responsible for the nervous form (Akkan *et al.*, 2009).

MVV leads to multi-systemic inflammatory disease. The four major tissues affected are lungs, mammary glands, joints and CNS. However, pathological lesions can also be seen in other organs including heart, liver, kidney, lymph nodes, blood vessels and bone marrow. MVV has a very long incubation period

and the clinical signs do not appear until the age of two years and many sheep remain asymptomatic carriers for their whole life (Sargan *et al.*, 1991; Herrmann-Hoesing *et al.*, 2010).

The clinical signs of Maedi are observed more commonly than those of Visna. The clinical signs of Maedi are cough, dyspnea, emaciation and mastitis. Abortions are also recorded but associated with severity of infection (Straub, 2004; Christodouloupoulos, 2006). Clinical signs of Visna are weakness in hind legs, arthritis, weight loss, mastitis and progression to complete paralysis. Sometimes CNS disorders are also observed. In both Maedi and Visna, the body temperature does not increase if there is not a secondary infection (Straub, 2004).

In clinical cases diagnosis is by the presence of the appropriate clinical syndrome, supported by the presence of a positive test for the viruses (Radostits *et al.*, 2007). The confirmative diagnosis must be performed by laboratory methods such as post-mortem examination, histopathological lesion, virus serology and isolation. Several methods are used for serological and molecular diagnosis of MVV including agar gel immunodiffusion assay (AGID), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), ristocetin-induced platelet aggregation (RIPA) and polymerase chain reaction (PCR) (Herrmann-Hoesing *et al.*, 2010; Asadpour *et al.*, 2014).

Molecular techniques of use in MVV diagnosis include the heteroduplex mobility assay (Germain & Valas, 2006). PCR procedures are the most commonly used to directly assess the presence of viral nucleic acid. Conventional PCR for diagnostic purposes has been more extensively studied than real-time PCR (Zhang *et al.*, 2000; Gudmundsson *et al.*, 2003).

The first conventional PCR protocols designed to detect MVV were published in the early 1990s (Zanoni *et al.*, 1990). PCR was considered initially successful in MVV diagnosis, due to its ability to directly detect the virus either in the infected cell as a provirus (DNA) or in exudates that contain free virus particles (RNA: reverse transcription-PCR) (Leroux, 1997). Different studies have shown the utility of conventional PCR to detect MVV infections in different animal samples, namely peripheral blood mononuclear cells (Alvarez *et al.*, 2006; Legina-goikoa *et al.*, 2009). Nowadays the conventional polymerase chain reaction technique is considered a confirmative diagnostic method and was extensively studied. These techniques are able to detect the free virus particles as well as provirus (DNA) in the infected cells (Zhang *et al.*, 2000; Gudmundsson *et al.*, 2003). Furthermore, the high genetic heterogeneity may impede the diagnosis of MVV (Peterhans *et al.*, 2004).

The target of sequences for PCR primers' design are widespread throughout the MVV genomes including: *gag*, *pol*, *env* and *LTR* regions (Carrozza *et al.*, 2010; Marinho *et al.*, 2018). Furthermore, positive PCR can be detected in seronegative animals (false negative).

The aim of this study was to detect the rate of diversity of MVV in Awassi-sheep showing respiratory signs in the Middle regions of Iraq.

MATERIALS AND METHODS

Two hundred and ten Awassi sheep of both sexes, aged from <1 to >6 years were used in this study, reared in the three governorates (Babylon, Karbala and AL-Diwaniya) in the middle regions of Iraq, December 2018 to August 2019. The his-

tory of each case was recorded in special chart prepared for this purpose. All sheep were suffering from respiratory signs, they were examined clinically and blood samples were taken from the jugular vein in EDTA-vacutainer tubes. The mononuclear cells were separated for detection of provirus. The PCR procedure was based on amplification of major surfaces of genes by using Genomic DNA mini kit extraction (Geneaid, USA), according to company instruction. The extracted DNA was used as a template to detect proviral (DNA) of MVV by single PCR. Four sets of specific genes primers were used for *gag*, *pol*, *env* and *LTR* (Table 1). PCR master mix was prepared by employing Accupower PCR PreMix kit also according to company instructions.

All PCR products were centrifuged at 3000 rpm (Exispin vortex centrifuge) for 3 minutes then placed in the thermocycler (THECHNE. USA). The PCR products of four genes were analysed by agarose gel electrophoresis (1%). They were visualised clearly by using UV transilluminator.

Sequencing

Sequencing analysis for PCR products of four genes was verified by comparing the result with the reference sequenced genes available in the Gene Bank at National Center for Biotechnology Information

(NCBI) database via Basic Local Alignment Search Tool (BLAST) algorithm.

Phylogenetic analysis

Phylogenetic tree analysis was achieved on partial sequence of four genes in local isolates of MVV. The evolutionary distances were used for computing the Maximum Likelihood Method by phylogenetic tree UPGMA method (MEGA 6.0 version).

Statistical analysis

Statistical analysis was done by Statistical Package for Social Science, version 23.0 (SPSS Inc, Chicago, USA). Significant differences were determined by Chi-Square and t-test at $P < 0.05$ and high significance was considered at $P < 0.01$ (Al-Ukaellii & Al-Shaeb, 1998).

RESULTS

Positive results of endpoint PCR were detected in 27 samples out of 210 total PBMCs extracted DNA (12.85%). The molecular percentages of MVV-positive samples for each place were 12.85% for Babylon, 17.14% for Al Diwaniyah and 8.57% for Karbala (Table 2).

All positive samples confirmed the presence of the one or more genes (*gag*, *pol*, *env* and *LTR*) (Table 3).

Table 1. The sequences of primers and flanked portion of MVV genome used in conventional PCR

Primer	Sequence	Amplicon	Origin
<i>gag</i> gene	F 5'-GTCTTTGCAGGCCACATTGG-3	516 bp	Designed with NCBI
	R 5-TGCCTTGCCTGATCCATGTT-3		
<i>pol</i> gene	F 5-TGCCTAGCACAAACAGAGGG-3	573bp	Designed with NCBI
	R 5-CTTGCCAATCCCCTGGATGT-3		
<i>env</i> gene	F 5-AGGATTTTCAGAGGGTGCAGC-3	582bp	Designed with NCBI
	R 5-TGTACACATCCTCCCGGACT-3		
<i>LTR</i> gene	F 5-AGTCATGTAACAGCTGACGC-3	202 bp	Designed with NCBI
	R 5-GCACGGAATTAGTAACGAATCCC-3		

Table 2. The percentages of MVV infectivity results by PCR of examination of 210 sheep samples

City	Infected	Non-infected	Total
Babylon	9 (12.85%)	61	70
Al Diwaniyah	12 (17.14%)	58	70
Karbala	6 (8.57%)	64	70
Total	27 (12.85%)	183	210

$\chi^2 = 2.29$; $P=0.317$

Table 3. The twenty seven MVV positive sheep according to sex, age, governorate and PCR results

Case No.	Governorate	Sex	Age (years)	<i>gag</i> gene	<i>pol</i> gene	<i>env</i> gene	<i>LTR</i> gene
1	Babylon	F	6		+		+
2	Babylon	F	8	+		+	
3	Babylon	F	6	+		+	
4	AL-Diwaniyah	F	6	+		+	
5	Babylon	F	7	+		+	
6	Karbala	F	8		+	+	+
7	Babylon	F	8	+	+		+
8	Babylon	F	10			+	
9	Babylon	F	7	+			
10	AL-Diwaniyah	F	4	+			
11	AL-Diwaniyah	F	5		+		+
12	Karbala	F	6	+	+	+	
13	Karbala	F	5		+	+	+
14	AL-Diwaniyah	M	2		+	+	
15	AL-Diwaniyah	F	5		+	+	+
16	Babylon	M	4	+	+		
17	AL-Diwaniyah	F	7	+			+
18	AL-Diwaniyah	F	5	+			
19	AL-Diwaniyah	F	6			+	+
20	Babylon	M	7	+			+
21	Karbala	F	5		+		
22	AL-Diwaniyah	F	4	+			
23	Karbala	F	1		+		+
24	Karbala	M	2	+		+	
25	AL-Diwaniyah	F	3		+	+	
26	AL-Diwaniyah	F	5		+		
27	AL-Diwaniyah	M	4				+
				14	13	13	11
				(6.66%)	(6.19%)	(6.19%)	(5.23%)

$\chi^2=0.397$ (NS); $P=0.941$

The samples producing bands of the expected size: 516 bp (Fig. 1), 573 bp (Fig. 2), 582 bp (Fig. 3) and 202 bp (Fig. 4) segments corresponding to the univer-

sal lader in size (100–1500 bp) were considered positive.

Eight MVV (*gag* gene) local isolates: MN611951.1, MN61195 2.1, MN6119

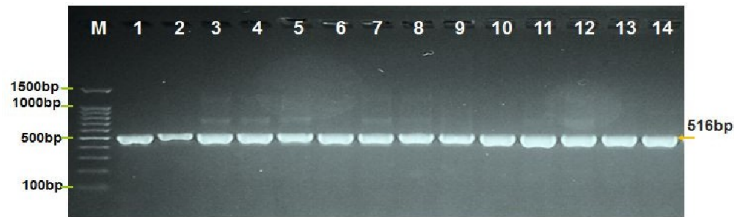


Fig. 1. Agarose gel electrophoresis image showing the PCR product analysis of *gag* gene in MVV from DNA extracted from PBMCs of infected sheep.

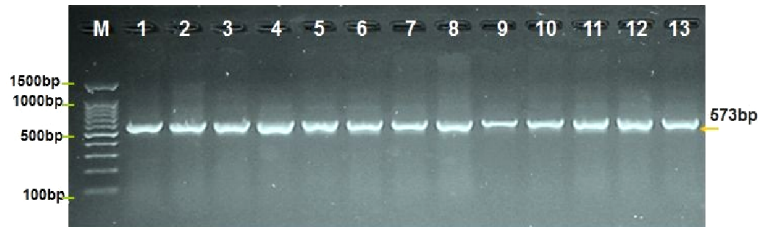


Fig. 2. Agarose gel electrophoresis image showing the PCR product analysis of *pol* gene in MVV from DNA extracted from PBMCs of infected sheep.

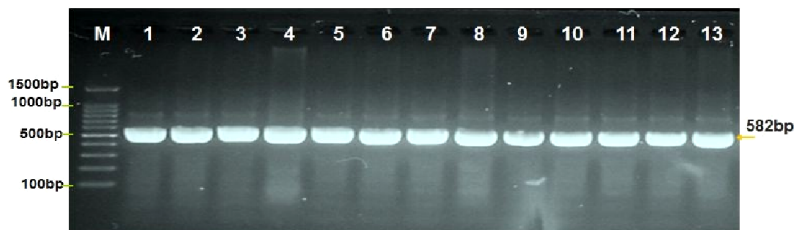


Fig. 3. Agarose gel electrophoresis image showing the PCR product analysis of *env* gene in MVV from DNA extracted from PBMCs of infected sheep.

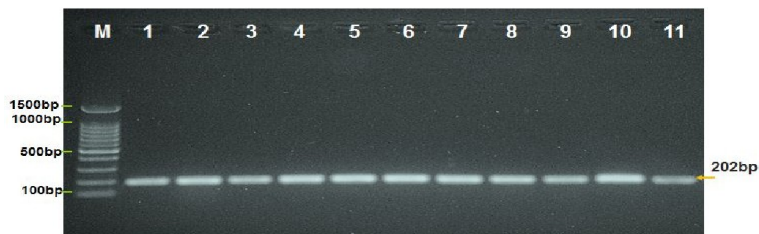


Fig. 4. Agarose gel electrophoresis image that showed the PCR product analysis of *LTR* gene in MVV from DNA extracted from PBMCs of infected sheep.

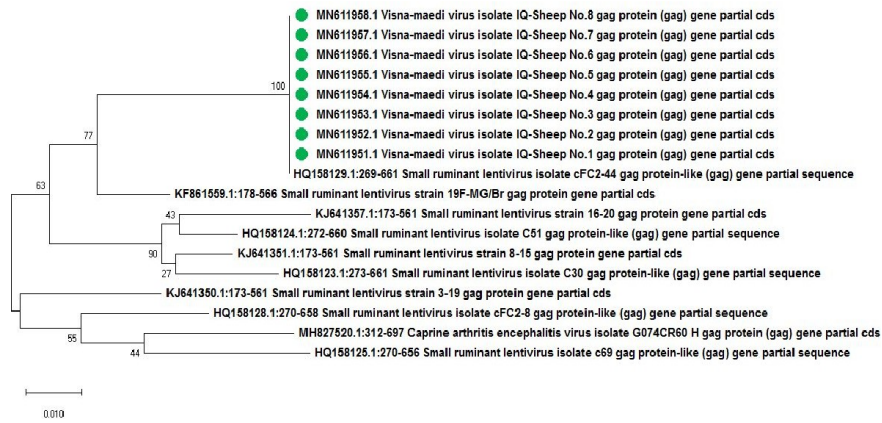


Fig. 5. Phylogenetic tree analysis of *gag* gene partial sequence in local Iraqi isolate of MVV in Awassi sheep, total genetic changes 0.010%.

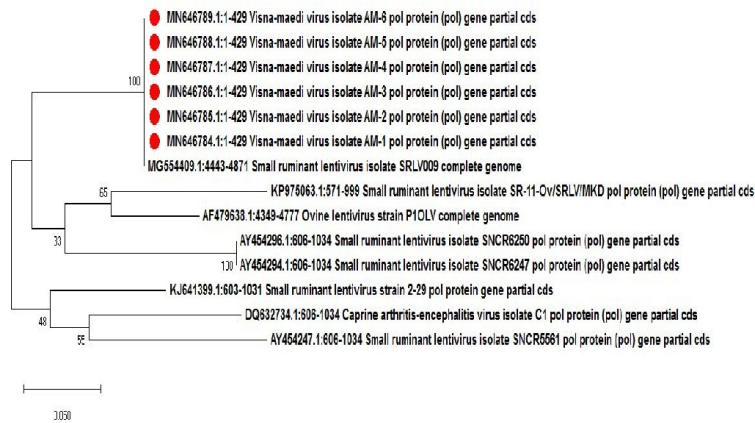


Fig. 6. Phylogenetic tree analysis of *pol* gene, partial sequence in local Iraqi isolate of MVV in Awassi sheep, total genetic changes 0.060%.

53.1, MN6119 54.1, MN611 955.1, MN 611956.1, MN611957.1 and MN611958.1 were used for analysis and comparison with the NCBI GenBank MVV isolates. The results of phylogenetic tree revealed that *gag* gene sequence in the local MVV isolates from all samples were closely (100%) related to the reference strain detected in Canada (Fig. 5).

The second detected gene was polymerase (*pol*). The results of phylogenetic tree revealed that sequence of *pol* gene in

the local MVV isolates from samples AM-1 (MN646784.1), AM-2 (MN646785.1), AM-3 (MN646786.1), AM-4 (MN646787.1), AM-5 (MN646788.1) and AM-6 (MN646789.1) were closely (100%) related to Small ruminant lentivirus *cFC2-44* isolate (HQ158129.1) in Italy (Fig. 6).

The third used gene *env* in nine MVV local isolates: 1 (MT001437.1), 2 (MT001438.1), 3 (MT001439.1), 4 (MT001440.1), 5 (MT001441.1), 6 (MT001442.1), 7 (MT001443.1), 8 (MT001444.1) and 9

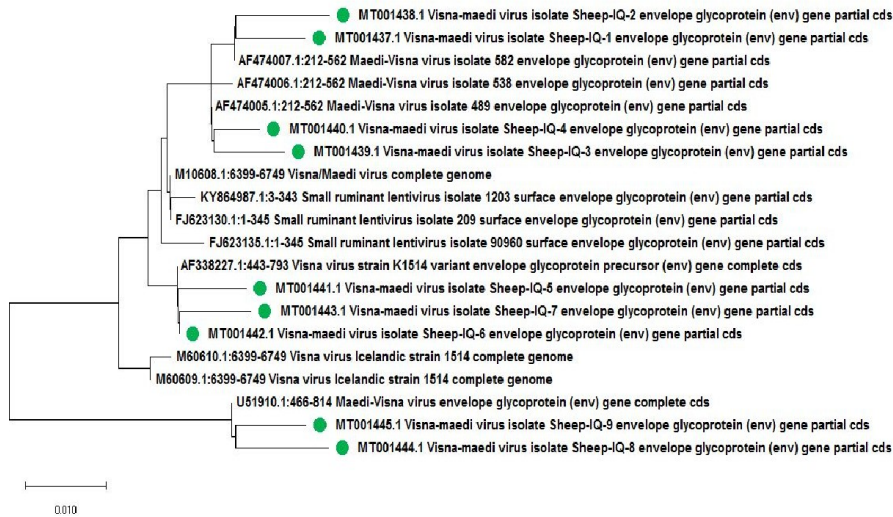


Fig. 7. Phylogenetic tree analysis of *env* protein gene partial sequence in local Iraqi isolate of MVV in Awassi sheep, total genetic changes 0.010%.

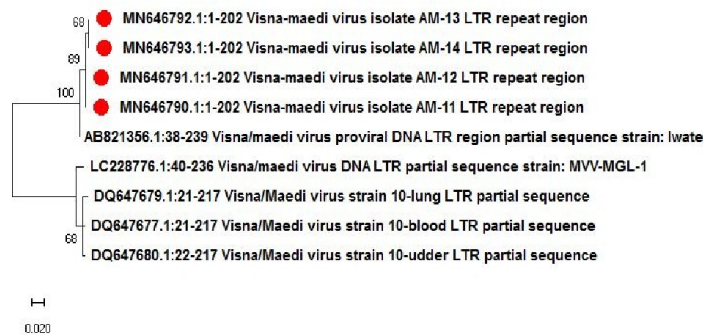


Fig. 8. Phylogenetic tree analysis of *LTR* gene partial sequence in local Iraqi isolate of MVV in Awassi sheep, total genetic changes 0.02%.

(MT001445.1) was compared with the NCBI GenBank MVV isolates. MVV IQ-1 and IQ-2 local isolates in the constructed tree were closely related to MVV isolate 582 envelope glycoprotein *env* gene (AF474007.1). IQ-3 and IQ-4 local isolates in the constructed tree were closely related to MVV isolate 489 envelope glycoprotein *env* gene (AF474005.1). IQ-5, IQ-6 and IQ-7 local isolates were closely related to Visna virus strain K1514 variant envelope glycoprotein precursor *env* gene

(AF338227.1). MVV isolates IQ-8 and IQ-9 were closely related to MVV envelope glycoprotein *env* gene (U51910.1) (Fig. 7).

Another gene tested in the present study was the *LTR* gene. The local isolates that carried it – AM-11 (MN646790.1), AM-12 (MN646791.1), AM-13 (MN646792.1) and AM-14 (MN646793.1) were highly related to each other and to MVV Iwate (AB821356.1) in Japan (Fig. 8).

DISCUSSION

Several studies have shown that the molecular diagnosis of MVV is increasingly sensitive (Oguma *et al.*, 2013; Marinho *et al.*, 2016; Sánchez *et al.*, 2016) being a suitable technique for monitoring animals, particularly those of high zootechnical value. In this study, the developed technique proved to be efficient for the simultaneous detection of sheep samples, allowing a faster and more sensitive diagnosis of MVV compared to ELISA.

Barquero *et al.* (2013) considered that there was no “gold standard” test for the diagnosis of MVV and with the advances in molecular biology, several PCR protocols have been developed. Therefore, it is difficult to use the same primers in different geographic regions due to the MVV genome heterogeneity.

The successful amplification of the *gag*, *pol*, *env* and *LTR* genes in MVV by PCR were at 516 bp, 573 bp, 582 bp and 582 bp respectively. However, a non-significant difference was noticed among gene variations of the MVV. All positive samples (27) in individual PCR tests for each set of primers were positive for one or more primers and negative for other primers.

The study agree with reported data (Marinho *et al.*, 2018) in Brazil about detection of *gag*, *pol* and *LTR* by duplex nested-PCR in whole blood leukocytes from sheep with clinical signs and clinically healthy sheep. Some of samples were positive for one or more primers. The partial sequence alignment for three genes performed in present study to detect compatibility with gene of the standard strain from GenBank by using BLAST and BioEdit software, indicated sequence compatibility or degree of similarity to a very high level meaning that these se-

quences were identical and provided greater confidence.

Carrozza *et al.* (2010) confirmed the present of *gag* and *pol* genes in MVV by real time PCR assays. The result showed that the *pol* assay detected in most cases lower numbers of viral molecules than *gag* and failed to detect some infected samples which were low copy positive with the *gag* assay, probably due to the higher sequence heterogeneity of *pol*. In Netherlands, Brinkhof *et al.* (2008) investigated the presence of proviral nucleic acid sequences of *gag* and *LTR* in MVV. The use of more primers in our study resulted from low virus load – one of the main problems for compromised PCR sensitivity in MVV detection. Therefore, the use of PCR protocol or primers from different regions (*gag*, *pol* and *LTR*) may be helpful for improving the sensitivity of PCR (Zanoni *et al.*, 1992).

Phylogenetic tree analysis was based on partial sequencing of *gag*, *pol*, *env* and *LTR* gene in local Maedi-Visna virus isolates used for confirmative genetic detection.

The expected value in each isolate of *gag* gene was 0.0 meaning that these sequences were identical giving greater confidence in reading the sequencing of *gag* gene nucleotides by using Blast and BioEdit programmes. Our current study contributed to other previously published data concerning the use of *gag* gene sequences as powerful method for the systematic classification of viruses (Asadpour *et al.*, 2014; Marinho *et al.*, 2018).

The important uses of *gag* gene sequences in this study are also supported by other cumulative results from a limited number of studies suggesting that *gag* gene sequencing provided a high degree of similarity among sheep MVV (Gil *et al.*, 2006; Olech *et al.*, 2019).

On other hand there is a relationship between MVV and caprine arthritis encephalitis virus *gag* gene. A group of genetically distinct ovine isolates, that are more similar to the caprine arthritis encephalitis virus (CAEV) prototype, has been described in France (Leroux *et al.*, 1997) and Italy (Grego *et al.*, 2005). There is also evidence of direct transmission of the Small Ruminant Lentivirus SRLV (CAEV-Co) from goats to sheep in mixed flocks (Pisoni *et al.*, 2007). Similarly, sheep-to-goat transmission has been established (Shah *et al.*, 2004).

In the present study, we described the sequence variation in a 573 nt region of the MVV *pol* gene from a population of naturally infected sheep from middle regions of Iraq. The amplified fragment of *pol* was a part of the retroviral RT coding region.

To determine the genetic relationship of Iraqi ovine isolates to other ovine and caprine lentiviral strains, a phylogenetic tree was constructed from the deduced *pol* sequences. The majority of our Iraqi isolates formed a related group with affinities to CAEV Co on a clearly separate branch from the MVV group consisting of the SRLV strains and ovine lentivirus. These results are in agreement with Leroux *et al.* (1995) who found that the comparison of nucleotide sequences showed only a moderate affinity between French isolates and CAEV Co as compared to the MVV group.

The use of envelope gene sequence in this study was also supported by Pisoni *et al.* (2007) who reported that *env* gene was present in almost all viruses. Function of the *env* gene over time has not changed. The author suggested that random sequence changes were a more accurate measure of time and the *env* gene, 582 bp being large enough for informatics purposes.

The high sequence similarity observed between members of the MVV (*env* gene) in the current study makes it difficult to differentiate between species of MVV on the basis of the *env* gene. Thus, the current study contributed to previously published data reported by Karr *et al.* (1996) and Mwaengo *et al.* (1997).

In a study by Mendiola *et al.* (2019) which included sequencing analysis of 28 (11 of sheep and 17 of goats) MVV isolates from sheep in Mexico, the BLAST analysis revealed that MVV strains detected in Mexico were highly related to each other with a nucleotide identity. The analysis of the SRLV *LTR* region showed two minimal changes in the nucleotide sequences (1 to 2 nucleotides). This finding could be important to better understand the function of region U3 of the *LTR* and its possible presence in the evolution of the disease.

In a previous study performed in Turkey, Muz *et al.* (2013) confirmed MVV by variable tests as well as PCR in 33 cases of different breeds of sheep. The phylogenetic analysis of 33 *LTR* sequences identified two distinct clades that were closely related to American and Greek *LTR* sequences.

In conclusion, as a result, MVV was seen in animals with clinical signs in middle Iraq regions. As this is first study performed in the region related with MVV, it provides a basis for further studies in the region. Moreover, further detailed studies should be performed, determining positive animals with the support of regional management.

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