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Original Article

Distribution of maedi-visna virus in different organs of the spontaneously affected small ruminants in India

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Abstract

Background: Maedi-visna (MV) is a small ruminant lentiviral (SRLV) disease affecting sheep and goats, and causes pathological alterations in various organs including lungs, pulmonary lymph nodes, mammary glands, joints, and CNS. **Aims:** Present study was focused to detect the MV virus (MVV) nucleic acid and MVV p28 antigen in different organs of the spontaneously MVV affected sheep and goats. **Methods:** Total of 657 samples were collected from sheep and goats (169 blood, 136 lungs, 96 pulmonary lymph nodes, 74 brain, 54 mammary gland, 78 joints, and 50 spleen) and screened for MVV nucleic acid using nested PCR assay. Serum samples were screened for SRLV antibodies by cELISA. Immunolocalization of MVV was demonstrated by using the polyclonal antibody against p28 antigen by immunohistochemistry in lungs, lymph nodes, mammary glands, and joint tissues. **Results:** Out of 657 samples, 10.7% (70) were found positive for MVV. Among different organs, lungs showed highest positivity (25.7%) followed by mammary glands (14.8%), blood (9.5%), joint tissues (7.7%), brain (5.4%), and pulmonary lymph node (1.0%). SRLV antibodies were detected in 29.2% of the serum samples of both sheep and goats by cELISA. MVV p28 antigen immunostaining was observed in lungs, lymph nodes, mammary glands, and joint tissues. However, the presence of MVV p28 antigen could not be demonstrated in the brain tissues. **Conclusion:** The highest positivity of MVV in lung tissues indicated higher predilection of the virus in the pulmonary tissue.

Key words: Immunohistochemistry, Maedi-visna, Seroprevalence, Small ruminants

Introduction

Maedi-visna (MV) was reported first time in 1952 in Iceland, where it caused huge mortality in sheep population (Sigurdsson and Palsson, 1954). MV is caused by MV virus (MVV) belongs to lentivirus group of family retroviridae (ICTV, 2022). The main route of transmission of disease is by colostrum/milk, aerosols and semen of infected animal (Larruskain and Jugo, 2013). MVV usually infect the cells of monocyte/macrophage lineage and cause lifelong persistent infections. Virus replicates in the monocytes during its differentiation to macrophage and disseminates further to different target organs (Blacklaws, 2012; Pinczowski *et al*., 2017). MVV infection leads to development of progressive interstitial pneumonia, meningioleucoencephalitis, mastitis, and polysynovitis/ arthritis in small ruminants.

Pathological lesions of the MV developed in various organs viz. lungs (interstitial pneumonia), mammary gland (lymphocytic mastitis), joints (arthritis), brain (encephalitis), etc. After infection of MVV, spread to other organs occurs through monocytes present in blood and it is reported that blood samples showed higher MVV positivity than other types of samples (Kumar *et al*., 2023). Diseases caused by small ruminant lentiviruses (SRLVs) are reported globally (except in Australia and New Zealand) including India, with variable rates of seropositivity (Gomez-Lucia *et al*., 2018; Mishra *et al*., 2020; Kumar *et al*., 2022). Studies based on serological and molecular detection of SRLVs in Indian small ruminant population revealed seroprevalence between 5-19% and PCR based MVV nucleic acid positivity between 0.27-10.41% (Dar, 2017; Mishra *et al*., 2020; Kumar *et al*., 2022). Mixed infection of MVV with other infectious agents such as jaagsiekte sheep retrovirus (JSRV) and mycoplasma has also been reported from the small ruminants (Valecha *et al*., 2023;

Yadav *et al*., 2024a).

Detection of the viral antigen in the tissues indicates the sites of the viral tissue tropism. So, to demonstrate the MVV proteins in the tissues, immunohistochemistry (IHC) can be used and further can be correlated with the active infection. MV is widely prevalent in our country and responsible for causing immuno-suppression, secondary bacterial/viral infections, and ultimately causes huge economic losses to the small ruminant industry. Diagnosis of the active or latent cases of the MVV in a herd can ring an alarm for the farmers to take the preventive measures for its control. Therefore, the present study was aimed to detect the MVV nucleic acids and antigen distribution in the different organs of the MVV affected sheep and goat.

Materials and Methods

Sample collection

Total 144 serum samples from sheep (n=86) and goats (n=58) were collected from industrial and rural farms of Rajasthan and stored at -20°C. MVV can infect multiple organs, so total 488 tissue samples were collected, which include 136 lungs (44 sheep, 92 goats), 96 lymph nodes (30 sheep, 66 goats), 74 brain (20 sheep, 54 goats), 54 mammary gland (18 sheep, 36 goats), 78 joints (20 sheep, 58 goats) and 50 spleen (20 sheep, 30 goats), from sheep and goat population of Rajasthan, and Uttar Pradesh states of northern India in the 10% buffered formalin for histopathology and IHC, and small pieces were stored at -20°C for molecular detection of MVV (Vaught *et al*., 2011).

Detection of SRLV antibodies in serum

Serum samples stored at -20°C were screened for the presence of SRLVs antibodies using SRLV antibody cELISA kit (VMRD, USA). The cELISA kit was able to detect the antibodies of both MVV and caprine arthritis encephalitis virus (CAEV). The procedure followed for the cELISA was as per the manufacturer's instructions.

DNA extraction from the tissue and blood samples

DNA was extracted from tissue samples (stored at -20°C) by using the commercially available tissue and blood DNA extraction kit (Qiagen) and kept at -20°C for further use.

Polymerase chain reaction (PCR)

Nested PCR was performed to detect the proviral MVV nucleic acid by using the specific primers (LTR region) described in earlier studies (Preziuso *et al*., 2013). Primers (external forward primer (Fex) 5´-TGA CAC AGC AAA TGT AAC CGC AAG-3´, external reverse primer (Rex): 5´-CCA CGT TGG GCG CCA GCT GCG AGA-3´, internal forward primer (Fin): 5´-AAG TCA TGT A(G/T)CA GCT GAT GCT T, internal reverse primer (Rin): TTG CAC GGA ATT AGT AAC G) were synthesized commercially and used in the PCR reaction. PCR assay was carried out in 25 µL reaction volume which comprised of 12.5 µL PCR master mix (Genetix), 0.5 µL each of both forward and reverse primer (0.4 µM), 8.5 µL of nuclease free water and 3 µL of DNA template. The contents were mixed thoroughly, spin briefly and then tubes were placed in thermocycler (Q-Cycler 96, Hains Lifesciences). Thermal profile for PCR reaction mixture includes initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 58°C for Fex and Rex and 50°C for Fin and Rin primers, and elongation at 72°C for 40 s, then final elongation at 72°C for 7 min. Amplified DNA fragments were visualized by transilluminator under UV light in 1.5% agarose gel by using Labsafe dye at 1 µL/10 ml (c150 Azure Biosystem, Gel Doc).

Sequencing of PCR products

PCR products were sequenced by Eurofins Genomics Company and analysed using BLASTn and DNASTAR software. Sequences were edited by Editseq (DNASTAR) and phylogenetic analysis of the sequences was performed by using ClustalW module of MegAlign (DNASTAR).

Histopathology

Formalin fixed tissue samples were washed overnight under tap water and paraffin-embedded tissue blocks were prepared by standard protocol. From paraffin blocks, 4-5 µm thick tissue sections were cut with the help of microtome and sections were taken on glass slides. Haematoxylin and eosin (H&E) staining was performed and the slides were examined for pathological changes under light microscope. Duplicate tissue sections of some of the selected cases were also processed for Masson's Trichome Staining (MTS).

Immunohistochemistry (IHC)

Duplicate sections were taken on APES (3-aminopropyl triethoxysilane) coated glass slides and immunostaining was performed to demonstrate MVV p28 antigen. Briefly, the tissue section taken on coated slides was deparaffinized in xylene and hydrated through graded alcohols. Blocking of endogenous peroxidase activity with H_2O_2 (3% in methanol) was performed for 30 min. Then sections were washed with phosphate buffered saline (PBS, pH 7.4) for 5 min (thrice) followed by antigen retrieval, by boiling the sections into citrate buffer (pH 6.2, 0.01 M) in microwave oven for 20 min. After washing with PBS for 5 min again, the sections were incubated with protein blocker (ab64226, Abcam) for 30 min at room temperature. The sections were then incubated overnight at 4°C with the mouse anti-MVV p28 primary antibody (VMRD, USA; 1:50 dilution), whereas PBS was used for negative control. Following PBS washings, the sections were incubated with secondary goat anti mouse IgG (ab6788, Abcam; 1:200 dilution) for 1 h, followed by incubation with streptavidin-HRP (ab7403, Abcam). After three washes in PBS for 5 min, the sections were covered with diaminobenzidine (DAB) chromogen (Immunocruz

Mouse ABC staining kit, Santa Cruz, USA). Sections were counterstained with Mayer's haematoxylin for 5-10 s. Repeat washings with tap water were given in the end to remove excess stain particles. After dehydrating in ascending grades of alcohol, tissue sections were mounted with coverslip (Immunohistomount). Then sections were examined under the light microscope for the dark brown positive immunosignals, if any. The positive signals or staining was assessed by observing the presence of dark brown signals with varied intensity (low/moderate/high).

Results

Seropositivity of SRLVs

Total seropositivity of 29.2% (42/144) was observed in both sheep and goats. Almost similar percentage of positivity was obtained in sheep 29.1% (25/86) and goats 29.3% (17/58). Seropositivity was non-significantly slightly higher in organized herd (29.6%, 24/81) as compared to unorganized herds (28.6%, 18/63).

Pathological lesions of MV

In the study, pathological lesions suggestive of ovine progressive pneumonia (maedi) were recorded in 33.1% (45/136) animals. Grossly, the lungs were heavy, non-collapsible, firm with meaty consistency and often with rib impressions on the dorsum. Microscopically, multifocal to diffuse thickening of the interstitium and interalveolar septa with infiltration of mononuclear cells (MNCs), fibroblasts and deposition of collagen fibrils were observed. Moderate to severe degree of lymphocytic hyperplasia was observed in the peribronchial and perivascular region or at the broncho-alveolar junctions, forming lymphoid follicle like structure. Lymph nodes showed lymphoid depletion mainly in the follicular region of the cortex and engorgement of blood vessels and capillaries in the cortical and medullary region. Spleen tissues showed lymphoid depletion, congestion and reactive hyperplasia. Mammary tissue revealed presence of atrophy, fibrosis, fatty changes and/or infiltration of mononuclear cells around the acini, in the glandular epithelium, interstitium, intra-lobular and interlobular tissues of the gland. Congestion, increased number of oligodendrocytes, meningitis, and mild peri-vascular cuffing were seen in brain tissues. Synovial membrane of the joints showed the thickening and mild infiltration of MNCs (Figs. 1A-F).

Detection and sequencing of MVV nucleic acid

Out of 657 samples (488 tissues and 169 blood samples) screened for MVV nucleic acid by nested PCR, 10.7% (70/657) were found positive and among these 13.6% and 8.5% samples belongs to sheep and goats, respectively. More specifically, percent positivity in lungs was found to be higher in sheep (38.6%) than in goats (19.6%).

Among different organs of both sheep and goats combined, lungs showed highest positivity (25.7%)

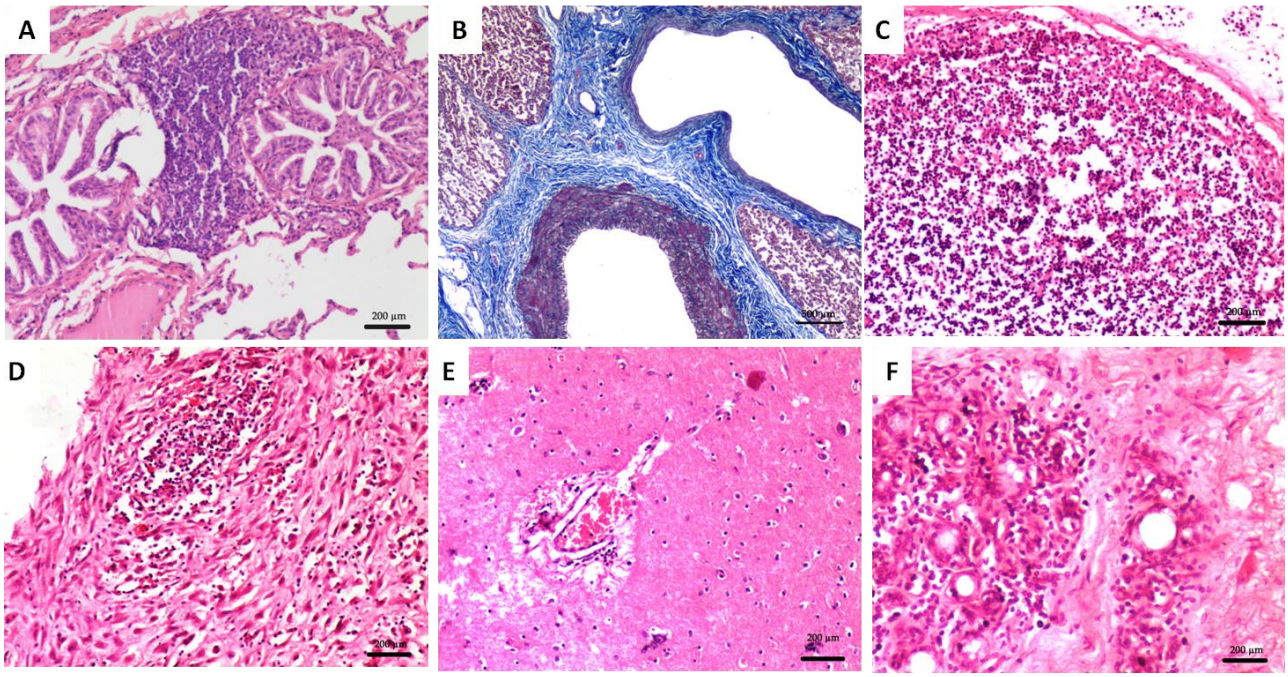

Fig. 1: Maedi: Lung tissue sections showing proliferation of bronchiolar epithelial cells with marked hyperplasia of lymphocytes forming follicle like structure in the peribronchiolar and perivascular area, thickening of the interstitium with infiltration of MNCs (**A**, H&E, scale bar, 200 µm), MTS stained tissue sections of lungs showing hyperplasia of smooth muscle cells in the peribronchiolar and perivascular area (**B**, MTS, scale bar, 500 µm), lymph nodes tissue section depicts lymphoid depletion (**C**, H&E, scale bar, 200 µm), joint showing infiltration of MNCs in synovial tissue (**D**, H&E, scale bar, 200 µm), brain showing mild perivascular cuffing with lymphocytes (**E**, H&E, scale bar, 200 µm), and sections of mammary glands showing degenerative changes with MNCs infiltration (**F**, H&E, scale bar, 200 µm)

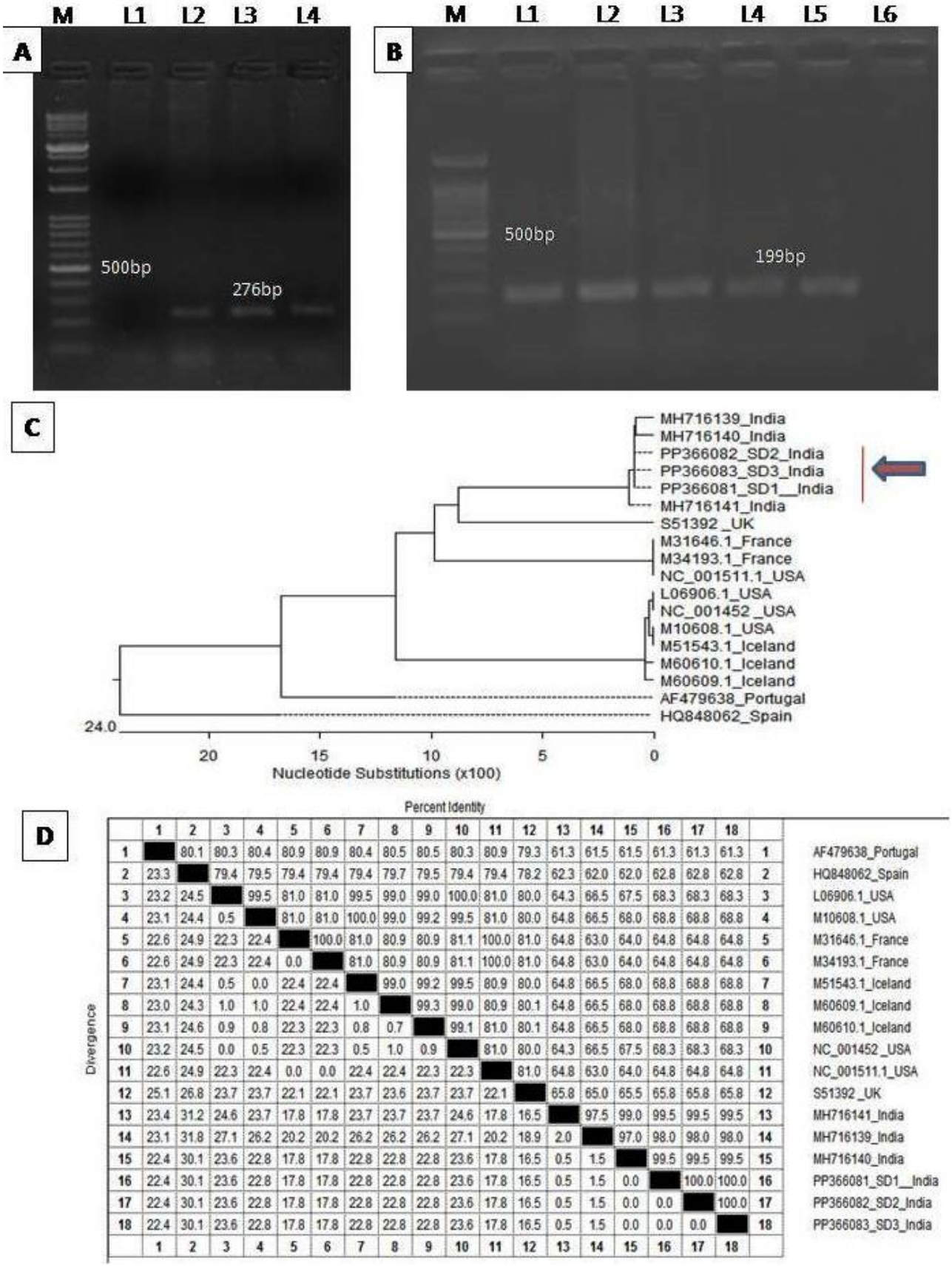

Fig. 2: Agarose gel electrophoresis (2%) showing PCR products of MVV with Fex & Rex (**A**) and Fin & Rin (**B**), M: Marker, NTC: No template control (A-L1; B-L6), S: Sample (A-L2 to L3; B-L2 to L5), Positive control (A-L4; B-L1), phylogenetic tree (**C**), and the divergence and percent identity table (**D**) of the MVV sequences generated (GenBank accession numbers: PP356081, PP356082, and PP356083) in the study

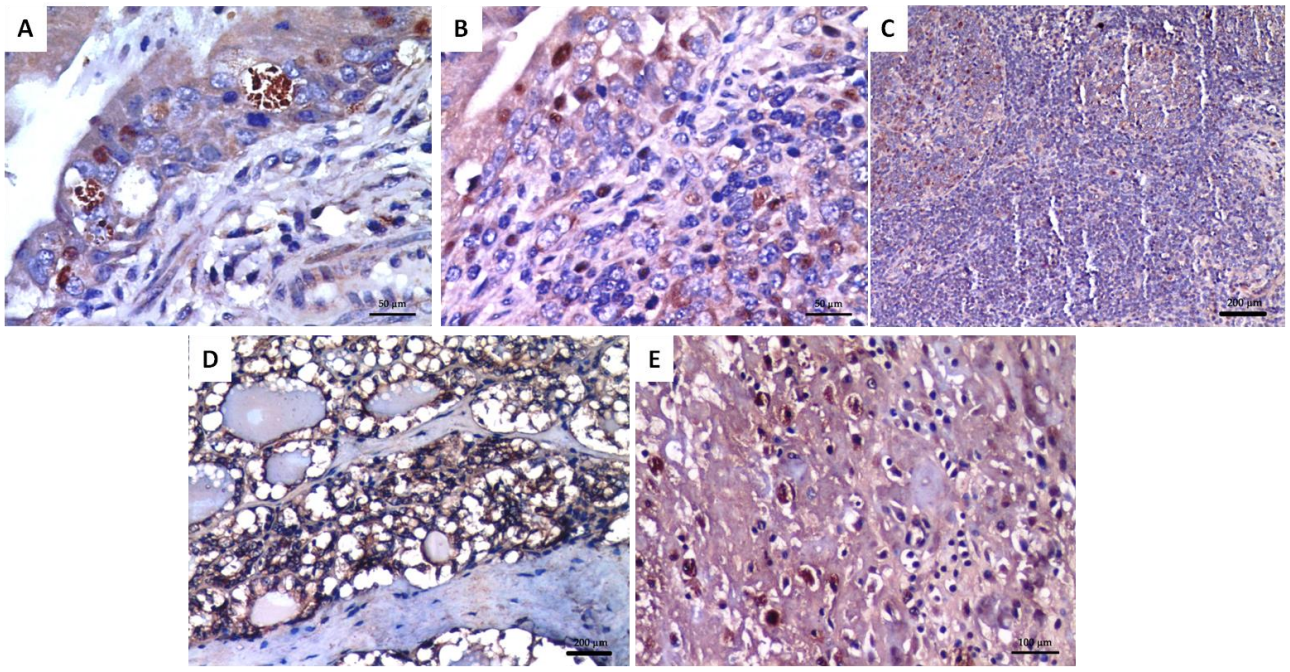

Fig. 3: Maedi associated lungs, lymph nodes, mammary glands, and joints presence of dark brown colour positive signals in the bronchiolar epithelial cells and macrophages (**A**, IHCxMayer's hematoxylin, scale bar, 50 µm), lymphocytes in the peribronchiolar area (**B**, IHCxMayer's hematoxylin, scale bar, 50 µm), and in follicles of lymph nodes (**C**, IHCxMayer's hematoxylin, scale bar, 200 µm); in cytoplasm of MNCs in the intralobular and interlobular area of mammary gland (**D**, IHCxMayer's hematoxylin, scale bar, 200 µm) and in MNCs and fibroblast like cells of joints (**E**, IHCxMayer's hematoxylin, scale bar, 100 µm)

followed by mammary glands (14.8%), blood (9.5%), joint tissues (7.7%), brain (5.4%), and pulmonary lymph node (1.0%). The MVV LTR region sequences (199 bp) of our samples (GenBank accession numbers: PP356081, PP356082, PP356083) showed 100% similarity among themselves. These sequences showed 98-99.5% similarity with earlier reported sequences from India. Among the sequences reported from other countries highest similarity was observed with strain of United Kingdom (Figs. 2A-D). Further, it was observed that lungs samples of males (45.5%) showed higher percent positivity for MVV than females (21.4%). It was also observed that MVV was more common in adult animals (>12 months of age) followed by growers (3-12 months) and young ones (<3 months) with frequency of occurrence being 40.2% (39/97), 19.4% (6/31), and 0%, respectively.

Distribution of MVV antigen in different organs

IHC staining showed dark moderate to high intense brown positive immunosignals in the cytoplasm of bronchiolar epithelial cells, peribronchiolar and perivascular lymphoid aggregates and in the alveolar macrophages. The presence of MVV p28 antigen was appreciated in the lymphocytes in the follicles of the lymph nodes, in the cytoplasm of the myoepithelial cells of the acini, in which variable vacuoles of the milk droplets were seen occupying most of the cytoplasm pushing the cytoplasm in the peripheral region and in the cytoplasm of macrophages and fibroblasts like cells of joint tissues (Figs. 3A-E). However, positive signals of MV viral antigens could not be demonstrated in the brain tissues in the present study.

Discussion

SRLV seroprevalence in the Indian small ruminant population was reported up to 19.58% in the earlier studies (Mishra *et al*., 2020, Kumar *et al*., 2022), which is lower than the seropositivity reported in present investigation. The higher seropositivity of SRLVs in animals of industrial farms reported in this study attributed to the intensive nature of the rearing in these farms, which provides condusive closed environment spread of the diseases among susceptible sheep and goats (Mishra *et al*., 2020). The percent occurrence of the lung tissues samples showing characteristic histological features of maedi i.e. lymphocytic aggregation in the interstitium, bronchus-associated lymphoid tissue (BALT) hyperplasia and interstitial pneumonia were higher in the present investigation as compared to the previous reports (Dar, 2017; Mishra *et al*., 2020; Valecha *et al*., 2023), which may be due to the variations in the climatic and geographical areas along with genetic makeup of the animals (breeds) from which the samples were collected in different studies. Such variation was recorded earlier also in the Indian small ruminant population with serological positivity (Kumar *et al*., 2022).

Detection of the MVV in the different biological samples by detecting the presence of nucleic acid detection has been used by many researchers (Lujan *et al*., 1991; Barquero *et al*., 2011). Also, the active infection of MVV in the animal has been diagnosed by

demonstrating MVV antigens in the interstitial fibroblasts, acinar epithelial cells, macrophages, endothelial cells, adipocytes, and desquamating epithelium and macrophages of mammary glands as performed in present investigation (Carrozza *et al*., 2003). Colitti *et al*. (2019) reported that spleen may also be one of the target organs for MVV. In present study, lungs showed higher MVV positivity in sheep than that in goats, which was similar to the previous studies (Mishra *et al*., 2020; Kumar *et al*., 2022). Among different organs, higher MVV positivity was detected in lungs, blood and mammary gland tissues as reported in earlier investigations also (Kumar *et al*., 2022), which indicated the predilection of the virus in these organs as the transmission of virus usually occurs through the aerosol route and colostrums/milk (Peterhans *et al*., 2004; Yadav *et al*., 2024b).

Detection of the MVV p28 antigen in the different organs in the present study indicates that the virus was replicating in those sites. Earlier workers have also reported demonstration of MVV antigen in the cytoplasm of MNCs especially macrophages (Benavides *et al*., 2006; Kumar *et al*., 2022). Alveolar macrophages and inflammatory macrophages harbour MVV capsid antigen and are the possible site of viral persistence (Herrmann-Hoesing *et al*., 2010). Immuno-positive signals in the pulmonary lymph nodes supports the opinion of it as a site of viral replication, persistence and reservoir (Kumar *et al*., 2022). MVV p28 signals were also detected in the mammary gland and joint tissue. MVV antigen has earlier been demonstrated in the epithelial cells of the mammary glands (Gayo *et al*., 2019). The demonstration of MVV antigen in the myoepithelial cells of the acini indicate the possibility of viral transmission through milk or colostrum and supports the earlier reports of the detection of viral antigen in the mesenteric lymph nodes of the lambs/kids fed with colostrum of maedi affected dam by previous workers (Preziuso *et al*., 2013). IHC demonstration of MVV antigen have not been reported earlier in joint tissues, however, it has been reported in the bone marrow cells (Preziuso *et al*., 2003). However, positive signals of maedi viral antigens could not be demonstrated in the brain tissues in the present study. The findings are in accordance with those of earlier workers, who also could not demonstrate MVV antigens in the brain and spinal cord tissues in experimentally infected animals (Preziuso *et al*., 2003).

In conclusion, it was established that maedi-visna is circulating in the Indian small ruminant population. Molecular and pathological screening for the MVV genome revealed specific amplification in lungs, brain, lymph nodes, mammary glands, joints as well as in blood. MVV antigens could be demonstrated in the bronchiolar epithelial cells, lymphoid cells, macrophages, Clara cells as well as in the MNCs of the lungs. Besides, viral antigens were also demonstrated in the lymphoid follicles of the pulmonary lymph nodes, myoepithelial cells and macrophages in the mammary glands, fibroblasts like cells and in the MNCs of the joints by IHC. The present study indicates the presence of infection in our country which further needs detailed and stratified sampling from different temporo-geographical regions and systematic sero-surveillance and routine monitoring to prevent the transmission and spread of infection in future.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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