DETECTION OF JAAGSIEKTE SHEEP RETROVIRUS IN RESPIRATORY TRACT FLUID AND LUNG TISSUE OF EXPERIMENTALLY INFECTED LAMBS

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Abstract

The possibility of jaagsiekte sheep retrovirus (JSRV) genome detection in peripheral blood leukocytes and respiratory tract fluid of ovine pulmonary adenocarcinoma (OPA) affected sheep was tested. Five of six lambs used in the experiment were infected with JSRV by intratracheal inoculation. The blood samples were taken from all the lambs at 10-d intervals for 3 months and the white blood cells were subjected to DNA isolation followed by PCR amplification for the detection of proviral DNA that gave negative results for all the samples. The respiratory tract fluid was collected from the nostrils of lamb No. 6, which developed clinical sign of OPA 2 months after the inoculation. The fluid was examined using PCR and reverse transcriptase PCR (RT-PCR) for the presence of proviral DNA or viral RNA. The presence of OPA in the lamb was subsequently confirmed by histopathological examination and the detection of proviral DNA in the lung tissue. Results of the standard PCR amplification performed on the DNA isolated from the nasal discharge from lamb No. 6 was negative, while the RT-PCR gave positive results confirming the presence of virions in the lung fluid. Results of the study show the RT-PCR technique may be a useful tool in ante-mortem diagnosis of OPA, especially in distinguishing it from other respiratory diseases.

Key words: sheep, ovine pulmonary adenomatosis, jaagsiekte sheep retrovirus, PCR, RT-PCR.

Ovine pulmonary adenocarcinoma (OPA), also known as ovine pulmonary adenomatosis or jaagsiekte, is a contagious tumour of sheep originating from the pneumocytes type II and Clara cells in the distal part of the lung after infection by jaagsiekte sheep retrovirus (JSRV). The virus belongs to the Retroviridae family, the subfamily Orthoretrovirinae, genus Betaretrovirus. Viral genome is composed of linear, positive, single-stranded RNA. The genome comprises four main genes, organised as 5' - gag-pro-pol-env -3', which encode the virus proteins. There is also additional open reading frame, named ORF-x that overlaps the 3' end of the pol gene. The integrated JRSV genome is flanked by the long terminal repeat (LTR) composed of the 3 non-coding regions U3-R-U5 that are essential for the virus replication. The LTR is the site of transcriptional initiation. The gene env encodes viral envelope protein, which is a major determinant of the transformation of differentiated lung epithelial cells: type II pneumocytes in the alveoli and Clara cells in the bronchioles (9, 11).

The disease is present worldwide, with the exception of Australia and New Zealand and it has been eradicated from Iceland. In Poland, the suspicion of its occurrence was reported in 2002 (1). OPA has a long incubation period. It takes at least 3 weeks in experimentally infected lambs, but usually lasts over 2 years in adult sheep to the occurrence of clinical signs of respiratory tract distress. Rapid breathing reflects the extent of tumour development in the lungs. Raising the back and lowering the head of the sheep may cause frothy mucoid fluid accumulated within the respiratory tract to leak out from the nostrils (13).

To date, identification of the disease has been possible only when clinical signs became apparent. There is no serological test for OPA due to the presence of endogenous viral sequences in sheep genome that causes lack of circulating JSRV-specific antibodies, as has been demonstrated by Western blotting (8, 14). The dissemination of JSRV was demonstrated, using reverse transcriptase PCR (RT–PCR), in tissues of the lymphoreticular system (LRS) and in blood mononuclear cells of terminally ill sheep affected with both naturally and experimentally induced adenocarcinoma (10). Results of PCR used for the detection of JSRV proviral DNA in blood before any clinical symptoms become evident were different and the method is still not enough sensitive to become a diagnostic method (4, 6). Diagnosis relies, at present, on clinical history and clinical signs that have to be confirmed by necropsy and by histopathology. The aim of our study was the experimental induction of OPA in sheep in order to study the possibility of early diagnosis of the disease in live animals.
Material and Methods

Sample collection. Five sheep, three-week-old, were experimentally infected with JSRV clone (kindly provided by Prof. M. Palmarini, University of Glasgow, Scotland) by intratracheal inoculation. Three sheep (one lamb and two eves) served as the control. Blood samples from the experimental animals were taken from the jugular vein to the tubes containing EDTA-K2 as an anticoagulant, at 10-d intervals. Fluid flowing from the nostrils of the lamb was collected for DNA and RNA isolation. The tissue samples were collected from four parts of the lungs during necropsy for histological examination and DNA isolation. Frozen lung tissue sample of non-affected sheep kindly provided by Prof. M. De Las Heras (University of Zaragoza, Spain) and lung tissue sample of non-affected sheep were used for DNA isolation and served as a positive and negative control for PCR.

Histological examination. Tissue samples were fixed in 10% neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax, and sectioned into 4-6-µm-thick slices. The sections were stained with haematoxylin and eosin and examined under light microscope for tumour lesions.

Peripheral blood leukocyte (PBL) isolation. Ten millilitres of each blood sample was centrifuged for 25 min at 3000 rpm, and then the buffy coat was collected and transferred to new tubes containing PBS. The tubes were centrifuged for 10 min at 1600 rpm. The pellet of PBLs (peripheral blood leukocytes) was subjected to DNA isolation procedure.

DNA isolation and PCR. DNA was isolated from 30 mg of collected tissue, respiratory tract fluid (taken pre-mortem from lamb No. 6) and from PBLs, using Genomic Mini Kit (A&A Biotechnology), according to manufacturer’s recommendations. The concentration levels were measured using spectrophotometer (Biometra). 0.5 µg of DNA was subjected to the mixture containing 2.5 µl of PCR buffer (100 µM Tris-HCl, pH 8.8, 500 µM KCl), 1.5 mM MgCl₂, 50 µM dNTP, 0.5 U of Taq polymerase, and 0.5 µM of each primer (JSRV-1 and JSRV-3 primer. The primers JSRV-1: 5’ TGGAGGCTTTTGGCATAAAGGC 3’ and JSRV-3: 5’ CACCCTTATTTACAATTCACCGG 3’, published by Palmarini et al. (10), were specific for viral LTR region. They were synthesised by IBB PAN (Warsaw).

The final reaction volume was 25 µl. The amplification has been performed by using Biometra thermocycler UNO II in the following conditions: 35 cycles, initial denaturation at 94°C – 2 min, denaturation at 94°C – 30 s, annealing at 59°C – 1 min, elongation at 72°C – 1 min, and final elongation 72°C – 3 min. PCR products were detected using UV light after gel electrophoresis (2% ethidium bromide stained agarose gel, 120 V). Aliquots of 10 µl of PCR products and 6 µl of DNA marker (Gene Ruler 100 bp DNA ladder Plus, Fermentas) were applied to the gel.

RNA isolation and RT-PCR: RNA was isolated from 30 mg of collected tissue and from respiratory tract fluid taken from nostrils of sheep No. 6, using Total RNA Kit (A&A Biotechnology), according to manufacturer’s recommendations. The amplification was done using Biometra thermocycler UNO II, in a final volume of 25 µl of reaction mixture containing: 2 µl of RNA, 0.125 µl of RNA-se inhibitor (40 U), 0.125 µl of AMV transcriptase (20 U), 2.5 µl of PCR buffer, 2.5 µl of MgCl₂ (25 mM), 2.5 µl of dNTP (2 mM), 0.25 µl of Taq polymerase (5 U), and 1.25 of each primer (20 pmol). Thermal cycling conditions were: 35 cycles, reverse transcription at 50°C – 30 min, initial denaturation at 94°C – 2 min, denaturation at 94°C – 30 sec, annealing at 59°C – 1 min, elongation at 72°C – 1 min, and final elongation 72°C – 3 min. RT-PCR products were analysed in UV light after electrophoresis in 2% ethidium bromide stained agarose gel. Two micro-litres of RT-PCR product were then subjected to standard PCR for the comparison with positive control.

Results

Two lambs (No. 2 and 6) showed clinical signs of progressive respiratory illness manifested by a rapid breathing associated with a noticeable movement of the abdominal wall. Lowering the head caused copious amounts of mucoid fluid to run from the nostrils in the advanced state of the disease. Lamb No. 2 showed clinical signs (cough) 6 weeks after inoculation and died the following week. Lamb No. 6 revealed signs of progressive respiratory illness two months after inoculation (cough, rapid, exaggerated breathing associated with a noticeable movement of the abdominal wall, loss of body condition, and discharge of mucoid fluid from the nostrils). The lamb died 3 weeks after the clinical signs occurred. The necropsy revealed the presence of frothy white fluid in the respiratory passages, flowing out from the trachea when it was incised. Tumour lesions were dispersed and found in many locations of the lungs. They appeared as small (1-5 mm), grey and light purple nodules. Histological examination revealed that the nodules were composed of single to multiple layers of cuboidal or elongated cells lining alveolar walls and frequently forming papillary projections (Fig.1). They were supported by a fine fibrovascular stroma. Multifocally haemorrhages and single, inflammatory, mostly perivascular nodules were also observed (Fig. 2).

DNA from Spanish cases of OPA and DNA isolated from healthy lung tissue were submitted to standard PCR at first to confirm the presence of ISRV proviral DNA in the OPA samples. Electrophoretic analysis revealed the presence of a band of expected size in the lane of OPA PCR product. The product was sequenced and was confirmed to be JSRV LTR sequence with expected length of 176 bp. The DNA served further as a positive control for the analysis of experimental samples.

The RT-PCR amplification products from the respiratory tract fluid of sheep No. 6 as well as from the lung tissue samples of sheep No. 2 and 6 analysed by
electrophoresis, revealed the presence of a band of an expected length of 176 bp (Fig. 2). The PCR amplification of cDNA obtained in RT-PCR also gave positive results confirming the presence of viral RNA in both samples (Fig. 3).

We could not confirm the presence of JSRV specific DNA material in PBLs isolated from blood samples of infected lambs (Fig. 2).

**Fig. 1.** The presence of variably sized nodules obscuring and replacing normal pulmonary architecture. Neoplastic nodules are frequently surrounded by slightly congested by otherwise normal lung parenchyma. HE, 10x.

**Fig. 2.** Uncapsulated nodules are composed of single to multiple layers of cuboidal to columnar cells that line alveoli and form papillary projections. They are supported by a fine fibrovascular stroma. Multifocally haemorrhages are observed. HE, 20x.
Fig. 3. Agar gel electrophoresis of RT-PCR products (A) and PBLs PCR products (B). (A): F and L are the product of amplification of cDNA from respiratory tract fluid (F) and lung (L) of lamb No. 6. (B): 1-6 are the PBLs samples of all 6 lambs. (+) - positive control, (-) - negative control.

Fig. 4. Agar gel electrophoresis of PCR products: 1 - DNA from healthy lung, 2 - DNA from respiratory tract fluid, 3 - cDNA from OPA affected lung, 4 – cDNA from respiratory tract fluid, (+) – positive control, (-) – negative control.

Discussion

In this paper, we presented the possibility of pre-mortem diagnosis of OPA in sheep. The study was focused on the examination by PCR and RT-PCR of different tissues and body fluids (lung tissue, blood, and nasal exudate) of experimental lambs, aiming at the detection of JSRV specific DNA sequences. Only two from five JSRV inoculated lambs developed clinical signs, while no other lamb even started to show any signs of the disease during that period. The samples were examined for the presence of proviral DNA then the lung fluid (collected as soon as clinical signs occurred) was tested for the presence of viral RNA. For the study, we used PCR technique to amplify LTR region, which is found to be unique for JSRV virus (2, 10).

Positive results of RT-PCR amplification may reflect the fact, that the respiratory tract fluid is rich in virions produced by affected lung cells. Standard PCR method did not give positive results that are probably due to low number of transformed proviral DNA-carrying cells present in the nasal exudate. According to Voight et al. (15), it is possible to find the proviral DNA in cell clusters present in lung fluid, but the collection of bronchioalveolar lavage requires medical immobilisation of the animal and intubation.

The examination for the presence of proviral DNA in peripheral blood leukocytes gave negative results even when clinical signs of the disease were developed. This is apparently due to a low frequency of infected cells in the peripheral blood leukocytes (estimated 1/2 400 000 cells) as is reported by Holland et al. (6). There are reports describing positive results of PCR method used for the examination of white blood cells for the
viral DNA presence (5, 10, 12); however, the studies were usually performed on larger groups of experimental animals, and included hemi-nested PCR (hnPCR) technique. The hnPCR was used to amplify JSRV-U3 region and was found to be more sensitive than single round PCR, but still not giving 100% of positive results during the examination of leukocytes from infected animals, especially when there were no clinical signs of OPA. However, according to De las Heras et al. (3), improved white blood cells isolation makes the one step PCR technique as effective as hnPCR. It also is less time-consuming than hnPCR and makes the one step PCR technique as effective as pre-mortem examina-
tion is so far, the only approach in diagnosing ovine pulmonary adenocarcinoma. There is still no approved laboratory method for pre-mortem diagnosis of the disease. Successful inoculation of the lambs with JSRV led us to find that the RT-PCR method may be a useful tool for positive diagnosis as soon as the first clinical signs of respiratory illness occur and in distinguishing it from other respiratory diseases. Finding the presence of JSRV in affected sheep lung tissue may therefore serve as a supplementary method to histological examination.

References