Detection of exogenous Jaagsiekte sheep retrovirus in Turkey

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ABSTRACT
The aim of this study is to detect the exogenous Jaagsiekte sheep retrovirus (exJSRV) in suspected cases of ovine pulmonary adenocarcinoma (OPA) from the Eastern Anatolian region of Turkey. Pathological examination and PCR were carried out with the lung, lymph nodule, brain, heart and liver tissues of four sheep with suspected OPA. Histology of the lung sections indicated the well-circumscribed, multifocal and unencapsulated gray to white masses (arrows) on the parietal surface of medial and caudal lobes. exJSRV was detected in all tissue samples except for brain by nested polymerase chain reaction (nPCR). In addition to the nested-PCR results, the presence of exJSRV into the clinical samples was confirmed with sequencing of two PCR-positive products for OPAV. This report highlights the first presence of exJSRV in the sheep suspected with OPA in Turkey. Furthermore, the results provide supporting evidence for the metastasis of exJSRV in extra-thoracic tissues.

Key words: exJSRV, OPA, PCR, Sheep.

INTRODUCTION
Ovine pulmonary adenocarcinoma (OPA, sheep pulmonary adenomatosis, Jaagsiekte) is a contagious tumor of the lungs that occurs in sheep. OPA has been reported in many countries in Europe, Africa, Asia and the Americas. The disease is an important animal welfare problem (De Las Heras et al. 2003). OPA tumors originate from type II pneumocytes and Clara cells. Metastases in intra-thoracic tissues have been reported in many studies (Garcia-Goti et al. 2000, Minguijon et al. 2013), but research related to extra-thoracic metastases has been limited. The most typical feature of clinical OPA is the discharge of a whitish foamy fluid from the nostrils. The incidence of OPA in sheep flocks is nearly 3.5% (Sharp and Herring 1983).

The aetiological agent of OPA is a beta-retrovirus known as Jaagsiekte sheep retrovirus (JSRV) (Palmarini et al. 1999). JSRV has two different forms: an endogenous form (enJSRV) and an exogenous form (exJSRV). Endogenous JSRVs are present in approximately 20 copies in the sheep genome (York et al. 1992). The majority of enJSVРs are defective in at least one viral gene. However, some enJSVR proviruses are potentially capable of encoding virus particles (Palmarini et al. 2001). enJSRVs are not oncogenic but their RNA and proteins are expressed in the female reproductive tract and in fetal tissue (Palmarini et al. 2001, Spencer et al. 2003). Exogenous JSRV is the aetiological agent of OPA. The amino acid sequences of exJSRV and enJSRV are between 90% and 98% identical (York et al. 1992, DeMartini et al. 2003). The U3 region of the JSRV long terminal repeat (LTR) is distinctly different between the endogenous and exogenous sequences (Palmarini et al. 1996). The enJSRV proteins interfere with the replication of exJSRV (Spencer et al. 2003, Arnaud et al. 2007).

Natural transmission of JSRV generally occurs via the aerosol route and a major risk factor for infection is close contact between animals. Lambs may be infected via the colostrum and milk from the JSRV-infected dams (Grego et al. 2008).

In sheep infected with exJSRV, there are no specific humoral immune responses to viral proteins (Sharp and DeMartini 2003, Summers et al. 2002). Serological assays are not useful due to a lack of JSRV-specific antibodies (Summers et al. 2002, Ortin et al. 1998). Clinical symptoms, histology, electron microscopy, immunohistochemistry, ELISA and polymerase chain reaction (PCR) have been used for the diagnosis of OPA. Anested PCR assay performed with the primers based on the U3 region of the viral LTR is able to distinguish between enJSRV and exJSRV (Palmarini et al. 1996).

The aim of this study is to detect exJSRV in different tissues of four sheep from the Eastern Anatolian region of Turkey suspected of having OPA.

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MATERIALS AND METHODS

Animals and necropsy examination: This study comprised four sheep from four different flocks with 150 to 500 animals each. The flocks were from Elazig and Tunceli in the Eastern Anatolian region of Turkey. The average animal losses of sheep flocks due to OP A were thought to range from 0.4–5%. Eight tissue samples were taken from the four sheep with suspected OP A for pathological examination and PCR (Table I).

The samples were presented for necropsy to the Department of Pathology, Faculty of Veterinary Medicine, University of Firat in Elazig. The animals ranged in age from 1.5 to 4.5 years and pulmonary lesions were identified at the time of necropsy. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax and stained with hematoxylin and eosin. Selected sections of the lung were stained with Masson’s trichrome.

Specific PCR for exJSRV: Paraffin embedded tissues were processed for PCR analysis as described by Wright and Mannos, (1990). Briefly, two 10µm sections were cut from wax blocks of the paraffin embedded tissues and placed into sterile Eppendorf tubes. The sections were then subjected to two steps of dewaxing by incubation in 1 ml of xylene for 30 min at room temperature, followed by centrifugation at 12000g for 5 min and disposal of the supernatant. The pellet was washed twice in absolute ethanol for 5 min, centrifuged again at 12000g for 5 min and the supernatant was discarded. Finally, the tissue was dried at 37 °C for 45 min before being digested overnight at 37 °C with 100µl of 0.8 mg of proteinase K. Total DNA was extracted by phenol-chloroform method (Sambrook et al. 1989). exJSRV-specific U3-hn-PCR was performed as described by Palmarini et al. (1996). PCR conditions were as follows: a preliminary denaturation at 96°C/3 min followed by 35 cycles at 94°C/45 sec, 50°C/1 min and 72°C/1 min and a final extension at 72°C/1 min with primers PI (forward 5’-TGGGAGCTCTTGGCAAAAGGCCAGAAAGGCC) and PIII (reverse 5’-CACCGGATTTTTACACATCA CCGG) to obtain a PCR product of 176 bp. A nested PCR was also conducted, with exactly the same conditions as detailed above, except that the annealing temperature was 57°C and the primers were PI and PIV (reverse 5’-GGCAGCTTCAAGAAAAATCAGGAAATCTGATT) to amplify a sequence of 133 bp. Products were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide (0.5 µg/ml).

Results and Discussion

Pathology and Histopathology: The lungs were heavy and moderately edematous, containing few multifocal, random, slightly elevated, fairly well-circumscribed and unencapsulated gray to white masses ranging from 0.5 to 3.0 cm in diameter, particularly in the caudal lobes (Figure 1).

There were fibrinous adhesions between the thoracic cage and the lung lobes. There was an excessive amount of frothy fluid accumulation in the trachea in all cases. The masses were pearly white in color, very hard in consistency and well demarcated from the surrounding parenchyma. Histology of the lung sections indicated the well-circumscribed, multifocal, highly cellular and unencapsulated neoplastic masses of epithelial cells were arranged mostly in cystic or papillary acinar-like structures (Figure 2) or, rarely, in solid aggregates.

Individual cells were cuboidal to low columnar epithelium with basally located round to oval nuclei with a central nucleolus and a vacuolated cytoplasm. Mitotic figures were rare and the mitotic index ranged from 1–3. The lumens of the alveoli around the neoplastic acini contained moderate

TABLE 1: Flock number, flock size, age, animal number in flock and death rate of sheep.

<table>
<thead>
<tr>
<th>Flock/Sheep number</th>
<th>Age (Years)</th>
<th>Death/Animal number</th>
<th>Death rates(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/799</td>
<td>3</td>
<td>5/150</td>
<td>3.33</td>
</tr>
<tr>
<td>II/758</td>
<td>4.5</td>
<td>20/500</td>
<td>4.0</td>
</tr>
<tr>
<td>III/699</td>
<td>3</td>
<td>25/500</td>
<td>5.0</td>
</tr>
<tr>
<td>IV/653</td>
<td>1.5</td>
<td>1/210</td>
<td>0.4</td>
</tr>
</tbody>
</table>
numbers of alveolar macrophages that characterized by a foamy vacuolated cytoplasm (Figure 3) and desquamated neoplastic cells, rarely lymphocytes and plasma cells. Multifocal areas of myxomatous degeneration were present within the connective tissue stroma. The bronchi adjacent to the tumor nodules were surrounded by mononuclear cell aggregates, primarily lymphocytes.

Gross and histological lesions of tissues other than lung were not examined.

**PCR and Sequencing:** Five tissues of a total of eight from four sheep were positive according to the first round of PCR using PI and PIII. 176 bp virus genomes were detected in whole liver and heart tissues. Seven tissues of a total of eight were JSRV positive by nested-PCR using PI and PIV. 133 bp virus genomes were detected in all liver tumors, heart and liver tissues. The virus was detected not only in lung tumor tissues of first flock of sheep but also in heart and liver tissues of the same animals (Table 2).

The sequences of two JSRV PCR-positive products revealed 100% homology. These gene sequences were compared to the sequences of OPAV obtained from GenBank database and 90–95% identity with exJSRV genomes was observed. In addition to the nested-PCR results, the presence of exJSRV into the clinical samples was confirmed with sequencing of two PCR-positive products for JSRV (Figure 4).

The Office International des Epizooties includes OPA as a listed disease to be considered in the international

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**TABLE 2:** The PCR results of the samples collected from four different flocks.

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Lung tumor</th>
<th>Lymph node</th>
<th>Heart</th>
<th>Brain</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+/-</td>
<td>*</td>
<td>+/-</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>II</td>
<td>+/-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<tr>
<td>III</td>
<td>+/-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>IV</td>
<td>+/-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*Samples were not collected
trade of sheep and ovine products (De Las Heras et al. 2005). Natural transmission of the disease occurs via aerosol routes and the lung fluids of affected animals spread the virus significantly (Sharp and Herring 1983, Sharp and DeMartini 2003). Additionally, lambs can be infected via the colostrum and milk from the JSRV-infected ewes (Arnaud et al. 2007). OPA has been recorded in the sheep ranging in age from 2 months to 11 years, although most clinical cases occur in animals aged 2–4 years (Hunter and Munro 1983, Salvatori et al. 2004). In the current study, OPA affected animals ranged in age from 1.5–4.5 years.

Diagnosis of OPA is possible when clinical signs or tumors are apparent (De Las Heras et al. 2003). However, the presence of JSRV can be detected in lung fluid or tumors by immunoblotting (De Las Heras et al. 2003), ELISA (Palmarini et al. 1995) or PCR (Palmarini et al. 1996) due to lack of circulating JSRV-specific antibodies during the preclinical period (Sharp and Herring 1983, Ortin et al. 1998). Nearly 20 endogen retroviruses have been diagnosed in the sheep genome. exJSRV as causative agent of OPA can be separated from enJSRV by PCR targeting the U3 gene part (Voigt et al. 2007). In this study, nested PCR was performed to detect the U3 gene part of exJSRV. Beytut et al. (2009) reported histochemically retroviral antigens from sheep suspected of having OPA. Therefore, our study is first detection of exJSRV in Turkey. Five tissues from atotal of eight from four sheep were found to be positive by first-round PCR in the current study. Four of these five tissues were lung tumor tissues. The nested-PCR stage detected two more positive tissues in addition to first-round PCR.

Metastases on regional lymph nodules were reported with OPA infection by previous studies but there were limited report data on metastases to extra-thoracic tissues (De Las Heras et al. 2003). Minguijon et al. (2013) reported the detection of JSRV by PCR on extra-thoracic tissues such as liver, kidney, skeletal muscle, digestive tract, spleen, skin and adrenal glands. In this study, the detection of the virus in regional lymph nodules of sheep belonging to flock IV confirms the occurrence of metastases on intra-thoracic tissues. Therefore, these results indicate that the virus was detected not only in lung tumor tissues of sheep from the first flock in this study but was also found in the heart and liver tissues of the same animals. Furthermore, contamination of JSRV to both intra-thoracic and extra-thoracic tissues via metastases might be possible.

This study showed the occurrence of OPA in four different sheep flocks in two cities of the Eastern Anatolia region of Turkey. This study is the first report of the presence of exJSRV in sheep suspected to carry OPA in Turkey. Furthermore, the results support metastasis to extra-thoracic tissues of exJSRV.

REFERENCES


