Comparison effect of fresh frozen and paraffin embedded samples in diagnosis of ovine pulmonary adenomatosis by PCR and immunohistochemistry

R. KHEIRANDISH1*, M. SAMI2,3, M. KHALILI1, E. MOHEBBI4, S. AZIZI1, N. ASKARI1

1Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Iran
2Food Security Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.
3Department of Food science and Technology, School of Nutrition and Food Science, Isfahan University of Medical Sciences, Isfahan, Iran.
4Researcher, Research center for modeling in health, Institute for futures studies in health, Kerman University of Medical Sciences, Kerman, Iran.

*Corresponding AUTHOR: kheirandish@uk.ac.ir

SUMMARY

Ovine pulmonary adenomatosis (OPA) is a contagious bronchio-alveolar carcinoma that is caused by Jaagsiekte sheep retrovirus. The virus induces transformation in Clara cells and pneumocyte type II. In the present study, 42 grossly suspected lungs of slaughtered sheep to OPA were collected. Lungs were enlarged, failed to collapse with distribution of firm greyish-white nodules on the cranioventral or diaphragmatic lobes and some of them contained foamy fluid in their airways. The lung samples were taken for histopathologic, PCR and immunohistochemistry investigations. Histopathologically, 16 lungs (n=16/42) showed neoplastic foci that were composed of proliferated pneumocytes type II and Clara cells arranged in the acinar or papillary growth pattern. Immunohistochemical analysis detected Jaagsiekte sheep retrovirus (JSRV) capsid protein (JSRV-CA) in the cytoplasm of pneumocytes type II and Clara cells. PCR technique was done on 16 paraffin embedded and fresh frozen tissues. Based on PCR technique targeting U3 region, amplicons of expected size (176 bp) were observed in all 15 paraffin embedded and 16 fresh frozen sections. No positive results were detected in the apparently normal lungs. It seems PCR can be used on paraffin-embedded tissues to reveal Jaagsiekte sheep retrovirus infection, but complementary studies on a large number of samples are required to determine sensitivity.

Keywords: Ovine pulmonary adenocarcinoma, sheep, immunohistochemistry, PCR, fixative

Introduction

Ovine pulmonary adenocarcinoma (OPA) is a bronchoalveolar carcinoma that causes by Jaagsiekte sheep retrovirus (JSRV). This disease occurs in sheep and rarely in goats [1]. In many sheep rearing countries, OPA is responsible for severe economic losses such as decreasing of growth rate, carcass weight, milk and wool production. Diagnosis of OPA is based on clinical signs and postmortem examination, but there is not effective laboratory method to diagnose the infection in clinically healthy animals [20]. Serological tests have not competency for diagnosis the OPA due to lack of circulating JSRV-specific antibodies. The virus replicates in Clara cells and pneumocytes type II and expresses endogenous retroviruses in the genome of sheep [15, 16]. Postmortem examinations are not sufficient for definitive diagnosis. Therefore, detection of provirus by molecular techniques is considerable. Polymerase chain reaction (PCR) on different samples including blood, bronchoalveolar fluid and lung tissues have been known as a reliable technique to detect JSRV [8]. PCR has this ability that detects the provirus in paraffin-embedded and fresh tissues. The fixation processes may affect the amplification of nucleic acids in paraffin-embedded tissue [3, 17] but some studies have described that there is not any differences between results of PCR technique on fresh frozen and paraffin-embedded tissues for detection of JSRV.

The present study was conducted on diagnosis of OPA in suspected sheep lungs by three methods including histopathology, PCR and immunohistochemistry. Both fresh frozen and paraffin-embedded tissues were used for PCR.
Materials and methods

SAMPLE COLLECTION

The forty-two suspected lungs of slaughtered sheep at Kerman slaughterhouse were grossly inspected for OPA lesions. The lungs were enlarged, swollen and not collapsed, and had grayish-white, firm nodules distributed on the different lobes. The airways of some sheep were contained foamy fluids. Sampling was performed from 42 suspected lungs for histopathological, immunohistochemistry and PCR method investigations. Samples for PCR were considered as both fresh frozen and paraffin embedded. Frozen tissues were stored at -20°C until starting examination time. The lung samples were kept in formalin for 10 days. PCR was performed after 30 days of providing paraffin blocks. Also, 10 apparently normal lungs were examined and used as controls. Positive sample tissues from our previous study were used for PCR and immunohistochemistry methods (Azizi et al. 2014).

PATHOLOGIC EXAMINATION

Lung tissues (1×1×0.5 cm³) were fixed in 10% neutral buffered formalin and embedded in paraffin for histopathological examination. Provided blocks were processed by routine standard procedures and 5 μm thickness samples were stained with hematoxylin and eosin (HE) and observed by a light microscope. Three slides with 50 μm space were provided from each sample and studied completely.

PCR PROCEDURES FOR DETECTION OF OPA PROVIRUS

PCR technique was done on 30-day-old paraffin embedded and fresh frozen tissues. Two 20 μm (25 mg) thickness were cut from each paraffin block and placed in a 1.5 cc tube. Paraffin was dissolved by adding 1 cc of xylene and place on a shaker for 15 minutes. Subsequently, the tubes were centrifuged at 12,000×g for 5 min. For removing the xylene, supernatants were disposed without disturbing of the pellets. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Quality of extracted DNA was investigated by agarose gel electrophoresis and ethidium bromide staining. The used primers and PCR procedure were designed according to the method described by Palmarini et al. (1996) [16]. The primers (5’TGATATTTCTGTGAAGCAGTGCC3) and (5CACCGGA TTTTTACACAATCACCGG 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 analyzed 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 deionized water) [12].

IMMUNOHISTOCHEMISTRY (IHC)

Sections from the suspected lungs and apparently normal lungs were labeled immunohistochemically by the avidin-biotin-peroxidase complex (ABC) technique for detection of JSRV capsid protein (JSRV-CA) (J.M. Sharp, Moredun Research Institute, Edinburgh, UK). The tissue sections were dewaxed in xylene, and hydrated through sequential immersion in the mixture of xylene and graded concentrations of ethanol. Endogenous tissue peroxidase activity was blocked by immersing in 0.5% H₂O₂-methanol for 30 min at room temperature. Antigen retrieval was performed in 100 ml of 0.1 M phosphate-buffered saline (PBS, pH= 7.4) containing 50 mg of protease XIV in 100 ml for 15 min at 37°C. Next, the

<table>
<thead>
<tr>
<th>Sheep Number</th>
<th>Mononuclear cell infiltration</th>
<th>BALT Hyperplasia</th>
<th>Alveolar macrophage</th>
<th>Stromal fibrosis</th>
<th>Myxoid</th>
<th>Other Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2+</td>
<td>-</td>
<td>2+</td>
<td>1+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>2</td>
<td>1+</td>
<td>1+</td>
<td>3+</td>
<td>1+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>3</td>
<td>2+</td>
<td>-</td>
<td>1+</td>
<td>2+</td>
<td>+</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>4</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>5</td>
<td>1+</td>
<td>-</td>
<td>3+</td>
<td>-</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>6</td>
<td>1+</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>7</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>8</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>3+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>9</td>
<td>2+</td>
<td>3+</td>
<td>2+</td>
<td>2+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>10</td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>11</td>
<td>3+</td>
<td>3+</td>
<td>1+</td>
<td>3+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>12</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>13</td>
<td>1+</td>
<td>-</td>
<td>3+</td>
<td>-</td>
<td>-</td>
<td>Muelleriosis</td>
</tr>
<tr>
<td>14</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>1+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>15</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
<tr>
<td>16</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>-</td>
<td>-</td>
<td>Secondary infection</td>
</tr>
</tbody>
</table>

*: (−) absence of the lesion, (+) mild, (2+) moderate, (3+) severe

Table I: Histopathologic scoring of lung tumors in sheep affected by ovine pulmonary adenocarcinoma.
slides were incubated for 30 min at room temperature with 4% normal goat serum. After washing three times in PBS for 5 min the slides were incubated, first with rabbit antiserum to recombinant JSRV-CA at a dilution of 1 in 100 in PBS for 60 min at room temperature. 1:50. Proper positive and negative control samples were stained. After washing three times in PBS for 5 min at room temperature, and then incubated with biotinylated goat anti-rabbit IgG. Following three PBS washes of 5 min each, the sections were incubated with avidin-biotin complex peroxidase (ABC-P, Dako, Denmark) solution according to the manufacturer’s instructions for 30 min at room temperature and washing three times in PBS for 5 min. The chromagen was applied by a final incubation with 3, 3'- diaminobenzidine-4HCl (DAB) 0.05% in PBS with 10 µl of hydrogen peroxide 50% according to the manufacturer’s instructions for 3 to 5 min at room temperature. These slides were counterstained with Mayer’s hematoxylin.

STATISTICAL ANALYSIS

To determine the reliability and the agreement among the three methods including PCR, IHC and histopathological examination, Kappa index (K) was measured. In this statistical test, complete agreement between data corresponds to K = 1, and lack of agreement corresponds to K = 0.

Results

HISTOPATHOLOGIC FINDINGS

Out of 42 grossly suspected lungs, 16 samples (n=16/42) showed microscopic characteristics of OPA (Table I). The affected lungs revealed multiple, variably size foci composed of neoplastic epithelial cells. The tumor cells including type II pneumocytes and Clara cells were cuboidal and columnar, respectively and arranged in the acinar or papillary growth pattern. Mitotic index was very low and pleomorphism was not observed (Fig. 1, 2).

IMMUNOHISTOCHEMISTRY ANALYSIS

Immunohistochemical technique detected JSRV capsid (JSRV-CA) protein in the cytoplasm of pneumocytes type II and Clara cells of 16/42 of fresh frozen and paraffin embedded sections (Table II). Positive staining was also observed in the alveolar macrophages, lymphocytes and plasma cells of the neoplastic regions and surrounding tissues (Fig. 3, 4, 5).
DETECTION OF OPA PROVIRUS IN THE LUNG TISSUES BY PCR

Based on PCR technique targeting U3 region of JSRV, amplicons of expected size (176 bp) were observed in 15 paraffin blocks and 16 fresh frozen sections. No positive results were detected in the apparently normal lungs (Fig. 6).

Only one paraffin embedded section was negative in the PCR (16/42 of fresh frozen and 15/42 of paraffin embedded section; K=0.9). The comparison of histopathological test and PCR indicated that there was acceptable agreement (K=0.81).

Discussion

Ovine pulmonary adenocarcinoma (OPA) or Jaagsiekte, is a contagious tumor of sheep that is caused by a Betaretrovirus belongs to the Retroviridae family. The disease has widespread distribution with the exception of Australia and New Zealand. The incubation period is long and occurs most in adult sheep aged 2–4 years. OPA transmits between animals by direct contact mainly through inhalation the virus [15, 16]. In the early stages, animals are clinically healthy or with mild respiratory signs that may be confused with other respiratory diseases. In advanced stage, moist respiratory sounds are caused to diagnosis of OPA. In the absence of excessive lung fluid, post-mortem examination associated with other complementary methods is useful [12, 15].

In the present study, ovine pulmonary carcinoma (OPA) was investigated by histopathology, immunohistochemistry and PCR technique. PCR was performed on paraffin embedded and fresh frozen tissue samples. Out of 42 grossly suspected lungs, 16 samples (n=16/42) showed microscopic characteristics of OPA including proliferation of pneumocytes type II and Clara cells as acinar or papillary growth pattern similar to the previous reports [5, 14]. Some researchers classified pathologic features of OPA as classic and atypical forms [2, 9]. Classic form in contrast to the atypical one has a wet appearance and is associated with a large amount of foamy fluid in the airways.

We detected JSRV capsid (JSRV-CA) protein in the cytoplasm of pneumocytes type II and Clara cells of 16/42 of fresh frozen and also paraffin embedded sections by immunohistochemical technique. Positive staining was also observed in the alveolar macrophages, lymphocytes and plasma cells. PCR technique targeting U3 region of JSRV, amplicons of expected size (176 bp) were determined in 15 paraffinized tissues and 16 fresh frozen sections. Similar immunohistochemical results were reported in previous researches. Oda and Youssif (2011) indicated positive immunoperoxidase staining for JSRV-CA in the all tumor epithelial cells, normal pneumocyte type II cells and few bronchiolar epithelial cells, alveolar macrophages, lymphocytes, and plasma cells [14]. Beytut et al. (2009) demonstrated immunolabelling staining in the apical cytoplasm of the neoplastic cells, papillary proliferations within the bronchioles and desquamated tumor cells in the acinar proliferations. Normal epithelial cells or tumor stromal cells did not show any positive reactivity. Few numbers of type II pneumocytes and alveolar macrophages in tissue near to the neoplastic foci were positive [5].

In this study, PCR was aimed on 42 samples of formalin-fixed paraffin embedded and fresh frozen tissues for detection of JSRV. Amplicons of expected size (176 bp) were observed in 15 paraffin blocks and 16 fresh frozen sections. PCR is known as a highly specific and sensitive DNA amplification method for diagnosis of diseases [2, 17, 21]. The literature review shows that the efficiency of PCR on paraffin embedded tissues influences by variable factors including type and duration of fixation, age of the paraffin block, DNA extraction procedure, length of DNA fragment for amplifications, concentration of target DNA amplified, PCR protocol and optimization of PCR [6, 10, 19]. The sensitivity of PCR decreases with long length of the amplified fragment rather than short ones [11, 13, 18]. The best results have been obtained with amplicons between approximately 80-120 bp [4]. Fixation time is a significant factor that influences the sensitivity of PCR. Inoue et al. (1996) suggested that the tissue specimens should be prepared just after 1 day fixation.
Dedhia et al. (2007) indicated that DNA obtained from paraffin embedded section is highly fragmentable. They amplify DNA fragments up to 250 bp, and higher length failed to amplify [7]. Another factor that should be considered is the storage time of paraffin-embedded tissues. Greer (1991) succeeded to amplify short fragments (270 bp) of the human beta-globin gene after storage of paraffin blocks for about 15 years, while fragments greater than 500 bp after 1-2 years failed to amplify [10]. In the present study the 176 bp product of JRSV was amplified successfully from 30-day-old blocks (15 out of 16 blocks) with 10 days fixation time. So, PCR negative results of a paraffinized sample may be related to destructive effects of formalin on the macromolecules such as DNA.

In conclusion, our results showed a high agreement between three used methods including histopathology, immunohistochemistry and PCR technique for diagnosis of OPA. It seems PCR as a specific and sensitive method can be used for the diagnosis of OPA in paraffin embedded sections. Nevertheless, further studies should be considered with different fixative, fixation time and age of paraffin block for achievement to better results with PCR technique on fixed tissues.

Acknowledgements

The authors would like to thank the Vice Chancellor of Research at the Shahid Bahonar University of Kerman for their financial support and cooperation.

References

7. - DEDHIA P., TARALES, DHONGDEG., KHADAPKAR R., DAS B.: Evaluation of DNA extraction methods and


