

Ovine pulmonary adenocarcinoma in slaughtered sheep: A pathological and polymerase chain reaction study

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Ovine pulmonary adenocarcinoma (OPA) is a contagious tumour in sheep caused by jaagsiekte sheep retrovirus (JSRV). This tumour originates from the pneumocyte type II and Clara cells and grossly appears as hard, prominent nodules in different lobes. The clinical signs of the disease are similar to those of other chronic respiratory diseases and are not pathogenomic. Therefore, *post mortem* examinations and histopathological studies are the most reliable ways to diagnose OPA, particularly subclinical cases of this neoplasm. In this study, out of 1000 sheep lungs grossly inspected, 50 animals were suspected of OPA. The suspected lungs as well as 25 apparently normal lungs were examined by histopathological and PCR methods. The proviral DNA was detected in 1/25 apparently normal lungs and 8/50 of the suspected lungs and subsequently confirmed by histopathological studies. The PCR-positive lung samples from five sheep revealed lesions of 'atypical' OPA and those from three sheep showed the 'classic' form of the disease. The tumours were multifocal and the masses were distributed throughout the cranioventral and diaphragmatic lung lobes. The stroma of the tumours in the atypical cases was more severely affected with inflammatory cell infiltration and connective tissue proliferation. The histopathological characteristics of maedi including hyperplasia of the perivascular and peribronchiolar lymphoid cells, interstitial lymphoplasmacytic infiltration and smooth muscle hyperplasia were also associated with OPA, especially the atypical form of this adenocarcinoma. Atypical OPA was more prevalent than the classic form. Geographic and climatic conditions, duration of exposure to the virus and the immune status of individual animals might be responsible for the differences between the two pathological entities of OPA.

Introduction

Ovine pulmonary adenocarcinoma (OPA), also known as sheep pulmonary adenomatosis (SPA) or jaagsiekte, is caused by an oncogenic retrovirus and leads to death of animals within a few weeks to several months (Greenlee *et al.* 2000; Leroux & Mornex 2008; Palmarini *et al.* 1999). Histopathologically, this transmissible tumour of sheep originates from the alveolar type II pneumocytes and Clara cells (bronchiolar non-ciliated epithelial cells) of the lungs (Palmarini & Fan 2003; Platt *et al.* 2002).

Ovine pulmonary adenocarcinoma is responsible for severe economic losses to the sheep industry in many sheep rearing countries and the subclinical form of the disease affects growth rate, carcass weight, and milk and wool production. The incubation period is long and it may take years after infection with the viral agent for clinical signs to develop. The affected animals show signs of respiratory distress, and there is no treatment for the affected sheep. There is no detectable specific circulating antibody to OPA because the viral sequences are endogenous in the sheep genome. Therefore, serological tests fail to diagnose the disease at ante-mortem examination (Ortín *et al.* 1998; Summers *et al.* 2002) and the diagnosis can only be made after death by necropsy and histopathological evaluation (Kycko, Jasik & Reichert 2008).

The few available reports have described two different morphological patterns for OPA, namely classic and atypical, (De las Heras *et al.* 1992; García-Goti *et al.* 2000). Therefore, the present study was undertaken to determine the frequency of OPA, to describe the pathological features of OPA with reference to the morphological patterns of the disease and to use the PCR assay to detect the provirus in the lungs of the affected sheep.

Materials and methods

Geographic area

Chaharmahal va Bakhtiary Province is located in south-western Iran, in the central part of the Zagros mountains, and Shahrekord is its centre. This province has a total area of 16 533 km² and its

geographical latitude is 31°4' to 42°4'N; its longitude is 49°39' to 51°21'E. Because of its geographic location, the province is exposed to various climatic zones. The origin of rainfall in this province is mostly Mediterranean and Sudanese atmospheric flows, which enter from the west and south and affect the area for eight months of the year (October to May). It receives an average annual rainfall of about 560 mm.

Sample collection

The lungs from 1000 native breed sheep, originating from different farms in this province and slaughtered at Sharekord slaughterhouse were grossly examined for OPA lesions. Following careful gross inspection, those lungs that were enlarged, failed to collapse, had firm greyish-white nodules located on the cranioventral or diaphragmatic lobes and contained foamy fluid in the respiratory tract were collected for further histopathological and PCR investigations. Sterile instruments were used to take the tissue samples for PCR and histopathological examination. The tissue samples were frozen and kept at -70 °C until commencement of the PCR procedure.

In addition, 25 grossly normal lungs were similarly examined histopathologically and for detection of OPA provirus as the control group.

Detection of the Ovine pulmonary adenocarcinoma provirus in the lung sections by long terminal repeat polymerase chain reaction

The primers and PCR procedure used for detection of OPA provirus were designed according to the method described by Palmarini *et al.* (1996). The primers (5'TGATATTTCTGTGAAGCAGTGCC3) and (5 CACCGGA TTTTACACAATCACCGG 3) were used to amplify the 176 bp fragment of the viral long terminal repeat (LTR) region. The amplification was performed under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles, denaturation at 94 °C for 30 s, annealing at 59 °C for 1 min, primary extension at 72 °C for 1 min, and final extension at 72 °C for 3 min. The PCR products were analysed in 2% gel electrophoresis containing ethidium bromide by UV light. Each set of the samples was run with a known positive and negative control sample (sterile deionised water) (Kycko, Jasik & Reichert 2008).

Histopathological investigation

Tissue samples 1×1×0.5 cm³ in diameter were taken from 50 suspected lungs and fixed in 10% neutral buffered formalin for histopathological examination. The tissue blocks were then processed by standard procedures. Sections of 5 µm in thickness were stained with haematoxylin and eosin and examined by light microscope (Olympus, Tokyo, Japan).

Results

Detection of Ovine pulmonary adenocarcinoma provirus in the lung sections

Amplicons of the expected size (176 bp) for OPA provirus were detected in 8/50 (16%) of the suspected

animals (Figure 1). One apparently normal lung was also LTR-PCR-positive.

Pathological findings

Histopathological lesions of OPA were observed in all the PCR-positive samples. The pulmonary lesions were categorised as atypical and classic forms. Five out of the eight affected sheep showed the atypical form of OPA. Grossly, greyish-white, multiple, subpleural nodules of variable sizes from 0.5 cm to 5.0 cm, having a hard consistency, were dispersed on the dorsal surfaces of the diaphragmatic lobes in the atypical form. These nodules had distinct borders and their cut surfaces were dry, and no mucoid fluid was present in the airways. Macroscopically the multifocal nodules in three lungs were observed only in the cranioventral lobes; some of them coalesced to form larger masses. Substantial amounts of mucoid and foamy material filled the airways. These lesions were classified as the classic form of OPA. Purulent bronchopneumonia and abscess formation made the diagnosis of the classic form more complicated.

The histopathological features of all the affected lungs were almost similar and revealed papillary projections of cuboidal to low columnar neoplastic cells in the lumen of the alveoli and bronchioles respectively. The neoplastic foci were supported by a sparse connective tissue stroma. The stroma was infiltrated by more mononuclear cells and connective tissue in the atypical form, and swollen and foamy macrophages were observed in the alveoli and bronchioles in the vicinity of the neoplastic lesions. The classic form showed lesser degrees of macrophage and lymphocyte infiltration, but instead, neutrophils and fibrin casts were dominant constituents in the alveolar and bronchial lumens (Figure 2).

Enlargement of the mediastinal lymph nodes was evident but no metastasis was detected. Pulmonary lesions comparable to those of maedi, such as proliferation of the lymphatic tissues around the small airways and microvessels and infiltration of macrophages and lymphocytes in the alveolar and bronchiolar lumens were observed in four affected lungs with adenomatosis and were more predominant in the atypical form of OPA (Figure 3). Maedi was confirmed by LTR-PCR in a previously published study (Azizi *et al.* 2012).

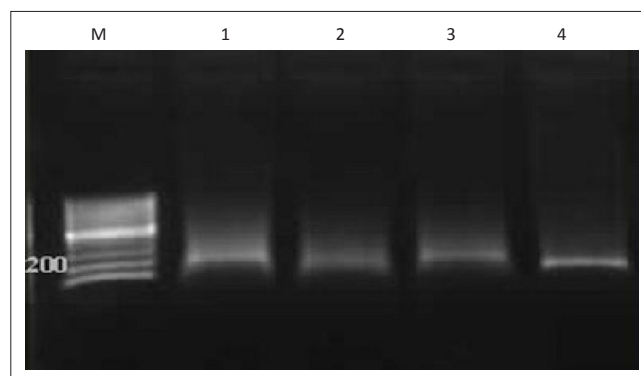


FIGURE 1: Detection of ovine pulmonary adenocarcinoma proviral DNA in the lung tissues by long terminal repeat-DNA. M.: 100 bp molecular weight markers; Lane 1–4: positive amplification (176 bp).

Other suspected lungs ($n = 42$) that were negative according to pathology and PCR showed other forms of multifocal interstitial pneumonias such as verminous pneumonia by *Mullerius capillaris* ($n = 32$) and diffuse interstitial and broncho-interstitial pneumonia ($n = 10$).

Discussion

Ovine pulmonary adenocarcinoma is a transmissible lung cancer of sheep. The clinical signs of OPA comprise cachexia, moist respiratory sounds, dyspnoea and coughing that are not specific for OPA. In classical OPA, the presence of oedematous fluids and copious mucoid secretions in the airways is a pathognomonic symptom. An important differentiating feature from maedi can be observed if the pelvic limbs are raised; thin, mucoid fluid produced by the neoplastic cells in the lungs pours from the nostrils of some of the animals with pulmonary adenomatosis. This clinical aspect of OPA is named the wheel-barrow test (Kycko *et al.* 2008; Sharp & De las Heras 2000). In the absence of excessive lung fluid, a *post-mortem* examination is the best way to make a diagnosis, particularly in the absence of a reliable serological test for detecting OPA in live animals (Ortin *et al.* 1998; Summers *et al.* 2002). In addition, detecting OPA provirus in the lungs of the affected sheep can serve as a supplementary method to histopathological investigation (Voigt *et al.* 2007; Kycko *et al.* 2008).

The present study was designed to make a preliminary estimate of the frequency of OPA based on pathological findings and LTR-PCR detection of the provirus in slaughtered sheep. All the eight PCR-positive lungs showed histopathological lesions of OPA. In addition, one apparently normal lung was also PCR positive for OPA but did not show any histopathological lesions. Therefore, it is likely that the LTR-PCR detected the provirus sequence in one control sample before histopathological lesions developed. This finding also indicates that some of the apparently normal lungs may also be affected by pulmonary adenomatosis. The results of the present study are in accordance with those of González *et al.* (2001) who detected jaagsiekte provirus in the grossly normal lungs and peripheral blood leucocytes of apparently unaffected sheep from OPA-positive flocks. They concluded that the jaagsiekte provirus could be identified in naturally infected sheep before the clinical signs appear or the neoplastic lesions develop. The PCR technique has successfully been applied in many studies for diagnosis of OPA in live animals or abattoir cases. Voigt *et al.* (2007) applied hemi-nested PCR for detection of OPA provirus in the blood and broncho-alveolar lavage (BAL) samples and concluded that PCR testing of BAL is useful for identifying suspected sheep and can be useful in control or eradication programmes. In a study by García-Goti *et al.* (2000), the proviral OPA was detected by hemi-nested PCR in all lung tumour samples.

The pathological features of OPA have not been classified in most instances and the investigators only described pulmonary lesions such as the presence of firm, greyish-

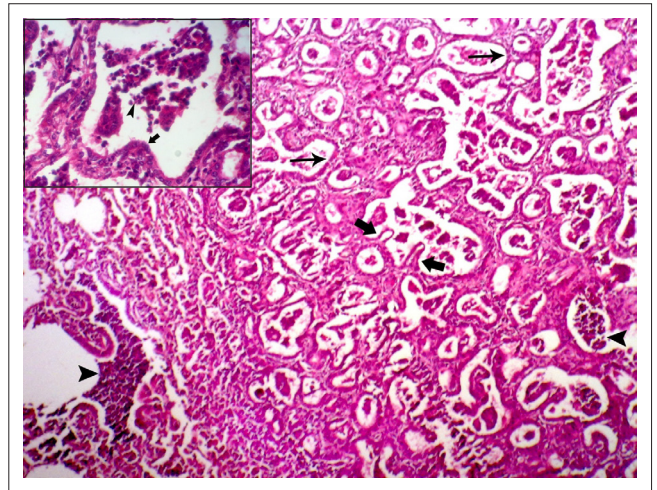


FIGURE 2: Section from a lung with classic ovine pulmonary adenocarcinoma. Note: acinar (thin arrows) to papillary (thick arrows) proliferation of alveolar epithelial cells is superimposed on the purulent bronchopneumonia (arrow heads) (H&E, $\times 100$). Cellular details are shown at a higher magnification in the top left hand corner ($\times 400$).

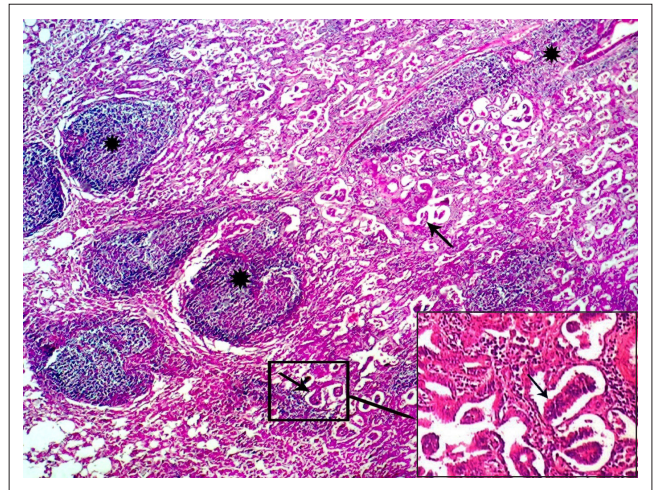


FIGURE 3: Marked perivascular and peribronchiolar lymphoid hyperplasia typical of maedi (asterisks) associated with the lesions of pulmonary adenomatosis (thin arrows) (H&E, $\times 100$). Note: Lesions are shown at higher magnification in the bottom right hand corner of the figure ($\times 400$).

white nodules in different lobes and observation of a high volume of mucoid fluid in the air passages (Hudachek *et al.* 2010; Kycko *et al.* 2008; Woldemeskel & Tibbo 2010). Some reports attributed these lesions to the classic form of the disease that is associated with typical clinical signs. In contrast to classic OPA, a different 'atypical' morphological form has also been reported (De las Heras *et al.* 1992; García-Goti *et al.* 2000; González *et al.* 2001). It seems that atypical OPA is the subclinical form of this disease and is usually recognised in the slaughterhouse.

The atypical form was grossly recognised by the solitary or multifocal nodules that were frequently located in the diaphragmatic lobes. These nodules were hard, white in colour and dry on cut surface. In the present study, the pathological lesions of five PCR positive lung samples were in conformity with the atypical form of OPA and those of three cases agreed with the classic form of the disease. Histopathologically, the basement membrane of the alveoli

and bronchioles was covered by the cuboidal to columnar epithelial cells and demonstrated acinar or papillary growth patterns in both the classic and atypical forms. However, the stroma of the atypical form was infiltrated by more mononuclear inflammatory cells and connective tissue as previously described (De las Heras *et al.* 1992; García-Goti *et al.* 2000). The pulmonary alveoli and bronchioles in classic OPA were filled with neutrophils and fibrin exudates. The presence of neutrophils in the classic form of OPA may indicate secondary bacterial infection of the affected lungs (Beytut, Sözmen & Erginsoy 2008; Kiran *et al.* 2000).

In this study, the lung lesions of maedi were identified in four lungs affected with OPA. The microscopic features of maedi were mostly associated with the atypical form of OPA. The provirus of maedi has been detected by LTR-PCR in a previous study (Azizi *et al.* 2012). A non-oncogenic retrovirus of the lentivirus subfamily is the aetiological agent of maedi. Differentiating maedi from pulmonary adenomatosis is usually difficult because both diseases mostly coexist in the same flock or even in the same animal (McGavin & Zachary 2007). Previous studies have reported concurrent occurrence of maedi and OPA mainly using serology or pathology (Dawson *et al.* 1990; Pritchard & Done 1990; Weikel 1991; Woldemeskel & Tibbo 2010). González *et al.* (1993) studied the coexistence of maedi and OPA in 161 flocks serologically. They stated that OPA is effective in the spread of maedi virus infection. Rosadio *et al.* (1988) studied the natural occurrence of maedi and OPA in sheep suffering from dyspnoea and showed that four sheep affected with OPA were serologically positive to ovine lentivirus and revealed pulmonary lesions of maedi. They detected type D retrovirus in the lung fluid and in the tumour mass by radioimmunoassay and immunoblotting. Their pathological study revealed conspicuous peribronchiolar and interstitial lymphoid hyperplasia together with fibromuscular proliferation associated with acinopapillary growth. It has been reported that a synergistic effect increases the spread of both diseases in herds infected with both viruses (Pritchard & Done 1990). Other pathological lesions such as different types of interstitial pneumonia induced by viral agents and verminous pneumonia may be confused with OPA.

The present study showed that the LTR-PCR had adequate sensitivity and specificity to detect OPA provirus in the histopathologically positive lungs, and also in an early stage of infection when the histopathological lesions had not yet developed. In addition, atypical and classic OPA showed differences in the distribution pattern of the neoplastic lesions at gross inspection, and demonstrated differences in mononuclear cell infiltration and fibroplasia in the stroma microscopically. In some studies such as that by Hunter and Munro (1983) it has been mentioned that the classical form was the common type in Scotland. In some other countries such as Spain, both forms have commonly been diagnosed (González 1990). In contrast, in this study, the atypical form of OPA appeared to be more common than classic OPA. It is not easy to know in natural infection

whether these two pathological types are simply due to two different developmental stages of the same disease or whether they remain different throughout the course of the disease. In addition, in the present study, because the authors were not aware of the background of these animals, it is also not clear whether the immune status of the infected animals contributed to the distribution pattern and type of inflammatory cell infiltration and connective tissue proliferation. Based upon the very few studies available in the literature, it is not possible to state that the geographic area and climatic differences may influence the distribution pattern and type of reaction to OPA. By designing further experimental studies and knowing the exact time of infection and testing the animals under various climatic conditions, it may be possible to answer some of the above questions.

Conclusion

Based on the results of the present study, it can be concluded that OPA is prevalent in native breeds of sheep in the western areas of Iran. Although macroscopic and histopathologic methods are diagnostic for OPA, molecular studies are more reliable tests in confirming even scanty amounts of the viral particles in the lung parenchyma and pulmonary discharges.

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Competing interests

The authors declare that they have no conflict of interest and financial disclosure to anybody or any organisations.

Authors' contributions

S.A. (Shahid Bahonar University of Kerman) was the project leader, designer and pathologist and wrote the article. E.T. (Islamic Azad University) carried out the molecular techniques. F.F. (Islamic Azad University) was responsible for collecting the samples and doing some of the experiments.

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