

## Prevalence of Jaagsiekte Sheep Retrovirus Infection in North-West Iran

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**Abstract:** Ovine pulmonary adenocarcinoma (OPA) is a chronic respiratory disease with a long incubation period. This disease is common in sheep and it is most often observed in older animals (over three years old). The disease is caused by a betaretrovirus called Jaagsiekte sheep retrovirus (JSRV) and is transmissible between sheep. OPA has long been recognized in many countries. This is the first study to obtain data on the prevalence of JSRV infection in sheep in North-West Iran. Blood samples were collected from 167 sheep. The location, age and sex of each sheep were recorded. The blood samples were tested for JSRV infection by applying JSRV- specific PCR to DNA extracted from the blood cells. 30 samples tested out of 167 were positive (18% 95 CI:12.5-24.6%). This study has demonstrated that there is a high prevalence of JSRV infection in sheep in the North-West of Iran. To increase the efficiency and profit from the sheep industry in this area it would be advisable to put in place measures that may limit the spread of this virus. Without vaccines and good diagnostic techniques control measures will rely on appropriate best practice management and biosecurity.

**Key words:** Adenocarcinoma • Retroviruses • Sheep • PCR

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### INTRODUCTION

Ovine pulmonary adenocarcinoma (OPA), also known as jaagsiekte (driving sickness), pulmonary adenomatosis and epizootic adenomatosis is a transmissible lung tumor of sheep caused by jaagsiekte sheep retrovirus (JSRV) [1- 3]. JSRV is transmitted by the respiratory route but can also be transmitted to lambs by colostrum and milk [4]. The tumor arises from secretory epithelial cells of the lung and can grow to occupy a large proportion of the lung and thereby block lung function (Figure 1). Often a large amount of fluid is produced by the OPA- affected lung and this exacerbates the loss of lung function. OPA is invariably fatal and there are no known treatments for OPA, although antibiotic treatment may prolong life by controlling the secondary bacterial infections which are a common further complication of OPA [5].

OPA has been reported in almost all countries of Europe, Africa, Asia and the Americas but is absent from Australia, New Zealand and Iceland [1]. It is not a new disease. For example, it was first described in the UK in

1888. The incidence of the disease is usually 2-5% but in some flocks can reach 10% [3]. OPA was reported for the first time in Iran in 1972 [6]. A recent study in Fars province of Iran reported that 21 samples out of 944 sheep (0.22%) had OPA lesions at slaughter [7]. In a separate slaughterhouse study in Chahar Mohal Bakhtiari province of Iran, OPA was reported in about 3% of sheep more than 3 years old [8]. Despite the disease having been known for several centuries and having been the subject of scientific research for decades, there is still no cure or vaccine for OPA. Clinical examination fails to identify early stage OPA [9] and diagnosis is only possible at late stage when clinical signs are observed or tumors can be detected by X-ray or CT-scanning [10, 11]. For a definitive diagnosis, post mortem examination of the lungs and histopathological conformation is required. In OPA-affected animals JSRV can be found in tumor cells of the lung and in pulmonary fluids. JSRV antigen can be detected by immunohistochemistry, Western blot or ELISA methods [12, 13]. JSRV can be detected by RT-PCR in pulmonary fluids where the virus is found at high concentration [14], or by PCR in its proviral (DNA)

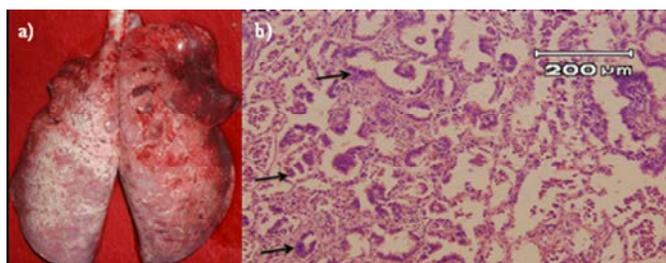


Fig. 1: OPA-affected lungs. a) Gross pathology; Note that the lungs are enlarged, the tumour is grayish in colour and of solid texture and may take over a large area of the lung. In this case many small tumours are visible. b) Histopathological slide (H&E×40). OPA tumour cells are visible as darker, cuboidal cells replacing the alveolar and bronchiolar epithelium. Some of these are indicated by arrows

form in infected cells [15, 16]. Although the production of copious amounts of pulmonary fluids is pathognomonic of OPA, not all OPA cases produce abundant fluid [14].

It has proven difficult to establish a diagnostic test to identify JSRV-infected animals during the preclinical period as infected animals do not produce circulating JSRV-specific antibodies, nor detectable JSRV proteins outside the tumor [17]. Reported laboratory abnormalities in sheep with OPA include neutrophilia, lymphocytopenia and reduction in the CD4/CD8 ratio in peripheral blood [18], none of which are conclusive indicators for OPA. The best available tests are based on PCR detection of JSRV-infected cells in blood [19- 23]. Whilst these tests each have slightly different PCR conditions they are all based on detection of JSRV proviral DNA in white blood cells. A PCR test for JSRV infection using bronchioalveolar lavage (BAL) samples instead of blood is probably more sensitive but the sample collection method is not easily amenable to field studies [24].

The aim of the present study was used to evaluate the extent of JSRV infection in sheep in the North-West of Iran for the first time by PCR- based blood.

## MATERIALS AND METHODS

**Blood Sample Preparation:** Blood was taken from jugular vein via a Venoject (WeMed®, Hamburg, Germany, with EDTAK3) from 167 sheep on the farms in the Northwest of Iran. The farms were located around Spiran and Mayan in the suburbs of Tabriz, the central city of the province of East Azerbaijan of Iran and around the towns of Pesian, Ebrahim-Samy and Ozan in the Kalaybar district in the North East of the province (Figure 2).

The blood tubes were centrifuged and the buffy coat layer was collected and transferred to new tubes, pelleted and stored at -20°C prior to DNA extraction.

**DNA Extraction:** Genomic DNA isolation was performed according to the phenol /chloroform method. The blood cell samples pellet (stored at -20°C) were thawed, suspended in 1 ml of 0.5% SDS and 1 mg/ml proteinase K solution and mixed gently. The mixture was incubated at 37°C for 24 hours, then at 60°C for one hour to inactivate the proteinase K. The mixture was phenol extracted once with a phenol/chloroform/isoamyl alcohol solution and after centrifugation the aqueous layer was removed to a fresh 1.5 ml tube. The DNA was ethanol precipitated, resuspended in buffer and then ethanol precipitated a second time. Once the pellet was dried, the DNA was resuspended in distilled water. The genomic DNA concentration was determined by the biophotometer measurement at 260 nm.

**Positive Control for JSRV PCRL:** Plasmid pJS21-VR3-U3, which comprises nucleotides 5350-7383 of JSRV genome (Genbank AF105220) inserted into pGEM-T plasmid, was used as a positive control. The concentration of the plasmid was 200 ng/μl and 3 μl was used in each PCR positive control reaction.

**Amplification and Identification of JSRV Blood Samples:** Amplification of a 175 bp segment of the JSRV genome was performed using the primers PIII and PI as forward and reverse primers, respectively. The primers sequences were 5'GCACAAGCGGTGGAGCATGT3' for PIII and 5'CCCGGGAACGTATTCACC3' for PI primer as described previously [15]. PCR was as follows; 1x buffer (Fermentas), 15 pmol each primer, 10 mM each deoxynucleoside triphosphate (dNTP), 0.5 μl of Taq polymerase (Fermentas) and 60ng whole blood extracted DNA as the template, made up to a final reaction volume of 25 μl with dH<sub>2</sub>O. PCR was carried out using a Mastercycler Personal (Eppendorf, Germany) with the following program: initial denaturation at 94°C for 2 min;



Fig. 2: Map showing the sampling areas in this study (<http://www.anobanini.ir/travel/fa/azarbaeijan-sh/>). Tabriz is the central city of the province of East Azerbaijan of Iran. Pesian, Ozan and Ebrahim-Samy are villages/towns located in the North East of the region close to the border with Armenia (shown in the ellipse)

30 cycles of denaturation (25 s at 94°C), annealing (30 s at 65°C) and extension (30 s at 72°C); followed by a final extension at 72°C for 5 min. Plasmid DNA (described above) was used as positive control and the negative control had sterile distilled water as template. The PCR was performed in triplicate for each sample. The PCR products were analyzed by electrophoresis of a 3 µl aliquot through a 1% (w/v) agarose gel, with 80 V for 20 min and then stained with ethidium bromide and visualized. A 100 bp DNA ladder was used as size marker. The result was considered as a positive PCR test if one or more of the triplicate reactions generated a product of the appropriate size.

**Statistical Analysis:** Confidence intervals were calculated as Clopper-Pearson exact confidence intervals [25]. Genstat v14.0 was used to calculate p values using the Chi-squared test evaluated using random permutations.

## RESULTS

In this study 167 blood samples were collected from sheep in North-West Iran and subjected to PCR for detection of JSRV infection. Typical results from positive and negative controls and samples are shown in Figure 3. Thirty of the 167 sheep tested were positive in the PCR blood test for JSRV infection. This gives a mean observed

prevalence of 18% (95 CI 12.5-24.6%) Analyzing the data by animal age, (Table 1) the groups aged over 2 years had the highest number of positive animals with 23 of 121 (21%) animals testing positive compared to the age under 2 where only 4 of 46 (8.7%) animals tested positive. However, because the group sizes were small, the 95% confidence intervals were wide and the Chi-squared test showed there was not a statistically significant difference between age groups ( $P>0.05$ ). The prevalence of sheep testing positive was higher from the villages of Pesian and Ebrahim-Samy than from Ozan or the suburbs of Tabriz (Table 2), but again the 95% confidence intervals were wide and the differences were not statistically significant ( $P>0.05$ ).

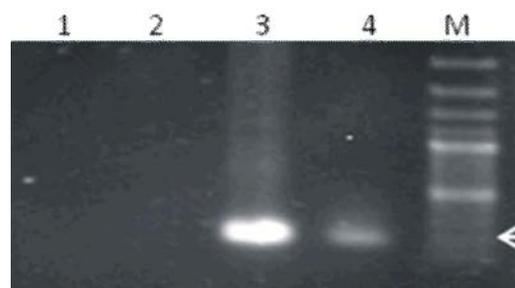


Fig. 3: Typical PCR results. Lane 1: Negative sample, 2: negative control (water), 3: positive sample, 4: positive control (plasmid), M: DNA ladder. The arrow indicates the 179bp PCR product

Table 1: Results of the JSRV PCR blood test according to the age of the sheep

Age group	Under 1 year old	1-2 years old	2-3 years old	Over 3 years old
No. animals positive /no. tested	2/30	2/16	7/28	19/93
% positive	7%	13%	25%	20%
95% CI	(0.8-22%)	(2-38%)	(11-45%)	(13-30%)

Table 2: Results of the JSRV PCR blood test in sheep from different areas in North-West Iran

Area	Tabriz (Spiran & Myan)	Pesian	Ozan	Ebrahim-Samy
No. animals positive /no. tested	16/105	7/24	2/20	5/18
% positive	15%	29%	10%	28%
(95% CI)	(9-24%)	(13-51%)	(1-32%)	(10-53%)

## DISCUSSION

OPA has been reported in many countries world-wide and has been recognized by the OIE as an important disease in the international trade of sheep and ovine products (<http://www.oie.int>). OPA is not a statutory notifiable disease and therefore accurate data on the prevalence of OPA is not collected in any countries. Annual mortality range has been reported from negligible up to 10% in affected flocks [3].

A previous slaughterhouse study in Iran examined the lungs of 944 sheep from the Fars province and identified OPA in 21 (0.22%) of these sheep [26]. They selected the samples first by observation of gross lesions and then confirmed by histopathology. In a similar slaughterhouse study in a suburb of Tabriz, the prevalence of OPA was 2.57% of 468 lungs that were inspected (Rezazadeh et al. unpublished data). The only other abattoir study reported was in Edinburgh, UK, in 1964 which recorded visible OPA lesions in 52 of 280,000 (0.02%) sheep examined [27]. More recent data has not been gathered. The two reports of 0.22% or 2.57% of sheep from Iran having OPA lesions are considerably lower than the blood test results we report here where 18% of sheep tested positive for JSRV infection. This agrees with previous descriptions that, as with many other viruses, the number of animals infected with JSRV is very much higher than the number that will ever develop disease [1, 19, 21]. Although OPA disease is uncommon in animals less than one year old, JSRV infection in these animals had previously been reported [1, 5] and was also shown in our study. Our study suggests that older sheep (2 years and above) are more likely to be infected with JSRV, in agreement with previous report [19].

A recent study in the UK, using a similar JSRV PCR blood test to the one reported here, tested samples from

125 flocks in Scotland (total 3374 sheep) and found an observed prevalence of 38% of flocks (95 CI: 29-47%) but only 3.6% of total population tested [23]. Statistical analysis of the data suggested low sensitivity of the assay. This low sensitivity at the level of individual animals had previously been noted [20] and suggests that the 18% testing positive reported in this study is likely to be an underestimate of the true prevalence of infection, (assuming that the specificity is also similar to that of the previous studies). Conversely, it is well established that not all infected animals develop OPA within their normal lifespan [1, 21]; therefore the proportion of sheep having positive PCR results in this study is likely to be an overestimate of the proportion of animals that will eventually develop clinically significant OPA disease. In the absence of a more accurate diagnostic test, the data presented is at least an indicator of a high prevalence of JSRV infection in sheep in North-West Iran and underlines the need for a better diagnostic test.

The prevalence of OPA varies depending upon the breed of sheep and the type of flock management [28]. There has not been any study in Iran on OPA prevalence in different breeds but as breeds and farming practice vary between the different regions of Iran it is probably not possible to separate breed effects from management effects. Sheep farming in the North West of Iran is traditional and as this is in the cold temperate area of Iran the sheep are housed indoors for 7-8 months of the year and are therefore in close contact. This will favour transmission between sheep within flocks. The practice of herding sheep from different flocks together and the use of shared watering facilities between different flocks will facilitate between-flock transmission. In other areas of Iran, alternative commercial sheep farming practices are followed and the within- and between-flock transmission risks will be different.

## CONCLUSION

This study has demonstrated that there is a high prevalence of JSRV infection in sheep in the North-West of Iran. To increase the efficiency and profit from the sheep industry in this area it would be advisable to put in place measures that may limit the spread of this virus. Without vaccines and good diagnostic techniques control measures will rely on appropriate best practice management and biosecurity.

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