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RESEARCH ARTICLE

Pathological and Molecular Based Study of Naturally Occurring Lentivirus Infection

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ABSTRACT

The present study describes the multicentric lymphosarcoma associated with lymphoid interstitial pneumonia in indigenous breeds of sheep and goats in Pakistan. Serum samples from sheep (n=93) and goats (n=129) were screened for ovine lentivirus using agar gel immunodiffusion test. Overall, 7.52 and 3.87% seroprevalence was recorded in sheep and goat, respectively. During necropsy of sheep (n=3) and goats (n=4), gross lesions including dark color liver with multifocal whitish areas, unilaterally lungs consolidation with granular appearance of cut surface were observed. Mediastinal lymph nodes were swollen and arranged in chain like fashion. Histopathologically, liver parenchyma exhibited extensive proliferation of neoplastic cells of lymphocytic series. Metastatic cells in the form of follicular pattern in the lungs, spleen and mediastinal lymph nodes were also observed. Brain tissue exhibited degenerative changes in the neuron and perivascular cuffing. The PCR product size approximately 300 bp from lung tissue confirmed viral infection.

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INTRODUCTION

In Pakistan, sheep and goat production systems are nomadic, agro-pastoral, and sedentary. These small ruminants play an important role in rural economy of arid and semi-arid regions in the country. Several viral pathogens are responsible for the respiratory diseases in small ruminants in tropical and subtropical regions of the world. Infections due to lentiviruses including maedi-visna virus and caprine arthritis-encephalitis virus are serious economic threats to sheep and goat farming (Ahmed and Khosa, 2010; Valas *et al*., 2011). Multi-systemic and chronic infections in mammary glands, lungs, joints and central nervous system due to retroviruses have also been reported (Hananeh and Barhoom, 2009).

Bovine immunodeficiency virus (BIV) is known as bovine lentivirus and bovine leukemia virus (BLV) (Meas *et al*., 2000). Lentivirus is a common disease entity of sheep and goats with characteristic clinical signs including respiratory distress, coughing, marked cachexia, dyspnea and pronounced enlargement of the mandibular lymph nodes (Extramiana *et al*., 2002). Affected lungs in naturally and experimentally induced lentivirus infection revealed interstitial lymphoproliferative pneumonia, alveolitis and lymphocytic follicles (Preziuso *et al*., 2003). Multifocal and degenerated areas, perivascular cuffing in brain and transient viremia (McNab *et al*., 2010) in animals due to these viruses have been reported. Lentivirus-associated lymphomas have also been recognized in nonhuman primates and ungulates (Shuljak, 2007) and pulmonary adenocarcinoma has been reported in ovine (Hofacre and Fan, 2010).

Pulmonary form of the disease has been reported from several countries such as ovine progressive pneumonia in United States (Biescas *et al*., 2005), Spain (Barquero *et al*., 2011), Great Britain (Synge and Ritchie, 2010), central Ethiopia (Woldemeskel and Tibbo, 2010) and in Egypt (Oda and Youssef, 2011). Presence of antibodies to BIV in Pakistan has been documented in water buffalo and cattle populations (Meas *et al*., 2000). Anti-BIV p26 antibodies were detected by Western blotting in 10.3 and 15.8% water buffaloes and cattle, respectively, whereas anti-BLV antibodies determined by immunodiffusion test were found in 0.8% water buffaloes and no cattle were found positive (Meas *et al*., 2000). Such information in sheep and goat population in Pakistan is missing. The present study describes diagnosis and pathological changes in naturally occurring cases of lentiviurs infection in sheep and goats in Pakistan.

MATERIALS AND METHODS

Study animals and sample collection: After the death of three sheep and four goats, a total of 93 sheep and 129 goats kept at the Livestock and Dairy Farm, University of Agriculture, Faisalabad, Pakistan were screened for the presence of serum antibodies against lentivirus infection. At necropsy, the lungs of sheep and goats were suspected for the presence of lentivirus infection and were investigated using histopathological and molecular techniques. Blood was collected without EDTA from all live sheep and goats and serum was separated for the screening of animals. For this reason all the sheep, male (n=59) and female (n=34) were divided into three age groups, i.e., >1 year (n=30), <1-3 years (n=27) and above three years (n=36). Similarly, 129 goats, males (n=76) and females (n=53) were divided into three age groups, i.e., >1 year, <1-3 years and above three years containing 56, 25 and 48 animals, respectively (Table 1). Information about history and husbandry conditions of the goat and sheep were recorded. Animals showing clinical signs were treated with systemic administration of antipyretic, steroids, and antibiotics.

Table 1: Overall prevalence of lentivirus in sheep and goats on the basis of agar gel immuno-diffusion (AGID) assay

Species/age groups	No. of Animal	AGID		95% CI	Odd Ratio/ P value
		No.	%		
Sex					
Female	87	7	8.05	3.6-15.27	2.27
Male	135	5	3.70	1.37-8.02	
Age groups (Years)					
<1	86	1	1.2	0.06-5.60	P<0.01
1-3	52	3	5.8	1.5-14.90	
>3	84	8	9.5	4.52-17.28	
Species					
Sheep	93	7	7.53	3.35-14.32	2.02
Goat	129	5	3.88	1.43-8.38	

Table 2: Prevalence of lentivirus infection in sheep and goats of different age groups

Species /age groups	No. of Animal	Positive		95% CI	Odd ratio/ P value
		No	%		
Sheep					
Female	34	4	11.76	3.8-25.98	2.49
Male	59	3	5.08	1.3-13.21	
Age groups					
< 1 Year	30	1	3.33	0.17-15.36	P>0.23
>1-2 Year	27	2	7.40	1.26-22.3	
Above 3 Years	36	4	11.11	3.63-24.66	
Goats					
Female	53	3	5.66	1.46-14.63	2.22
Male	76	2	2.63	0.44-8.42	
Age					
< 1 Year	56	0	0.00	0.00	P< 0.05
>1-2 Year	25	1	4.00	0.20-18.19	
Above 3 Years	48	4	8.33	2.70-18.89	

Agar gel immune-diffusion assay (AGID): The presence of virus infection was detected by using AGID. Briefly, 0.7% agar plates were prepared and five millimeters wells were made after solidification. 40µl of diluted ovine lentivirus (OvLV) antigen (Allied Monitor, Fayette, MO, USA) was inoculated to the central well. The serum samples were pored in remaining wells. Finally, results were observed after 24 and 48 hours (Herrmann-Hoesing, 2010).

Paraffin method: For histopathological observations, tissue samples from lungs, liver and brain of sheep (n=3) and goats (n=4) at necropsy were fixed in 10% neutral buffered formalin and processed through paraffin embedding technique. Sections (5µm thick) were cut, stained with hematoxylin and eosin (H&E) and examined under light microscope (Bancroft and Gamble, 2008).

PCR: For confirmation of infection, DNA from suspected lungs samples of both sheep and goats was extracted using DNA extraction kit (Vivantis, USA) according to manufacturer's protocol. The PCR technique was optimized to confirm the pathogen. The LTR gene was amplified using specific set of primer (LTR-R 5-TGA CAC AGC AAA TGT AAC CGC AAG-3, LTR F 5- CCA CGT TGG GCG CCA GCT GCG AGA-3). Thermal cycler conditions optimized. The amplified PCR products were analyzed using 2% agarose gels containing ethidium bromide as a staining dye.

Data analysis: The collected data was subjected to statistical analysis by applying Chi-square test. 95% C.I. and odd ratio was also determined. The $P<0.05$ was considered as significance level.

RESULTS AND DISCUSSION

During the present study, the overall non-significant difference in seroprevalence of lentivirus infection in sheep (7.52%) and goats (3.87%) was recorded (Table 1 and 2). The variable prevalence of lentivirus has been reported from 2.47 to 25.10% in Canada (Shuaib *et al*., 2010; L'Homme *et al*., 2011), 17 to 25.7% in Ethiopia (Woldemeskel and Tibbo, 2010) and 22% in Iran (Azizi *et al*., 2012). Association of ovine lentivirus infection with chronic respiratory disease has been reported in Spain (Barquero *et al*., 2011), Brazil (Bandeira *et al*., 2009) and in Roccaverano goats (Grego *et al*., 2009).

A summary of gross and histopathological lesions of individual sheep (n=3) and goats (n=4) are presented in Table 3. Gross lesions including pleural adhesion, lung consolidation, and whitish color (Fig. 1) with granular cut surface were observed (Fig. 2). Similar enlargement and adherent of pleura with grayish raised foci were also reported in central Ethiopia (Woldemeskel and Tibbo, 2010). Previously, mottled white, brown and red lungs along with enlargement of mediastinal lymph nodes in goats (Rozear *et al*., 1998), a few multifocal gray to white masses slightly elevated, with pale and dry cut surface in lungs of sheep (Oda and Youssef, 2011) have been reported.

Histologically, lung parenchyma exhibited extensive lymphocytic follicular pattern of neoplastic cells penetrating alveoli, interstitium and bronchioles (Fig. 3). Among neoplastic cells, macrophages and plasma cells were also observed (Fig. 4). Lymphoid follicle formation in lung tissue observed in the present study could be due to immunological cellular proliferation in response to the replication of lentivirus in lung tissue. The persistent proliferation and constant insult in lungs parenchyma could be responsible for the transformation of normal cells in to neoplastic ones due to production of free oxygen or nitrogenous species which induce mutation to genome of lymphocytes as a result of chronic inflammatory process. Similar histological changes have been reported in experimentally infected sheep with maedi-visna virus (Preziuso *et al*., 2003), Palestinian sheep and goats (Hananeh and Barhoom, 2009) and in lambs (Hudachek *et al*., 2010). Induced pulmonary lesions

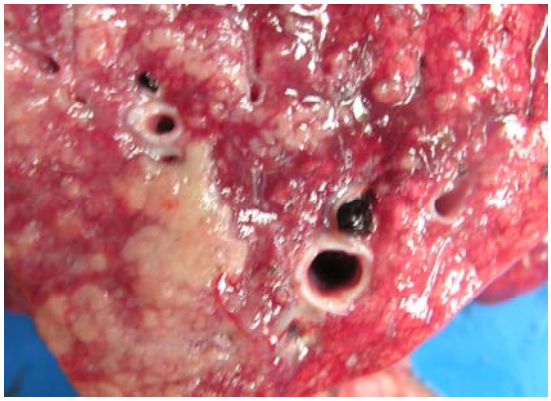

Fig. 1: Grossly lung exhibiting whitish papilliform ingrowths.

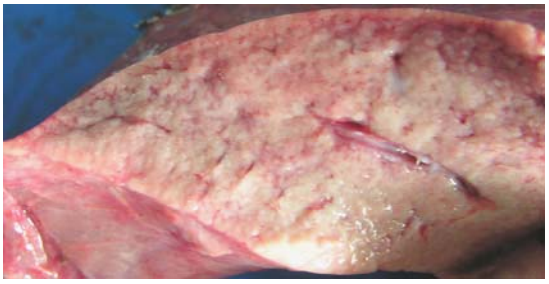

Fig. 2: Lungs consolidated, white in color with granular cut surface.

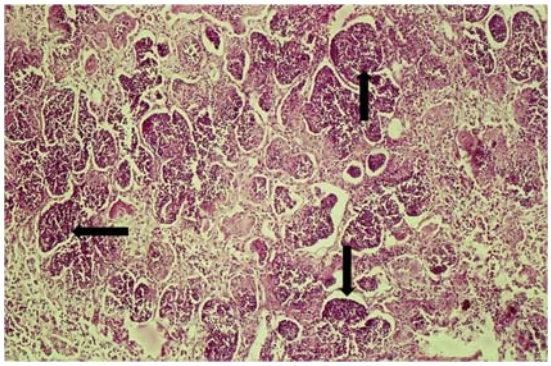

Fig. 3: Lung parenchyma showing extensive lymphocytic follicular pattern of neoplastic cells penetrating alveoli (Arrow) H&E. X-200.

in rhesus monkeys infected with simian immuno-deficiency virus have also been reported (Baskin *et al*., 1991). Similarly, numerous interstitial lymphocytic follicles, increase number of macrophages in lungs of naturally infected sheep with visna virus have been reported (Extramiana *et al*., 2002). Atypical T-Cell lymphosarcoma in calf and lymphoid follicle in lung tissues of North American sheep (Biescas *et al*., 2005) and in Iranian sheep (Azizi *et al*., 2012) have been observed.

Grossly, multiple whitish necrotic areas were observed over liver. Microscopically, massive invasion of carcinomatous cells replacing most of the hepatocytes and enclosing only islands of few healthy hepatocytes were observed (Fig. 5). Metastasis of these carcinomatous cells was also observed in mediastinal lymph node and spleen. Extensive proliferation of these neoplastic cells destroyed the normal architecture of these tissues. These histological findings have never been reported in the local breeds of Pakistan. However, from the accessible literature,

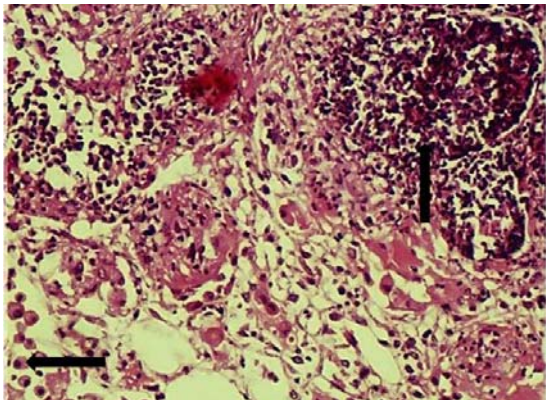

Fig. 4: Neoplastic cells lymphocytic follicles involving alveoli, interstitial areas and bronchioles plasma cells (Arrow) H&E. X-400.

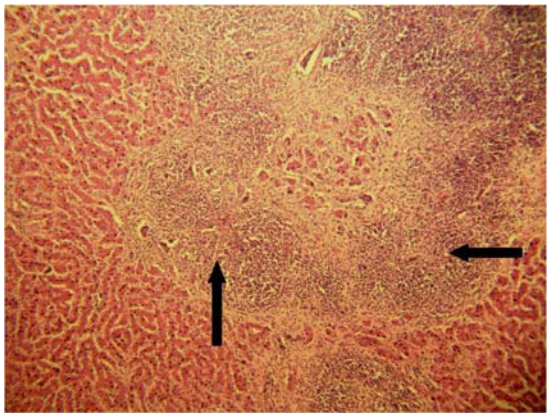

Fig. 5: Carcinomatous cells replacing hepatocytes and enclosing only islands of few healthy hepatocytes. (Arrow) H&E. X-200.

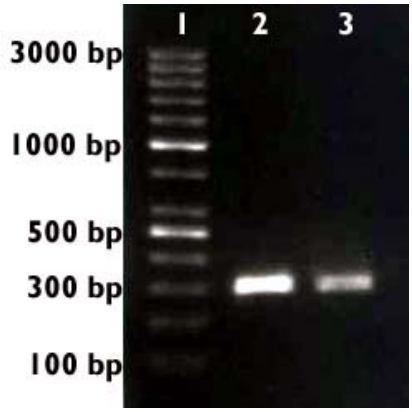

Fig. 6: Confirmation of lentivirus infections in lung tissue of sheep and goats. M:100 bp molecular marker and lane 1, 2 positive (about 300 bp).

metastasis has been reported in boar (Vo *et al*., 2004). Degenerative changes in neurons, perivascular cuffing and aggregation of mononuclear cells were observed in brain of infected animals. Similar findings were also reported in brain of rats infected with lentivirus (Jakobsson and Cecilia, 2006).

The PCR products size approximately 300 bp (Fig. 6) were obtained from the lung tissue in 5 animals (sheep, n=3 and goats, n=2). Previously, PCR procedures have been used for lentivirus detection in tissue samples (Preziuso *et al*., 2003). The viral entities have also been confirmed by PCR technique in infected ovine lambs

Table 3 Gross and histopathological lesions observed during necropsy of sheep and goats

Species/No.	Organs			
	Lungs	Liver	Brain	Lymph nodes
Sheep/Gross lesions				
1	Unilateral consolidation	Multifocal whitish areas	Congestion	Swollen
2	Consolidation, edema	Swollen	Mild congestion and petechiation	Arranged in chain fashion
3	Nodular appearance	Hard and whitish areas	Cerebral edema	Consolidated
Histological lesion				
1	Follicular pattern of neoplastic cells	Extensive neoplastic cells	Degeneration of neuron and perivascular cuffing	Extensive neoplastic cell growth
2	Proliferation of fibroblast and macrophages	Islands of hepatocytes	Congestion	Destruction of parenchyma
3	Pulmonary edema	Billary hyperplasia	Mild congestive changes	Normal
Goat/Gross lesions				
1	Pulmonary edema and congestion	Peri-hepatitis	Congestion	Normal
2	Consolidation	Congested	Normal	Normal
3	Hard and whitish in color	Normal	Mild congestion	Swollen
4	Pulmonary edema	Normal	Normal	Normal
Histological lesions				
1	Eosinophilic and proteineous fluid in alveoli	Mild necrotic changes in hepatocytes	Congestion	Normal
2	Thick interalveolar septae	Mild congestion	Mild degenerative changes	Congestion
3	Connective tissue proliferation	Neoplastic cell proliferation and multinucleated giant cells	Mild congestion	Extensive proliferation of neoplastic cells
4	Accumulation of neutrophil	Normal	Normal	Normal

(Alvarez *et al*., 2006) and infected synovial fluid, milk and blood samples from goats (Azizi *et al*., 2012).

Clinical signs, postmortem and histopathological findings recorded in our study are useful in establishing presumptive diagnosis. In the light of present investigations carried out in sheep and goats in tropical conditions, it can be concluded that these animals died due to viral infections. Therefore, further studies under tropical conditions are needed.

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