Global Veterinaria 15 (6): 564-569, 2015
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2015.15.06.95289

Effect of Freezing of Lung Tissue and Lung Fluid Samples on the Detection of Jaagsiekte Sheep Retrovirus by Heminested PCR

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Abstract: Ovine pulmonary adenomatosis (OPA), also known as Jaagsiekte, is a contagious progressive pulmonary neoplasia of sheep and rarely goat. The disease is caused by a beta retrovirus called Jaagsiekte sheep retrovirus (JSRV). In this study we employed three methods for extraction of DNA to ensure the optimum amplification of proviral DNA and then we investigated the effect of freezing of the samples on hnPCR detection of JSRV provirus in different freezing periods. The sensitivity of hnPCR test on frozen samples regardless to the sample types were calculated as 100%, 91%, 79%, 62% and 54% in different freezing periods, respectively. Estimate of test sensitivity was 100%, 100%, 100%, 75% and 75% for lung tumor samples compared with 100%, 83%, 58%, 50% and 33% for lung fluid samples in different freezing periods. The results of the present study showed that freezing of the lung tissue and lung fluid samples of OPA affected sheep can decrease the sensitivity of hnPCR test and this effect increases with duration of the freezing. Also probably due to higher number of infected cells in lung tissue than in the lung fluid, freezing has less effect on the sensitivity of hnPCR test in lung tissue samples.

Key words: Jaagsiekte · Hnpcr · Freezing · Ovine

INTRODUCTION

Ovine pulmonary adenomatosis (OPA), also known as Jaagsiekte, is a contagious progressive pulmonary neoplasia of sheep and goat [1], with long incubation period caused by a betaretrovirus called Jaagsiekte sheep retrovirus (JSRV) [2]. The disease is present worldwide, with the exception of Australia and New Zealand and it has been eradicated from Iceland [3]. It usually takes over 2 years in adult sheep to show clinical signs of respiratory tract distress [4]. A pathognomonic symptom of the OPA is accumulation of copious amounts of frothy fluid within the respiratory tract that emitting from the nostrils of the affected animals in the final stages of the disease [3]. At necropsy grayish and firm masses of tumor is observed in the lung of affected sheep. Histopathological examination reveals non-capsulated neoplastic foci that originate from the pneumonocytes type II and Clara cells in the lung to form acini and papillary proliferation. These foci are supported by fibro-vascular connective tissue and large number of alveolar macrophages is observed in the alveolar spaces around the neoplastic foci [5].

Because of lack of the circulating JSRV-specific antibodies there is no diagnostic serological test available for OPA [6, 7]. In the past, diagnosis of the disease was based on the observation of clinical signs, post mortem findings and histopathological examination. Recently, viral RNA and proviral DNA have been detected in lung tissue, lymph nodes, lung fluid and mononuclear blood cells of infected sheep by using of PCR technique [8-11]. Since both tumor cells and broncho-alveolar fluid have been shown to contain a high proviral load [11], PCR testing of lung tissue and lung fluid is a useful method for accurate diagnosis of OPA in sheep that have histopathologic lesions of the disease.

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In some instances, immediately PCR testing of the collected samples is not possible and must protect them from DNA degradation until tested. There are many techniques for preservation of tissue samples, such as freezing specimens at low temperature and preservation in100% ethanol [12], but freezing is the common method of liquid sample preservation and has been used routinely in many studies.

In the present study we employed three methods for extraction of DNA from a day frozen lung tissue and lung fluid samples to ensure of optimum amplification of proviral DNA and then we investigated the effect of freezing of the samples on hnPCR detection of JSRV provirus in different freezing periods. We also compared the results of the hnPCR on the frozen lung tissue and lung fluid samples of the slaughtered sheep which had gross and histopathologic lesions of OPA.

MATERIALS AND METHODS

Sampling: All cases upper than 2 years that had clinical signs of OPA were selected from a slaughterhouse in central area of Iran. The signs were much mucosal fluid discharge from the nostrils, respiratory disorders and progressive weight loss. After slaughter, lungs were inspected carefully for visible lesions of OPA and lung fluids were collected by sterile technique. Also, sterile technique and separate sterile instruments were used to remove tissue samples from four parts of the lungs that had copious amounts of frothy fluid and white grayish nodule or masses in their different lobes, for histopathological examination and PCR test. 10 lung tissue and lung fluid samples from sheep that had no clinical signs and gross lesions of OPA were used as a negative control for PCR test. All samples were divided to five parts for evaluation of the effect of different freezing periods on the test results and transferred to lab immediately.

Histopathology: Tissue samples were fixed in 10% neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax and sectioned into 5im thick slices. The sections were stained with haematoxylin and eosin and examined under light microscope for OPA lesions.

Preparation of Samples for Freezing: Each lung fluid samples were divided to five parts by sterile method and laid at -80°C until tested. Five tissue samples of each lung of approximately 1 g were minced with a sterile blade and transferred to 6 ml of TE buffer (1M Trisaminomethan, 0.5 M EDTA),. Sample was homogenized in a stomacher 80 for 3 minute and homogenate was stored at -80°C in 400 µl aliquots that were thawed prior to DNA extraction [20].

Table 1: Primer sequences for amplifying of genomic proviral of JSRV by PCR.

Primer	Sequence 5′→3′	*position?
P2	ATACTGCAGCYCGATGGCCAG	1875-1896
P3	GCTGCTTTRAGACCTTATCGAAA	1598-1621
P4	TGATATTTCTGTGAAGCAGTGCC	7316-7338

*nucleotide position reference.,Palmarini*et al.*,1996a,b.

DNA Extraction: Genomic DNA was extracted from frozen lung tumor and lung fluid samples in1 day after freezing by Phenol chlroform extraction (PCE) method, modified Phenol chlroform extraction (modified PCE) method(20)and by use of high pure viral nucleotide acid kit (HPVN kit) (kiagen, Korea). After carrying out of the PCR test in the 1 day frozen samples, only HPVN kit was used for extraction of DNAs from frozen lung tissue and lung fluid samples in 1 week, 1, 3 and 6 months after freezing the samples.

PCR: Extracted DNAs from lung tissues and lung fluids were amplified by primers that were specific for viral LTR region (Table1).

U3 5′ LTR hnPCR: Sense primer P3 was paired with P4 as antisense primer in first round of PCR for amplifying almost whole JSRV proviral genome. Two hundred to 400 ng DNA of each sample was added to 23µl of PCR reaction mixture containing: 2.5µl PCR buffer (50 mMKCl and10 mMTris-HCl (pH: 8.4), AMS buffer containing 20 mM ammonium sulfate, 75 mMTris-HCl (pH: 8.8)) 1.5µl MgCL2, 2% (vol/vol) dimethyl sulfoxide, 200mM each dNTP, 10pM of each primer, 2 µl templateDNA and 0.3 U of Taq polymerase (SmarTaq, Fermentas). Amplification was done by previous published procedure [9]. Second round amplified under above condition but by primer P3 as sense and P2 as antisense primer. One micro liter of first round PCR product used as DNA template in second round. The size of second round PCR product was 229 bp that visualized by electrophoresis in 2% agarose gel (Figure 1, 4).

Statistical Analysis: Data obtained from hnPCR testing of frozen samples were analyzed with Kruskal-Wallis one way ANOVA and Chi-square test at $p<0.05$ significant level. All calculations were performed using SPSS software.

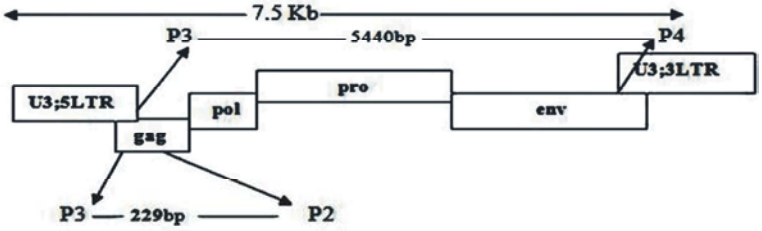

Fig. 1: Organization of JSRV proviral genome. The *gag*, *pro*, *pol* and *env*genesencode the core proteins, the protease, the enzymatic activities and the envelope glycoproteins, primer P3 and P4 in first round of hnPCR amplified 5540 bp products. P3 and P2 amplified 229bp in second round of hnPCR.

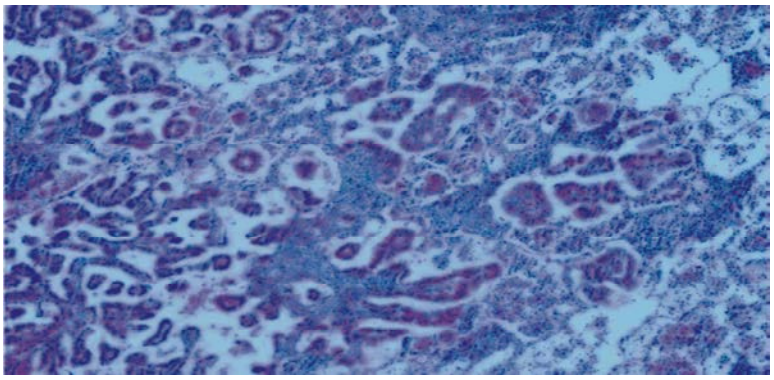

Fig. 2: Variable sized un-capsulated tissues are composed of cuboidal cells that line alveoli to form acini or papillary projections. They are supported by fibro-vascular connective tissue and localized infiltrate of macrophages around the neoplastic foci are seen. H&E.

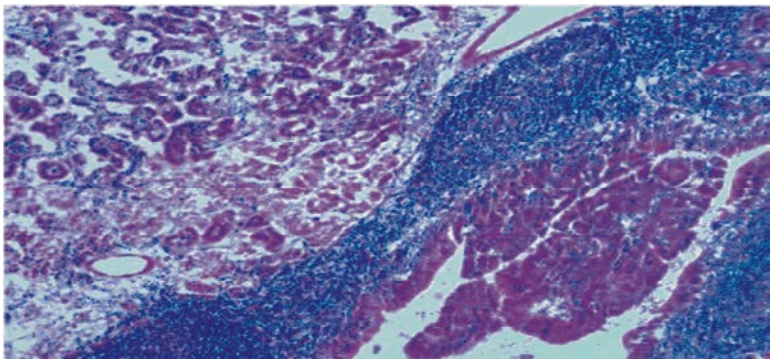

Fig. 3: Papillary projection of Proliferatedcolumnar cells in a bronchiol and peribronchiolar lymphoid hyperplasia is seen. Note the infiltration of macrophages around the neoplastic bronchioi and alveoli.

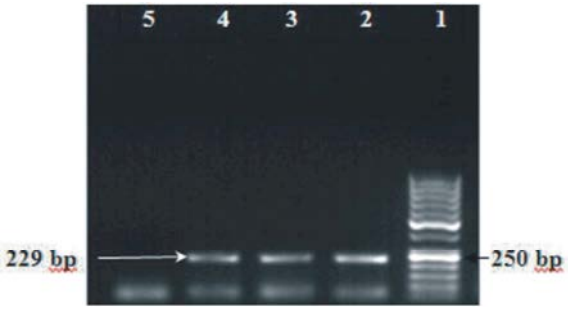

Fig. 4: Agar gel electrophoresis of hnPCR products: Lane 1: 50 bp DNA ladder. Lane 2: DNA from lung fluid extracted by HPVN kit. Lane 3: DNA from lung tissue extracted by modified PEC method. Lana 4: DNA from lung tissue extracted by PCE method. Lane 5: DNA extracted from healthy lung

Table 2: Results of PCR on 1 day frozen lung tissue and lung fluid DNA samples isolated by 3 extraction methods

DNA extraction method	Sample type	Positive sample/totaltested sample
PCE	Lung nodule	12/12
	Lung fluid	12/12
Modified PCE	Lung nodule	12/12
	Lung fluid	
	12/12	
HPVN kit	Lung nodule	12/12
	Lung fluid	12/12

Table 3: Detection of genomic JSRV in lung tissues and lung fluids in different times after freezing

Freezing period	Sample type	Positive sample/total tested sample
1 day	lung nodule	12/12
	lung fluid	12/12
1 week	lung nodule	12/12
	lung fluid	10/12
1 month	lung nodule	12/12
	lung fluid	7/12
3 months	lung nodule	9/12
	lung fluid	6/12
6 months	lung nodule	9/12
	lung fluid	4/12

RESULTS

In the histopathological examination 12 cases had lesions consistent with OPA. Affected lungs revealed papillary proliferation of cuboidal cells in the alveoli and columnar cells in the bronchioi that had been surrounded by fibrovascular connective tissue. Also, accumulation of macrophages in the adjacent alveoli was observed (Figure 2, 3).

All of DNA samples isolated from lung tumors and lung fluids in 5 different periods after freezing examined by standard and hemi-nested PCR using primers P2, P3 and P4 to confirm the presence of JSRV proviral DNA. Expected length of products was 229 bp in heminested PCR (Figure 4).

The results of PCR test on isolated DNA from lung tumors and lung fluids by 3 extraction methods in 1 day after freezing, samples has been shown in Table 2. As detailed in Table 2, all PCR reactions on isolated DNAs from frozen lung tumor and lung fluid samples were positive one day after freezing and there were not any differences among these three DNA extraction methods.

The effect of freezing periods on PCR results has been shown in Table 3. Comparison of the effect of freezing periods regardless to the sample type showed that most positive results were achieved after one day and one week post freezing. False negative results were increased significantly after that time. The sensitivity of hnPCR test on frozen samples regardless to the sample types were calculated as 100%, 91%, 79%, 62% and 54% in different freezing periods, respectively.

Also, comparison of the PCR results on lung tumor and lung fluid samples showed that there is not any significant difference between them in 1day after freezing, but in other times there is significant difference between them and lung tissue had more positive results in PCR test. Estimate of test sensitivity was 100%, 100%, 100%, 75% and 75% for lung tumor samples compared with 100%, 83%, 58%, 50% and 33% for lung fluid samples in different freezing periods.

None of 10 negative lung tissue and lung fluid samples that examined as negative controls were positive in PCR.

DISCUSSION

A problem frequently encounters in collecting material for DNA detection is degrading of DNA during long period keeping it in freezer. Use of proper preservation methods can be preventing of DNA degradation in different samples. Degradation of DNA might be due to presence of nucleases. These enzymes cleave DNA strands into small fragments which affect on many laboratory results. Preventing the action of nucleases is usually achieved through cryopreservation (by dry ice, liquid nitrogen or freezer) at low temperature where most enzymatic activity stopped. In the present study, we had tested the effect of freezing period of the lung tumor and fluid samples of sheep, which had gross and histopathologic lesions of of OPA, on detectable genomic DNA of JSRV using PCR method. We used ahnPCR assay using primers based on the U3 region of viral long terminal repeat (LTR) for detection of exogenous JRSV proviral DNA. The hnPCR protocol has been widely used to amplify JSRV-U3 region [9, 11]. This method is more time consuming than one step PCR and has a higher risk of false positives due to contamination, but it is very sensitive and specific and can detect low amount of provirus [13].

We used three DNA extraction methods (Phenol chloroform extraction (PCE), modified PCE, high pure viral nucleotide acid Kit) to ensure of optimum amplification of JRSV provirus. The results showed that all three methods had identical performance and all samples were positive in hnPCR test in one day after freezing. Thus, we employed only high pure viral nucleotide acid Kit for extraction of DNA from frozen samples in other freezing periods.

Results of hnPCR, regardless to type of the samples, showed significant decrease of positive cases at five freezing periods. Although freezing is the conventional way to preserve samples for many studies, our results indicate that freezing of lung tumor and lung fluid samples in deep freeze (-80°C) can cause damage of DNA and this effect increase with the duration of freezing the samples. Actually freezing at ultralow temperatures can sometimes cause severe damage of DNA. Serious shearing of DNA has been reported in blood samples frozen at -70°C for six months. It is also reported that freezing of samples targeting the *Flavo-bacterium column were* destroyed destroyed DNA and caused most of the *F. columnare* DNA undetectable by PCR [14]. Degradation of DNA in frozen samples may be happen due to formation of intracellular ice crystals.

Comparison of PCR results of the lung tumor and lung fluid samples showed no difference in 1day after freezing, but in contrast to the lung tumor PCR testing, lung fluid PCR testing showed lower positive results and sensitivity in other freezing periods when compared to histopathological examination. These differences in results of genomic JSRV detection between lung tumors and lung fluids might be due to higher load of JSRV proviral DNA in the lung tumors than in the lung fluids. Previous studies, using immunological and molecular techniques demonstrated that the lungs, particularly the epithelial tumor cells are the major site of JSRV replication [15]. Also it has been showed that tumor cells and lung fluids of OPA infected sheep contain JRSV provirus [11]. Previously the presence of tumor cell clusters in lung fluids has been demonstrated, but these were only observed in less than 50% of 43 advanced OPA cases examined [16]. It has been reported that PCR examination of lung tumor give more positive results than lung fluid in the first round and after the heminested protocol [16]. Also it has been showed that use of standard PCR method on the nasal discharge of experimentally JSRV infected lambs did not give positive results [17]. These finding indicate that the number of JSRV infected cells in the lung fluids is less than in lung tissues. In the present study, the estimated sensitivity of the hnPCR in different freezing periods showed that JSRV proviral DNA is detected at much higher levels in lung tumors than in lung fluids of histopathological positive cases and due to high amount of infected cells in the lung tissue, freezing has had less effect on the PCR results of the lung tumor than on the lung fluid samples.

Another reason for these differences might be use of different techniques for freezing of the lung tissue and lung fluid samples in the present study. Use of preserving buffer solutions can be prevent of DNA degradation in samples stored at low and ambient temperatures for extended periods. Also better preservation is achieved when tissue samples are minced before being immersed in the preserving buffer solution so that all that cells are rapidly in contact with the preservant [18]. In the present study we laid all lung fluid samples at -80°C without any further processing, but lung tumor samples were finely minced and immersed in the preserving solution contain EDTA that can reduce activity of the nucleases at high concentration. Thus, we can conclude that frozen lung tissue samples have contained higher contact JSRV provirus than lung fluid samples at the time of PCR testing. In another study we demonstrated effect of storage condition on detecting JSRV transcript in Iranian sheep flocks by Real-time PCR [19].

In summary although quantitative assays were not employed in the present study, the estimated sensitivity of the PCR assays in different freezing periods confirmed that JSRV provirus was detected at much higher levels in OPA lung tumors than in the lung fluids. Also due to damaging of DNA by nucleases, we suggest immediate testing the frozen samples as soon as possible for better detection of JSRV provirus by PCR method.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. N. Ahmadi and Mrs. F. Yektaneh for their kindly cooperation in this study. This work was supported by research deputy of Shahrekord University

REFERENCES

1. De las Heras, M., A. Ortín, D. Salvatori, M. Pérez de Villareal, C. Cousens, Miguel and L. Ferrer, 2005. A PCR technique for the detection of Jaagsiekte sheep retrovirus in the blood suitable for the screening of ovine pulmonary adenocarcinoma in field conditions. Research in Veterinary Science, 79: 259-64.
2. Palmarini, M., J.M. Sharp, M. De Las Heras and H. Fan, 1999. Jaagsiekte sheep retrovirus is necessary and sufficient to induce a contagious lung cancer in sheep. Journal of Virology, 73: 6964-72.
3. De las Heras, M., A. de Martino, M. Borobia, A. Ortín, R. Alvarez and L. Borderías, 2014.Solitary Tumours Associated with Jaagsiekte Retrovirus in Sheep are Heterogeneous and Contain Cells Expressing Markers Identifying Progenitor Cells in Lung Repair. Journal of Comparative Pathology, 150: 138-47.

4. Sharp, J. and J. DeMartini, 2003. Natural history of JSRV in sheepJaagsiekte Sheep Retrovirus and Lung Cancer. Springer, 12: 55-79.
5. Garcı´a-Goti, M., L. Gonzalez, C. Cousens, N.Cortabarrı´a, A. Extramiana and E. Minguijon, 2000. Sheep pulmonary adenomatosis: characterization of two pathological forms associated with jaagsiekte retrovirus. Journal of Comparative Pathology, 122: 55-65.
6. Ortı´n, A., E. Minguijón, P. Dewar, M. Garcı´a, L.M. Ferrer and M.Palmarini, 1998. Lack of a specific immune response against a recombinant capsid protein of Jaagsiekte sheep retrovirus in sheep and goats naturally affected by enzootic nasal tumour or sheep pulmonary adenomatosis. Veterinary Immunology and Immunopathology, 61: 229-37.
7. Summers, C., W. Neill, P. Dewar, L.Gonzalez, R. van der Molen, M. Norval and J.M. Sharp, 2002. Systemic immune responses following infection with Jaagsiekte sheep retrovirus and in the terminal stages of ovine pulmonary adenocarcinoma. Journal of General Virology, 83: 1753-7.
8. Caporale, M., P. Centorame, A. Giovannini, F. Sacchini, M. Di Ventura, M. De las Heras and M. Palmarini, 2005. Infection of lung epithelial cells and induction of pulmonary adenocarcinoma is not the most common outcome of naturally occurring JSRV infection during the commercial lifespan of sheep. Virology, 338: 144-53.
9. Palmarini, M., M.J. Holland, C. Cousens, R.G. Dalziel and J.M. Sharp, 1996. Jaagsiekte retrovirus establishes a disseminated infection of the lymphoid tissues of sheep affected by pulmonary adenomatosis. Journal of General Virology, 77: 2991-8.
10. Holland, M.J., M. Palmarini, M. Garcia-Goti, L. Gonzalez, I. McKendrick, M. de las Heras and J.M. Sharp, 1999. Jaagsiekte retrovirus is widely distributed both in T and B lymphocytes and in mononuclear phagocytes of sheep with naturally and experimentally acquired pulmonary adenomatosis. Journal of Virology, 73: 4004-8.
11. Gonzalez, L., M. Garcı´a-Goti, C. Cousens, P. Dewar, N. Cortabarrıa, A.Extramiana, A. Ortin, M. De Las Heras and J.M. Sharp, 2001. Jaagsiekte sheep retrovirus can be detected in the peripheral blood during the pre-clinical period of sheep pulmonary adenomatosis. Journal of General Virology, 82: 1355-8.
12. Post, R., P. Flook and A. Millest, 1993. Methods for the preservation of insects for DNA studies. Biochemical Systematics and Ecology, 21: 85-92.
13. Zhang, K., H. Kong, Y. Liu, Y. Shang, B. Wu and X. Liu, 2014. Diagnosis and phylogenetic analysis of ovine pulmonary adenocarcinoma in China. Virus Genes, 48: 64-73.
14. Suomalainen, L.R., H. Reunanen, R. Ijäs, E.T. Valtonen and M. Tiirola, 2006. Freezing induces biased results in the molecular detection of Flavobacterium columnare. Applied and Environmental Microbiology, 72: 1702-4.
15. Palmarini, M., P. Dewar, M. De las Heras, N. Inglis, R. Dalziel and J. Sharp, 1995. Epithelial tumour cells in the lungs of sheep with pulmonary adenomatosis are major sites of replication for Jaagsiekte retrovirus. Journal of General Virology, 76: 2731-7.
16. Voigt, K., M. Brügmann, K. Huber, P. Dewar, C. Cousens, M. Hall, J.M. Sharp and M. Ganter, 2007. PCR examination of bronchoalveolar lavage samples is a useful tool in pre-clinical diagnosis of ovine pulmonary adenocarcinoma (Jaagsiekte). Research in Veterinary Science, 83(3): 419-27.
17. Kycko, A., A. Jasik and M. Reichert, 2008. Detection of jaagsiekte sheep retrovirus in respiratory tract fluid and lung tissue of experimentally infected lambs. Bullefin of the Veterinary Insfitute in Pulawy, 52: 9-13.
18. Seutin, G., B.N. White and P.T. Boag, 1991. Preservation of avian blood and tissue samples for DNA analyses. Canadian Journal of Zoology, 69(1): 82-90.
19. Azizi, S., E. Tajbakhsh and F. Fathi, 2014. Ovine pulmonary adenocarcinoma in slaughtered sheep: A pathological and polymerase chain reaction study. Journal of the South African Veterinary Association, 85(1): 01-5.
20. Extramiana, A., L. González, N. Cortabarrıa, M. Garcıa and R. Juste, 2002. Evaluation of a PCR technique for the detection of Maedi-Visna proviral DNA in blood, milk and tissue samples of naturally infected sheep. Small Ruminant Research, 44(2): 109-18.