PCR-BASED METHODS FOR DETECTION OF JSRV IN EXPERIMENTALLY AND NATURALLY INFECTED SHEEP

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Abstract

Application of real-time PCR using Taqman probe was tested for jaagsiekte sheep retrovirus (JSRV) detection. Sensitivity of real-time PCR and hemi-nested PCR methods was compared using plasmid DNA. The methods, along with RT PCR and real-time RT PCR, were tested for the possibility of JSRV genome (LTR region) detection in biological material from experimentally and naturally infected sheep. The experimental group of eight animals was used, including five lambs infected with JSRV by intratracheal inoculation at the age of 2 weeks. The samples collected from the animals ante-mortem included blood and respiratory tract fluid. Lung tissue, mediastinal lymph nodes, spleen, and liver were collected post-mortem. The field studies included blood samples collected from sheep from Polish flocks and lung samples obtained from slaughterhouse. In addition, DNA samples isolated from blood of sheep from the abroad located farms with history of ovine pulmonary adenomatosis (OPA) were also included. Lung samples were examined histologically for the presence of pulmonary adenocarcinoma. The sensitivity of PCR, hemi-nested PCR, and real-time PCR using Taqman probe was evaluated as 10^3, 10^2, and 10^2 viral copies, respectively. Both viral RNA and DNA were detected in the lung fluid taken from JSRV infected sheep showing clinical signs of OPA and in all neoplastic tissues. Proviral DNA was found in mediastinal lymph node of one experimental sheep. Five of the 66 DNA samples from the abroad located farms were positive for the presence of JSRV LTR. All blood and lung samples collected from Polish sheep were negative for the presence of JSRV LTR. The characteristic adenocarcinoma lesions were found in all lung sections of experimentally infected sheep. Implementation of the real-time PCR method is a good alternative to traditional PCR and hmpCR in JSRV detection and, apart from histopathological and immunohistochemical examinations, may be used as a confirmatory test in clinically suspected cases, or as a screening method in control or eradication scheme.

Key words: sheep, jaagsiekte, jaagsiekte sheep retrovirus, hemi-nested PCR, real time PCR.

Jaagsiekte sheep retrovirus (JSRV) is an aetiological agent of ovine pulmonary adenocarcinoma (ovine pulmonary adenomatosis, jaagsiekte, OPA) marked by neoplastic growth of epithelial secretory cells of the lung (Clara cells and type II pneumocytes). The disease occurs in sheep and rarely in goats. There are no efficient vaccination or treatment methods available. Diagnosis of jaagsiekte is based on clinical signs and post mortem examination, but there is still lack of effective laboratory method to diagnose the infection in living, clinically healthy animals (13, 21). The methods such as serological tests or virus isolation are not available for diagnosis due to the unique nature of JSRV infection, because the virus is able to replicate efficiently only in pulmonary secretory cells and expression of homologous endogenous retroviruses present in ovine genome causes lack of circulating JSRV-specific antibodies (14-16). The PCR technique using primers for fragments of long terminal repeats (LTR) allows specific JSRV detection. The LTR region is a component of JSRV genome that is essential for replication of the virus and is activated only in secretory cells of the lung. It is one of the regions most different in nucleotide sequence when compared to ovine endogenous retroviruses or closely related enzootic nasal tumour virus (ENTV) (4). JSRV DNA and RNA can be detected in tumour tissue and secretions produced by neoplastic cells; however, the detection of the viral genome in peripheral white blood cells is limited and it is reported to be more useful for OPA detection in flocks rather than in individual animals (5, 17).

Jaagsiekte has been diagnosed all over the world with the exception of Australia and New Zealand and it had been eradicated from Iceland. Kopczewski et al. (8) described in 1978 the appearance of the pulmonary adenomatosis in sheep of Texel breed imported from France. According to OIE, the last confirmed cases of OPA in Poland were reported in 1998 and suspected (but not confirmed) in 2002 (1). One of the reasons for that may be an economically justified rapid decrease in sheep population in the country in the last decades. On the other hand, this may confirm the fact that the available diagnostics (in practice post-mortem) are not able to assess the actual scale of the problem. The owners, due to the economic reasons (need to transport carcasses, research costs, etc.) often try to avoid the investigation of dead animals, which may occur in individual cases only, as the advanced
clinical signs of the disease often are visible only in small percentage of the infected flock.

In this study, we used a highly sensitive and specific PCRR technique - real time PCR for detection of JSRV LTR sequences. We compared the sensitivity of real time PCR with traditional PCR and hemi-nested PCR using plasmid DNA containing JSRV LTR fragment. We also used the PCR based methods to test the possibility of OPA detection in different samples taken from both experimentally and naturally infected sheep, and to examine DNA samples of sheep from Polish flocks for the presence of JSRV. Additionally, 140 ovine lung sections obtained from slaughterhouse were examined histopathologically for the presence of OPA lesions.

**Material and Methods**

**Material.** The experimental group of sheep involved eight animals. Five lambs (1-3 weeks old) were inoculated intratracheally with JSRV clone (kindly provided by Professor M. Palmarini from the University of Glasgow, Scotland). Three sheep (one lamb and two adult ewes) served as negative control. Blood was collected from the animals as described previously (9). The fluid from the nostrils was collected from the lambs showing signs of respiratory illness for RNA and DNA extraction. The animals died 1-4 weeks after the first visible signs of dyspnea had occurred. The remaining animals were euthanised after 1.5 year from the infection. Lung sections were collected during the necropsy for histopathological examination. The lung sections, mediastinal lymph nodes, spleen, liver were also collected and frozen at –70°C for further study.

Field samples included: 198 samples of DNA isolated from blood of sheep from Polish flocks, where the last cases of ovine pulmonary adenocarcinoma were noted, 66 samples of DNA isolated from blood of 3-4 year old sheep from Iranian flocks with jaagsiekte occurrence history, including two sheep with visible signs of respiratory illness (the samples were kindly provided by Dr A.F. Tafti from Shiraz University, Islamic Republic of Iran) and 140 lung sections of sheep removed from the flocks and slaughtered as a result of age-associated removal/replacement policy. The last samples were also used for DNA isolation and histopathological examination.

**Histopathology.** For histopathological examination, the collected samples were fixed in 10% buffered formalin and processed routinely to paraffin wax embedded tissues. Thin sections (5 µm) were cut from paraffin blocks and stained with haematoxylin and eosin (HE).

**JSRV LTR cloning and plasmid DNA preparation.** Total DNA isolated from lung tumour tissue of experimentally inoculated lambs was used as a template for JSRV LTR fragment amplification. The PCR was performed using pair of primers JSRV-1 and JSRV-3 published by Palmarini (15) (JSRV-1: 5’ TGGGAGCTCTTTGGCAAAAGC 3’, JSRV-3: 5’ CACCCGGATTTTTACACACACTCAGGG 3’). 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), 2 mM MgCl2, 100 µM dNTP, 1U of Taq polymerase, (Fermentas, Lithuania), and 0.9 µM of each primer. The final reaction volume was 25 µl. The amplification was performed by using thermocycler UNO II (Biometra, Germany) under the following conditions: initial denaturation at 94°C – 2 min (1 cycle), denaturation at 94°C – 1 min, annealing at 59°C – 1 min, elongation at 72°C – 1 min (40 cycles), and final elongation 72°C – 5 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, Lithuania) were applied to the gel. The expected product of 176 bp length was extracted from agarose gel using Qiaquick Gel Extraction kit (Qiaegen, Germany), sequenced by Genomed (Warsaw, Poland) and identified as JSRV LTR. The purified PCR product was cloned using Qiaqen Cloning Kit (Qiagen, Germany), then the plasmid DNA was purified using Qiaqrep Spin Miniprep Kit (Qiagen, Germany). The concentration of plasmid DNA was measured in the spectrophotometer (GeneRay UV Photometer, Biometra, Germany), and the amount of plasmid copies was calculated according to Qiaugen kits producer. Following the calculation of plasmid copies, 10-fold dilutions of plasmid DNA were made (with initial concentration of 10^9 copy of plasmid DNA) and submitted for hemi-nested PCR and real time PCR.

**DNA and RNA isolation.** The isolation of DNA and RNA from blood, lung fluid, and frozen tissues were performed as described previously (9) and collected samples were frozen in –20°C for further study. The DNA samples were submitted for hemi-nested PCR and real-time PCR and the RNA was analysed using RT-PCR and real time RT-PCR. All collected DNA and RNA samples were analysed for the presence of JSRV LTR.

**Hemi-nested PCR.** For the hemi-nested PCR method the following primers were used: JSRV-1, JSRV-2 and JSRV-6 (5’ TGGGAGCTCTTTGGCAAAAGC 3’, JSRV-3: 5’ CACCCGGATTTTTACACACACTCAGGG 3’). The sequences of the primers were published by Palmarini et al. (15) and were specific for viral LTR region. All the primers were synthesized in IBB PAN (Warsaw, Poland). The single round PCR (the first step of hemi-nested PCR) using primers JSRV-1 and JSRV-3 was performed according to procedure described above. In the following step, 1 µl of PCR product was subjected to the reaction mixture containing 2.5 µl of PCR buffer (100 µM Tris-HCl, pH 8.8, 500 µM KCl), 2 mM MgCl2, 100 µM dNTP, 1U of Taq polymerase, (Fermentas, Lithuania), and 0.9 µM of each JSRV-1 and JSRV-6 primer. The final reaction volume was 25 µl. The amplification was performed by using Biometra thermocycler UNO II in the following conditions, initial denaturation at 94°C – 2 min (40 cycles), denaturation at 94°C – 1 min, annealing at 57°C – 1 min, elongation at 72°C – 1 min (45 cycles) and final elongation 72°C – 5 min. PCR products were detected using UV light after gel electrophoresis (2% ethidium bromide stained agarose gel, 120 V). Aliquots of 10 µl of hemi-nested PCR products and 6 µl of DNA marker
Real-time PCR. For the real time PCR, the TaqMan probe S-JSRV was designed using online software Primer3Plus (available on the internet site www.bioinformatics.nl). The probe design was based on JSRV LTR sequence available in the GeneBank database (Nr NC_001494). JSRV-land JSRV-3 primers targeted a specific conserved 176 base pair region of the JSRV LTR sequence. The probe was synthesised in IBB PAN (Warsaw, Poland) and it was labelled at the 5' end with fluorescent reporter dye (FAM) and at the 3' end with quencher (TAMRA).

Real time PCR reaction mixture was prepared using QuantiTec Probe PCR kit (Qiagen, Germany) and it contained 2x QuantiTec Probe PCR Master Mix, 0.9 \( \mu \text{M} \) of primers JSRV-1 i JSRV-3, 0.2 \( \mu \text{M} \) of TaqMan probe S-JSRV (5' AGCTCCCTGTCCGGCCACCCTC 3'). 0.5 \( \mu \text{g} \) of matrix DNA was added to the final volume of 25 \( \mu \text{L} \) of each reaction mixture. The amplification process was carried out in ABI Prism 7500 FAST thermocycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 95°C – 15 min (1 cycle) denaturation at 94°C – 15 s, 60ºC for 1 min (45 cycles). The analysis of the amplification reaction was performed using the software associated with ABI Prism 7500 FAST (7500 System SDS Software). The software allowed for assignment of cycle threshold values \( C_T \) to positive samples (number of amplification cycle at which reporter signal of the sample reaches an intensity above background allowing for product detection). The reaction was performed three times for each dilution. The standard curve was generated in the software according to the plasmid DNA dilutions.

RT-PCR. The amplification process was conducted in a 25 \( \mu \text{L} \) final volume of reaction mixture containing: 2 \( \mu \text{L} \) of RNA, 0.125 \( \mu \text{L} \) of RNA-se inhibitor (40 U), 0.125 \( \mu \text{L} \) of AMV transcriptase (20 U) (Sigma), 2.5 \( \mu \text{L} \) of PCR buffer, 2.5 \( \mu \text{L} \) of MgCl\(_2\) (25 mM), 2.5 \( \mu \text{L} \) of dNTP (2 mM), 0.25 \( \mu \text{L} \) of Taq polymerase (5 U) (Fermentas, Lithuania), and 1.25 of each primer (20 pmol). Thermal cycling conditions were: 40 cycles, reverse transcription at 50°C – 30 min, initial denaturation at 95°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. The reaction was carried out in thermocycler UNO II (Biometra, Germany). The products of reaction were submitted for electrophoresis in agarose gel with ethidium bromide and analysed in UV light.

Real time RT-PCR. QuantiTec Probe RT-PCR kit (Qiagen, Germany) was used for the real time RT-PCR. The final volume of the reaction mixture was 25 \( \mu \text{L} \), and it contained 500 ng of RNA 1x QuantiTec Probe RT-PCR Master Mix, 0.2 \( \mu \text{M} \) of each primer JSRV-1 and JSRV-3, 0.2 \( \mu \text{M} \) of TaqMan probe S – JSRV, 0.25 \( \mu \text{L} \) QuantiTec RT Mix (containing reverse transcriptase), 0.25 \( \mu \text{L} \) of RNA-se inhibitor, and appropriate amount of RNA-se and DNA-se free water. The reverse transcription and amplification were carried out in ABI Prism 7500 FAST thermocycler under the following conditions: one cycle of reverse transcription at 50°C for 30 s, initial denaturation at 95°C for 15 min followed by 45 cycles of denaturation at 94°C for 15 s, and annealing at 60°C for 1 min. The amplification curves were analysed in 7500 System SDS Software (Applied Biosystems, USA).

Results

Four experimentally infected lambs showed clinical signs of respiratory illness (cough, exaggerated breathing associated with a noticeable movement of the abdominal wall, discharge of mucoid fluid from the nostrils) 1-4 months after virus inoculation. The animals died 1-2 weeks after visible signs of dyspnea had occurred. The remaining animals were euthanised 1.5 year after inoculation.

Analysis of the sensitivity of the PCR techniques using serial dilutions of plasmid DNA revealed that single-round PCR method allowed for the detection of 1,000 copies of plasmid DNA, while both hemi-nested PCR and real-time PCR were able to detect as few as 100 copies of plasmid DNA (Fig.1). In case of the real-time PCR, the R2 value corresponding to the standard curve generated in the 7500 System SDS Software was 0.99, which indicated a proper optimisation of the reaction (Fig.2). The \( C_T \) value above which the samples were considered as negative was selected to be 42. The \( C_T \) value for 100 copies of plasmid DNA was 41.

The analysis of DNA and RNA isolated from PBLs of experimentally infected lambs revealed lack of detectable viral sequences. The study of 198 DNA samples isolated from PBLs of sheep from Polish flocks showed negative results for all samples. Five of 66 DNA samples from Iranian sheep were positive for the JSRV presence (two of the positive samples came from sheep reportedly showing signs of respiratory illness). The analysis of RNA and DNA isolated from respiratory tract fluid collected from experimentally inoculated lambs revealed the presence of JSRV LTR in all samples.

DNA and RNA samples isolated from neoplastic lung tissues of five experimentally infected animals were positive for JSRV LTR sequences, as well as the DNA sample isolated from mediastinal lymph nodes of one of the lambs (lamb nr 4). The samples of the spleen, liver, and mediastinal lymph nodes of other JSRV inoculated sheep were negative, as well as all the samples collected from sheep that served as negative controls (Table 1). JSRV LTR was not detected in any of 140 DNA samples isolated from the lung sections obtained from abattoir.

In the histopathological study, neoplastic lesions characteristic for OPA were observed in all sections prepared from the lung samples of experimentally inoculated lambs. All sections prepared from the lungs of the sheep that served as negative controls, as well as 140 lung sections collected in the abattoir were negative for the presence of any neoplastic lesions.
Fig. 1. The sensitivity of PCR (I) and hnPCR (II) assays estimated using 10-fold dilutions of plasmid DNA ($10^0 - 10^{10}$ copies), K- - negative control, M - molecular weight marker (100 bp, Fermentas).

Fig. 2. Real-time PCR sensitivity: standard curve created in 7500 System SDS Software (Applied Biosystems, USA). Ct – cycle threshold, N – values representing dilutions of plasmid DNA ($10^2 - 10^{10}$ copies).
Table 1
The presence of JSRV DNA in different tissues and respiratory tract fluid of experimentally infected lambs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lungs</th>
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<th>Respiratory tract fluid*</th>
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1N-3N – negative controls, 1-5 – experimentally inoculated lambs: + positive results, - – negative results, 0 – lack of sample; *samples collected pre-mortem.

Discussion
Evaluation of the hemi-nested PCR and real-time PCR in amplification of JSRV LTR region demonstrated that sensitivity of these methods was similar, and at the same time higher than a single–round PCR.

The advantage of the real-time PCR over the hemi-nested PCR is generally approved (7), as the real-time PCR presents much lower risk of errors resulting from cross-contamination of the samples, which may occur when using the two-step method of hemi-nested PCR. Another advantage of real-time PCR is much shorter detection time, because the results are analysed simultaneously by the software. Detection of PCR amplification product may be performed during the early phases of the process, whereas in case of the traditional PCR the results’ analysis requires an additional step of electrophoresis in agarose gel using factors dangerous for human health such as ethidium bromide and UV light (6, 10, 12).

Positive results of detection of JSRV in all samples of respiratory tract fluid collected from nostrils of infected lambs are in agreement with the results described by Voigt et al. (19), who used PCR for the detection of virus genetic material in bronchoalveolar lavage (BAL) collected from 29 premedicated animals infected with JSRV. The PCR method, when applied to blood samples, gave in the same group of animals only four positive results. The authors suggested therefore that testing of BAL samples can be very useful for getting definitive diagnosis of suspected clinical cases of OPA, as well as for pre-clinical identification of infected animals in any flock with OPA history.

The cause of the negative results of blood testing using the methods based on PCR may lie in the insufficient sensitivity, or in the nature of JSRV infection. Low sensitivity of the methods used to detect viral DNA in blood may result from the inhibitory effect of many factors on the PCR, such as haemoglobin, anticoagulants, high concentration of DNA, leukocytes, and immunoglobulins present in the plasma (2, 3, 20). These assumptions are in agreement with the study by Lewis et al. (11), who used the real-time PCR as the second step of hemi–nested PCR to test blood samples from 125 Scottish sheep flocks. The authors concluded that observed sensitivity of the routine PCR method, used for testing blood for the presence of JSRV, was lower than the “analytical method” (applied on plasmids) due to low concentrations of target DNA in the blood of clinically healthy animals. Above conclusions are also in agreement with the results reported by Holland et al. (8), who have shown that frequency of infected cells in OPA affected animals was low (less than 1/240,000 PBMCs). Consequently, the proportion of negative PCR results was high and inevitable due to insufficient amount of white blood cells containing viral genetic material in circulating blood. Furthermore, Summers et al. (18), who examined the local immune response in sheep affected with ovine pulmonary adenocarcinoma, concluded that the infiltration of the tumour tissue by macrophages is inhibited by factors such as surfactant proteins abundantly produced by the neoplastic secretory cells of the lungs, which might explain a small amount of white blood cells containing virus particles. Moreover, De las Heras et al. (5) in their studies on the detection of virus in blood leukocytes in experimentally infected sheep, obtained positive results always when testing animals with well developed neoplastic changes in lungs, confirmed during autopsy, while in sheep, in which there were small lesions, few positive results were obtained. The PCR gave negative results in infected animals but without clinical symptoms of the disease. These authors also found that the numbers of blood mononuclear cells containing provirus DNA were higher in sheep in late stage of the disease, compared with infected sheep during the preclinical period (5).
The studies described above demonstrate the limitations of PCR techniques in the detection of JSRV in the blood, because of the high risk of false-negative results. Being of limited utility for the diagnosis of infection of individual animals, these methods, however, may be important in detecting infections in flocks (5). The examples cited above confirm the results of our analysis of DNA isolated from blood cells of Iranian sheep. Positive results were obtained only in five cases, including samples collected from over 3-year-old animals showing clinical signs of the disease. So far, preliminary screening of small number of blood and tissue samples of Polish sheep provided negative results, in regard to the presence of JSRV in Polish flocks. Although field samples (all in all 338) were collected from a risk group of animals, a negative result should not be interpreted as evidence that the JSRV infection does not occur in Poland. For better clarification of epizootic status of sheep in Poland more studies need to be conducted, using similar material and methods. Results obtained in this study justify testing the respiratory tract excretions of suspected sheep. In our opinion, PCR based approach, in particular real-time PCR, is as yet the best pre-clinical diagnostic method of JSRV infection and can be useful in future control and eradication programmes.

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References