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Original Article

Histopathological, serological, molecular and electron microscopy detection of Maedi-Visna infection in sheep population in the West of Iran

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Abstract

Background: Maedi-Visna (MV) is a progressive lymphoproliferative viral disease that affects multiple organs of small ruminants, including sheep and goats. The disease occurs primarily in the lung tissue and causes interstitial pneumonia. **Aims:** The aim of present study was to investigate the prevalence of ovine MV infection in Iranian sheep population through macroscopic, histopathological, serological, and molecular assays, as well as transmission electron microscopy (TEM). **Methods:** Lung and blood samples of one-hundred female sheep (≤ 2 years old) referred to the Kermanshah slaughterhouse with respiratory symptoms were collected for histopathological and molecular evaluations. Corresponding serum samples were also collected for serological examination. **Results**: Histopathological study showed the Maedi-like pulmonary lesions in 85% of the affected lungs, which included the interstitial pneumonia, smooth muscle hypertrophy of alveolar septa and around the blood vessels, interstitial lymphoplasmacytic infiltration and lymphofollicular hyperplasia. Specific antibodies against MV virus were detected in 7% of serum samples. Long terminal repeat (LTR) region of MV provirus was amplified in three (3%) DNA samples, extracted from the suspected lungs. Sequencing analysis of polymerase chain reaction (PCR)-positive samples confirmed the presence of MV provirus in the genome. No amplification was observed, neither in the DNA samples extracted from the blood samples of suspected sheep nor the control group. Transmission electron microscopy also confirmed the presence of MV virions inside the cytoplasmic membrane of MV-infected macrophages. **Conclusion:** Although histopathology can provide a preliminary estimation of Maedi in populations, definitive diagnosis of the disease needs to be approved by more sensitive techniques such as serological examinations and molecular analysis.

Key words: Electron microscopy, Maedi-Visna, PCR, Serology, Sheep

Introduction

Small ruminant lentiviruses (SRLVs) including ovine Maedi-Visna virus (MVV) and Caprine arthritis encephalitis virus (CAEV) mainly infect the monocyte lineage and develop slowly progressive fatal diseases in different countries (Brinkhof *et al*., 2010a). Ovine MVV which is also called ovine progressive pneumonia virus (OPPV), belongs to the family of *Retroviridae* and genus of *Lentivirus* and is a monocyte/macrophage-tropic non-oncogenic virus which induces persistent infection in sheep (Pepin *et al*., 1998, Brinkhof *et al*., 2010b, Herrmann-Hoesing, 2010). Maedi-Visna was found in 1939 in sheep flocks in Iceland, and eradicated in 1965 (Minardi da Cruz *et al*., 2013). Except Australia and New Zealand, Maedi is endemic throughout the world with varied seroprevalence depending on the area, age, breed, or gender (Lago *et al*., 2012; Zhang *et al*., 2013; Oguma *et al*., 2014; Minguijon *et al*., 2015; Norouzi *et al*., 2015).

Although Maedi is a progressive interstitial pneumonia, the virus can affect multiple organs including lung, mammary glands, central nervous system and joints and cause multisystemic diseases in sheep and goats (Brinkhof *et al*., 2010a; Carrozza *et al*., 2010; Minardi da Cruz *et al*., 2013; Preziuso *et al*., 2013; Asadpour *et al*., 2014). The disease process is chronic and slowly progressive with a long incubation period, and commonly occurs in sheep with age over two years (Peterhans *et al*., 2004). Economic losses of Maedi disease are considerable, that might vary among the flocks and include export restriction, decrease in weight of infected sheep, decrease in lamb birth weight and decrease in milk production due to the chronic mastitis (Peterhans *et al*., 2004; Fournier *et al*., 2006; Benavides *et al*., 2013; Nowicka *et al*., 2015).

Maedi disease cannot be easily detected in herds based on the clinical symptoms. Diagnosis of respiratory form of the disease is based on the recognition of typical gross and microscopic lesions in advanced cases. However, only a small proportion of infected animals develop the clinical signs and most of these pathological changes are not pathognomonic (De Andres *et al*., 2005). Difficulties in interpretation of the Maedi disease can arise in samples with mild interstitial pneumonia caused by other pathogens such as lungworms or mycoplasma

pneumonia, exudative pneumonias due to the secondary bacterial infections and ovine pulmonary adenomatosis (Caswell and Williams, 2016). In such cases, clinical symptoms are not helpful and use of an accurate diagnostic method seems necessary. Detection of specific antibodies against the MVV in the serum samples and definitive diagnosis using molecular techniques are among the most valuable methods employed for detection of MVV in the herds (Brinkhof *et al*., 2008, Herrmann-Hoesing, 2010; Preziuso *et al*., 2013; Villoria *et al*., 2013).

The objective of present study was to evaluate the prevalence of ovine MVV in Iranian sheep population through macroscopic, histopathological, serological, and molecular assays, as well as transmission electron microscopy (TEM). This study provides an approximate evaluation of morbidity of Maedi disease among sheep flocks in the west of Iran and compares the consistency between different diagnostic methods.

Materials and Methods

Geographical situation

Kermanshah province is located in the west of Iran at the central part of the Zagros Mountains. It has a moderate and mountainous climate with a total area of 24,998 km^2 and is located on the border with Iraq. The slaughterhouse is located 28 km away from Kermanshah in the countryside, which supplies more than 80% of mutton produced in this province.

Sampling

The study was conducted on 500 Iranian female sheep (≤2 years old) referred to the Kermanshah slaughterhouse, from January to April 2017. The lungs of 100 sheep that showed the respiratory symptoms were grossly examined for Maedi lesions and tissue samples were collected for pathological and molecular investigations. Blood samples of suspected sheep were collected from the Jugular vein and sera were separated and stored at -20°C until serological assay. Blood samples with Ethylene diamine tetra acetic acid (EDTA) were also collected for molecular analysis. Ten sheep without any respiratory signs and apparently normal lungs were considered as the control group. The study was approved by the Shiraz University Policy on Animal Care and Use.

Histopathological investigation

Tissue samples collected from the suspected lungs and control group were fixed in 10% neutral buffered formalin and processed by standard procedures for pathological examination. Finally, the 5 µm-thickness sections were stained with haematoxylin and eosin (H&E) and examined for Maedi lesions.

Serological examination

Specific antibodies against ovine MVV were evaluated by a qualitative indirect enzyme-linked immunosorbent assay (ELISA) using commercial sheep-specific kits (Catalog No.: ED01124Sh, Shanghai Crystal Day Biotech, Shanghai, China). Positive and negative controls were provided in the kit and average optical density (OD) value) of negative control ± 0.15 were considered as a Cutoff value. Samples with OD value ≥ Cutoff value were considered positive.

Detection of MV provirus

Genomic DNA was extracted from the whole blood and lung samples using DNA Extraction Kits (Genet Bio, Seoul, Korea) and the purity of the DNAs was confirmed by NanoDrop measurement (Thermo Scientific, USA) and agar gel electrophoresis. A 260/280 ratio of about 1.8 was obtained for almost all the samples. Maedi-Visna provirus was detected by polymerase chain reaction (PCR) of the long terminal repeat (LTR) region. The primers were composed of forward 5´-AAG CAA ATG TAA CCG CAA GT-3´ and reverse 5´-AAC GAA TCC CGA TAA TAA CC-3´ primers, which resulted in the amplification of 236 bp DNA fragment in the LTR region. Amplification was performed in a final volume of 25 µL containing 20 ng genomic DNA, 100 ng of each primer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 50 mM KCl and 20 mM Tris-HCl, and 1 U/µL *Taq* DNA polymerase (CinaClon, Iran). The thermal cycling profile was 1 cycle of 95°C for 2 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 56.2°C for 30 s and extension at 72°C for 30 s, with a terminal extension of 5 min at 72°C. The amplified PCR products were detected using electrophoresis on 2% agarose gel and visualized by ultraviolet (UV) light. Polymerase chain reaction product generated from MVV positive sample was used as a positive control for PCR amplification. DNA sample prepared from confirmed MVV-seronegative sheep was also used as negative control.

Maedi-Visna provirus sequencing

Polymerase chain reaction products from MVV-positive samples were purified and sent for sequencing using ABI 3730 XL automatic DNA sequencer (Macrogen Inc., Seoul, Korea). Sequences were aligned using the Clustal program available in the package of Bioedit software version 7.0.5.3 (http://jwbrown.mbio.ncsu.edu/Bioedit/bioedit.html).

Transmission electron microscopy

The lung samples of MVV-positive sheep confirmed by ELISA and PCR/sequencing were prepared for TEM. Samples were sectioned to blocks of 1 mm^3 in dimensions and fixed in cold 2.5% glutaraldehyde diluted in 0.1 M phosphate buffered saline (PBS) containing NaCl, NaH_2PO_4 and Na_2HPO_4 (pH = 7.2) for 5 h. The samples were rinsed twice in 0.1 M PBS, and then fixed in 1% Osmium tetroxide. They were then dehydrated in a graded series of increasing concentration of ethanol and subsequently embedded in an equal volume of propylene oxide and - EPON - resin 828 overnight and then in fresh 100% EPON for another 24 h. Finally, the blocks were polymerized in fresh EPON resin by leaving them in an oven at 65°C for 18 h. Using

a ultramicrotome (No. OMU3, C. Reichert, Austria), sections of 50-70 nm in thickness were provided and loaded on 200-mesh copper grids having carbon stabilized formvar support films. Finally, the sections were stained by immersion in saturated uranyl acetate for 10 min and lead citrate for 5 min and washed by distilled water, air dried and examined with an electron microscope (Philips CM 10, TEM, Eindhoven, Netherlands).

Results

Gross and histopathological findings

One-hundred lungs with gross lesions suspected to Maedi disease were selected for microscopic investigations. Lungs with hard grayish white mottled focal or multifocal lesions that failed to collapse and were heavier than normal lungs were considered as the suspected Maedi cases. The firm lesions were mostly found in the caudal lobes with fleshy consistency. The color of suspected lungs usually ranged from pink to white and the rib impressions were prominent on them. The cut surfaces of the lesions were dry, without fluid in the airways (Fig. 1).

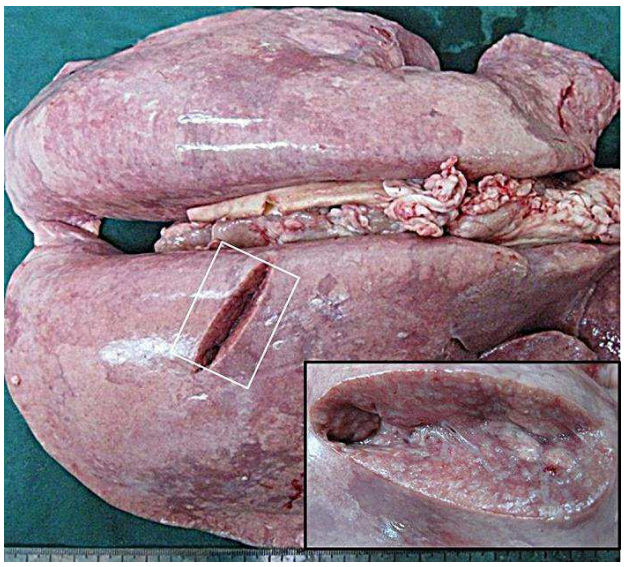

Fig. 1: Gross appearance of Maedi disease in the lung sample of sheep. The lung is enlarged, heavy, gray-white mottled pallor, and failed to collapse. Inset, thickened interstitial tissue and alveolar septa a rubbery texture

Microscopic examination revealed lympho-plasmacytic interstitial pneumonia, presence of peribronchiolar and perivascular accumulation of lymphoid cells and smooth muscle hyperplasia in 85% of the affected lungs. Chronic interstitial pneumonia was also characterized in the positive samples by diffuse thickening of the interalveolar septa due to the accumulation of lymphocytes, plasma cells and macrophages (Fig. 2). Lymphocytic cuffs were observed around the bronchi, bronchioles, blood vessels, and within the pulmonary parenchyma (Fig. 3). No significant lesions were detected in the lungs of control group.

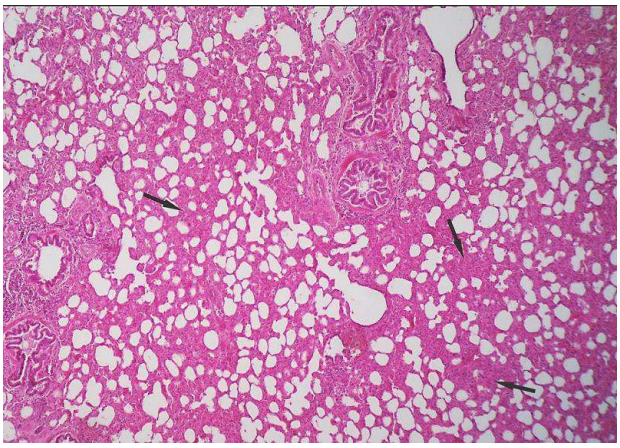

Fig. 2: Histopathologic characteristics of the MVV-infected lungs. The interalveolar septa are thickened by infiltration of mononuclear inflammatory cells and interstitial fibrosis (arrows), (H&E; ×40)

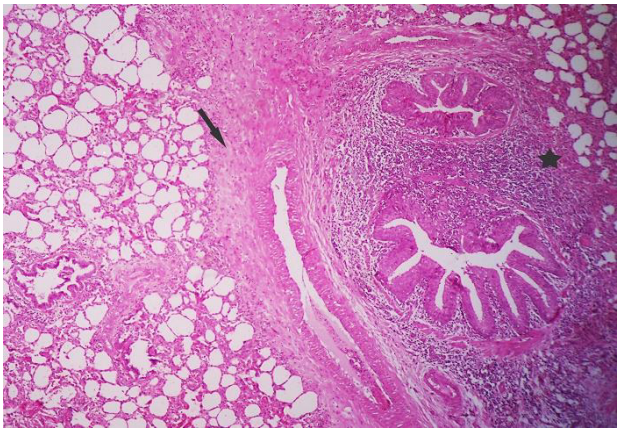

Fig. 3: Microscopic view of the lungs infected with Maedi-Visna virus. The bronchiolar-associated lymphoid tissue (BALT) surrounding the bronchioles (asterisk) and hypertrophy of the smooth muscle fibers around the blood vessel (arrow) are evident, (H&E; ×100)

Serology

Out of 100 serum samples collected from the suspected sheep, seven samples (7/100; 7%) had antibodies against MVV and showed the OD value greater than the Cutoff in ELISA technique. All control samples were MVV seronegative.

Detection of MV provirus

The LTR region of MV provirus was amplified in three (3/100; 3%) DNA samples extracted from lungs of suspected sheep (Fig. 4). Sequencing analysis also confirmed the presence of MV provirus in the three samples. No amplification was observed, neither in DNA samples extracted from the blood of suspected sheep nor the control group.

Ultrastructural findings

The ultrastructural appearance of the MVV after TEM analysis of the seropositive, PCR/sequencing

confirmed lung samples was shown in Fig. 5. Virions were seen budding the cytoplasmic membrane of MVV-infected macrophages (Fig. 5A). Furthermore, TEM analysis revealed the typical features of MVV and the viral particles produced in the macrophages (Fig. 5B).

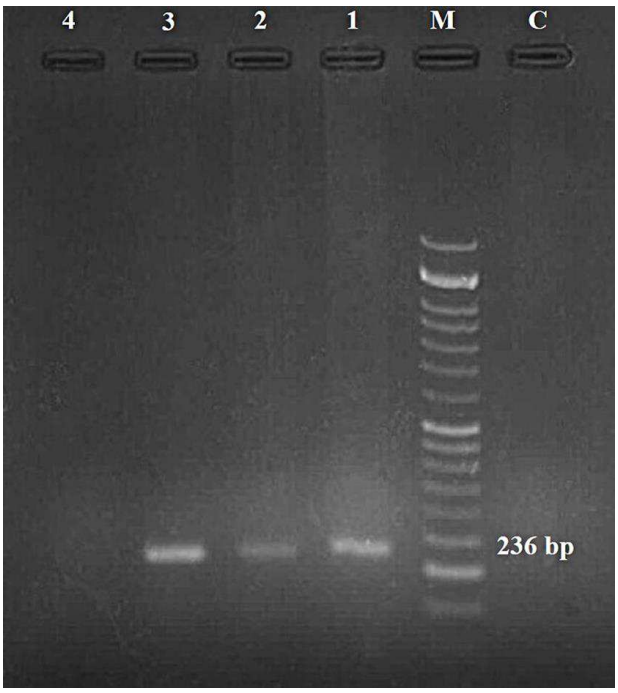

Fig. 4: Detection of Maedi proviral LTR in the lung samples. C: Negative control, M: Molecular weight markers, Lanes 1-3: Positive amplification (236 bp), and Lane 4: Negative amplification

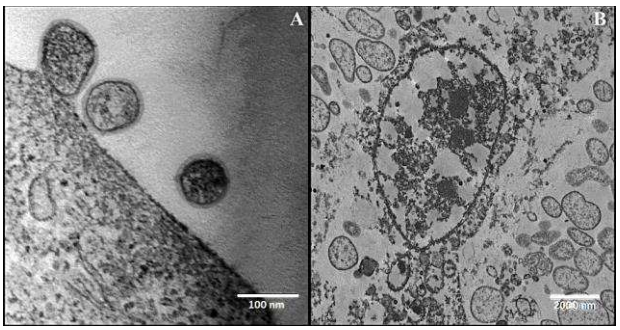

Fig. 5: Transmission electron micrographs of the MVV-infected lungs. (**A**) Viral buds were present at the cytoplasmic membrane of MVV-infected macrophages (scale bar, 100 nm), and (**B**) intracytoplasmic particles were seen in the macrophages (scale bar, 2000 nm)

Discussion

Maedi-Visna disease is caused by a non-oncogenic retrovirus and manifests with a long incubation period, from several months to years (Sakhaee and Khalili, 2010). Progressive clinical course of the disease in sheep is characterized by the infiltration of mononuclear cells and lymphoid hyperplasia in the central nervous system, pulmonary parenchyma and other organs (Sayari and Lotfi, 2001). The clinical manifestations of the disease in the respiratory system may take several years to be diagnosed and persistently infected (PI) cases remain as a source of viral transmission in the flocks (Sasani *et al*., 2013). Due to the long incubation period and high frequency of PI cases, the disease can cause considerable economic losses, including decrease in lamb birth weight, reduction of milk production, and export restriction, especially in the border cities (Leginagoikoa *et al*., 2010; Azizi *et al*., 2012; Benavides *et al*., 2013; Nowicka *et al*., 2015). Considering the chronic nature of the disease, identification of infected sheep without clinical signs has a critical role in preventing the spread of the virus and controlling the disease.

Diagnosis of Maedi disease based on the clinical manifestations is quite difficult. Moreover, histopathological lesions of the MVV are not pathognomonic and informative. Definitive diagnosis of Maedi is usually made based on the characteristic lesions that are found grossly and histopathologically along with a supportive clinical history of the disease; confirmed by serological examinations and molecular techniques (Sasani *et al*., 2013; Beigi Boroujeni *et al*., 2015; Norouzi *et al*., 2015; Caswell and Williams, 2016). In the present study, histopathological examination of lung samples from 100 sheep with respiratory symptoms indicated the Maedi-like lesions in 85% of the affected lungs. Serology and molecular analysis confirmed the MVV infection in 7% and 3% of cases, respectively. Maedi-Visna provirus was also identified through sequencing of LTR region and confirmed by TEM. The Maedi provirus was observed inside the macrophages of the infected lung tissues. Although lentiviruses infect various cell types including choroid plexus, fibroblasts, endothelial cells, monocytes, and epithelium of the mammary glands, the complete replication and persistence of the virus occurs in the cells of monocyte-macrophage lineage, particularly the mature macrophages (Preziuso *et al*., 2013; Caswell and Williams, 2016).

Different serological and molecular methods with different sensitivity and specificity levels have been applied to determine the Maedi disease status in sheep populations, including ELISA, agar gel immunodiffusion (AGID) immunofluorescence (IF), and PCR (De Andres *et al*., 2005; Sasani *et al*., 2013; Norouzi *et al*., 2015). Sayari and Lotfi (2001) applied the AGID method for detection of MVV antibody in 400 serum samples collected from sheep population in Ahvaz province, in Southern Iran and all the samples were seronegative. However, histopathology examination showed the Maedi-like lesions in 7.25% of the corresponding lungs. A study conducted on culled ewes in Alberta also reported 26.8% prevalence of MVV infection based on the histopathological findings, as compared with 13.0% by AGID test (Fournier *et al*., 2006). Leginagoikoa *et al*. (2010) showed 77% seroprevalence of Maedi disease in North Spain which had reached up to 80% in some herds. Zhang *et al*. (2013) investigated the seroprevalence of Maedi disease in 672 sheep from 12 regions of China

using ELISA method and reported the seroprevalence of 4.60 to 50% in different areas. Prevalence of MVV in 757 sera and 911 blood samples collected from 23 sheep flocks in Turkey has been reported 50.05% through the ELISA and 54.7 using LTR-PCR. The MVV prevalence increased with age, while PCR positivity was higher in young animals (Muz *et al*., 2013). Brinkhof *et al*. (2008) also confirmed the presence of virus in 44% of seropositive sheep through LTR-PCR. Variation in the prevalence rate of the disease among different populations can be attributed to the different factors including sensitivity of the diagnostic tests, sample size, weather conditions, and management systems (Beigi Borujeni *et al*., 2015).

A direct relationship seems to exist between the proviral load and antibody titers in serum samples. Higher viral loads are associated with increased viral antigens exposed to the immune system and resulted in elevated immune responses. However, lentiviruses tend to replicate and remain inside the macrophages and may expose low level of antigens to the immune system. Therefore, serological assays like ELISA or AGID may fail to identify all the positive cases (Herrmann-Hoesing, 2010; Crespo *et al*., 2016). On the other side, investigations of MVV in sheep populations have demonstrated that serological tests can produce false-positive results due to the cross reactivity of antibodies with other viruses from the same families (Muz *et al*., 2013; De Souza *et al*., 2015; Crespo *et al*., 2016). Extramiana *et al*. (2002) examined the LTR-PCR for detection of MVV proviral in blood, milk, and tissue samples. They reported 100% specificity and 98% sensitivity of LTR-PCR technique for detection of the MV provirus in tissue samples, in comparison with the two serological methods, AGID and ELISA. According to the data obtained in this study, TEM detection of MV provirus inside the macrophages of all PCR-positive lung samples also confirm the reliability of the results achieved by LTR-PCR. It has been suggested that a combination of serology with more sensitive techniques like PCR and sequencing may represent the more accurate and reliable data for detecting the infected cases, specially the PI animals (De Andres *et al*., 2005; Brinkhof *et al*., 2008; Azizi *et al*., 2012).

The results of this study showed the presence of MVV infection among the sheep population in Kermanshah province, western Iran. Although the high incidence of disease was estimated by histopathology, serological and molecular tests showed the low prevalence of 7% and 3%, respectively. Consistency between TEM findings and PCR results represents that molecular analysis of LTR region is a highly sensitive and specific method for detecting the Maedi provirus in the lung samples. However, Maedi provirus was not detected in DNA samples extracted from blood samples. Since the complete replication and persistence of the virus mostly occurs in the mature macrophages, tissue samples are more sensitive specimens for diagnosing the disease, in comparison with the blood or milk samples.

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Conflict of interest

The authors do not have any particular conflicts of interest to declare.

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