First molecular diagnosis of ovine pulmonary adenocarcinoma in Awassi sheep in Iraq

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Abstract

Background: Ovine pulmonary adenocarcinoma (OPA), formerly known as sheep pulmonary adenomatosis is a contagious tumor of the respiratory epithelium.

Aim: The aim of this study to investigate about ovine pulmonary adenocarcinoma (OPA) in Iraqi Awassi sheep.

Material and methods: One hundred and twenty blood samples from Awassi sheep clinically had respiratory problematic were collected as well as sixty tissues samples. included twenty of each of (lung, lung fluid, mediastinal lymph nodes) were collected from lungs with abnormal pathological lesions , all samples tested by RT-PCR by using specific primers and probe were designed in this study by using conserved region in envelope protein gene (NCBI-Gen Bank Code: KT279065.1) .

Results: Ten sheep (5.55%) were found to be positive for JSRV by RT-PCR with higher infection rate in lung fluids 20% , mediastinal lymph nodes 10%, lung tissues 5% , and the less infection rate in blood . 2-5 years aged sheep were more susceptible for infection than other age ,but gender of examined sheep not affected on APO infection .

Conclusion: The first detection of Ovine pulmonary adenocarcinoma (OPA) in Iraq in Awassi sheep in field and abattoir.

Keywords: **Adenocarcinoma, Jaagsiekte, Sheep, RT-PCR, Iraq.**

Introduction:

Ovine pulmonary adenocarcinoma (OPA) is a contagious tumor disease affecting epithelial cells of lungs of sheep that caused by Jaagsiekte retrovirus (JSRV). The disease was firstly recorded in South Africa at 1890 (1) .It affected sheep in many rearing sheep countries around the world as in Europe, Africa, Asia and America except Australia, New Zealand and Iceland (2,3). OPA is caused by a retrovirus known as Jaagsiekte sheep retrovirus. Which induces oncogenic transformation of alveolar and bronchiolar secretory epithelial cells. Sheep is highly susceptible for JSRV but goat and mouflon are less and with rarely OPA incidence. (4). The disease can transmitted vertically from dam by colostrum and milk to their lambs, although the aerosols transmission is the principal horizontal routes for spreading virus during inhalation the infected aerosols (5). Clinically, the disease in sheep characterized by progressive respiratory signs including struggle in breath especially after exercise with signs of pneumonia without response to treat with antibiotic as well as emaciation. The pathognomonic clinical features is production of copious clear frothy fluid and sometimes pinkish in color from lung and drain from nostrils when tested with wheelbarrow test. (6,7). Diagnosis of OPA depend upon clinical signs especially at last stage and post-mortem examination with histopathology of affected lung ,While the JSRV can detected by Immunohistochemistry ,western bloat or Elisa methods (8). PCR tests are informative for epidemiological studies and for identifying infected flocks, the most successful method for identifying early OPA has been PCR
Testing (9).

Materials and Methods:

Collection of sampling:

One hundred and eighty (180) different samples were collected that included one

hundred and twenty (120) blood samples from Awassi sheep flock which showing marked pneumonic signs that become clearly visible after slightly work with case history of non-responsive to treat with antibiotic for long time. Another sixty (60) samples included specimens from lung (20) ,lung fluid(20), mediastinal lymph nodes(20) that collected from lungs which exhibited abnormal lesion in abattoir. All blood and tissues samples were taken under aseptic technique and frozen immediately until examines by RT-PCR , each sample was tested in duplicate and the reactions were considered positive when both of the duplicas was found to be positive. Other data about collected samples such as sex and age were recorded.

Viral RNA extraction:

The extraction of viral RNA from lung tissue, lymph node, blood, and lung fluid samples by using AccuZolTM RNA extraction kit Bioneer. Korea instructions. The samples were placed in 1.5ml microcentrifuge tube, and then 1ml trizol reagent was added and mixed well by vortex for one minute. After that, 200µl chloroform was added and mixed vigorously for 15 seconds, and then the mixture incubated on ice for 5 minutes. The tubes placed in cold centrifuge 4C° at 12000 rpm for 15 minutes. The supernatant was transferred to a new microcentrifuge tube, and 500µl isopropanol was added and the mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. The tubes back to centrifuge at 12000 rpm for 10 minutes, and then supernatant was discarded. The RNA pellet was washed by added 1ml 80% Ethanol with DEPC and mixed again, and then, placed in centrifuge at 12000 rpm for 5 minutes. After that, the supernatant was discarded and the RNA pellet left to air dry. Finally, 50µl DEPC water was added to elution of RNA pellet, and then the extracted RNA sample was checked by Nanodrop spectrophotometer and store in -80 C° freezer until used in RT-PCR assay.

Reveres Transcription Real-Time PCR:

RT-qPCR technique was performed for direct detection of Jaagsiekte sheep retrovirus according to method described by (10). Real-Time PCR primers and probe were designed in this study by using conserved region in envelope protein gene (NCBI-GenBank Code: KT279065.1) .These primer and probe were provided by(Bioneer Company-Korea), The forward primer sequence was **TTGGCTGCTTTTGGTCATGG** , and the reverse primer sequence was **GGCCTTGTATCAACATGAATGGG with product size 136 bp and the sequence of JSR probe** was **FAM- TGGGAG** The Reveres Transcription Real-Time PCR amplification reaction was done by using one step reaction kit (AccuPower^{TM} Dual star RT-qPCR master mix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instructions that included 5µL of RNA templet , 1µL from each one of(JSR Forward primer (10pmol) , JSR Reverse primer (10pmol), JSR probe (20pmol)) ,and 12µL of DEPC water to reach final volume as 20 µL **CAAATATGGTGATGTGGGA-BHQ1**..
These qPCR master mix reaction components were added into standard RT-qPCR premix tube, which contain (Rocket Script reverse transcriptase enzyme, DNA polymerase, dNTPs, and 10X buffer). Sterile white RT-qPCR strip tubes and transferred into Existing vortex centrifuge for 3minutes, the place in Mini Opticon Real-Time PCR system and Thermal cycling conditions were : 45 cycles, reverse transcription at 50°C\60 min, initial denaturation at 95°C\ 3 min, denaturation at 95°C\10 sec, annealing at 60°C\30 sec , elongation at 60°C\30 sec . RT-PCR products were analyzed by scanning.

Statistical analysis:

The obtained data was statically analyzed for means and significances between groups using ANOVA according to computerized SPSS program (version 7).

Results:

The RT-PCR results showed ten (10) samples were positive for JSRV as the first detect of ovine pulmonary adenocarcinoma (OPA) in Iraqi Awassi sheep. This infection rate 5.55% that summarized in table (1) is establishing the infection in all samples types with different level which significantly higher in lung fluid 20% (4\20) and mediastinal lymph nodes 10% (2\20). While in lung tissue 5% (1\20) and the lower infection of JSRV was observed in blood 2.5% (3\120) and figures (1, 2, 3, 4) represent the positive RT-PCR results of examined samples. OPA infection in abattoir 11.6% significantly higher than detected in field 2.5%

Table (1): pulmonary adenocarcinoma (APO) infection rates in sheep

Samples source	Samples no.	Positive RT-PCR	%	samples	Samples no.	Positive RT-PCR	%
Field	120	3	2.5 a	Blood	120	3	2.5 a
abattoirs	60	7	11.6 b	Lung fluid	20	4	20 b
				Lung tissues	20	1	5 c
				mediastinal Lymph nodes	20	2	10 a
Total					180	10	5.55

The different small letters refers to significant variations at ($p \leq 0.05$)

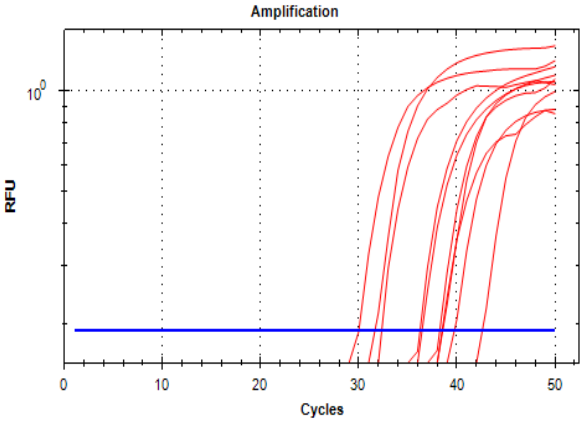

Figure (1): Real-Time PCR amplification plots for Jaagsiekte sheep retrovirus envelope protein gene positive blood samples.

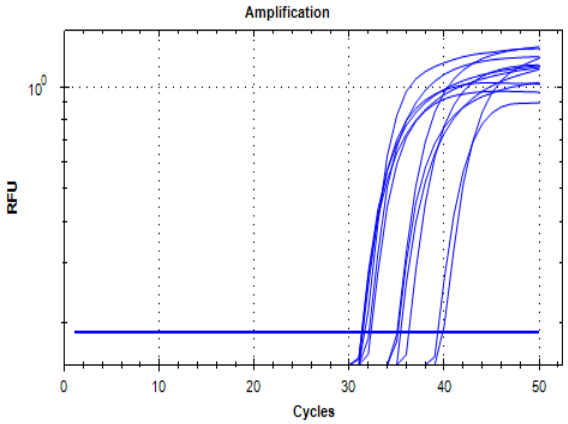

Figure (2): Real-Time PCR amplification plots for Jaagsiekte sheep retrovirus envelope protein gene positive lung fluids samples.

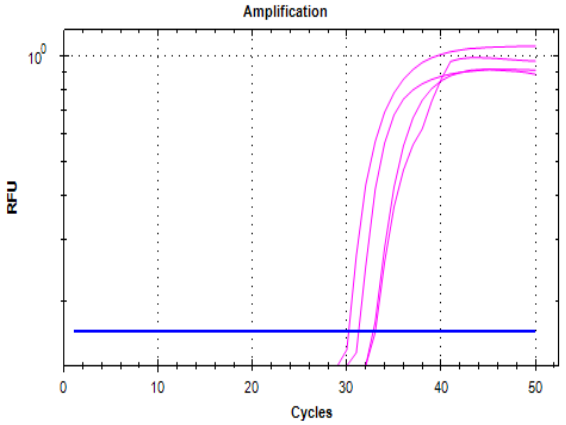

Figure (3): Real-Time PCR amplification plots for Jaagsiekte sheep retrovirus envelope protein gene positive lung tissue samples.

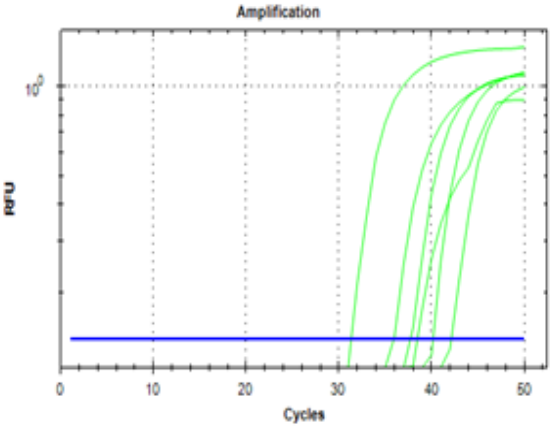

Figure (4): Real-Time PCR amplification plots for Jaagsiekte sheep retrovirus envelope protein gene positive lymph node samples.

The analyzing data according animal ages exhibited sheep with 2-5 years age are more susceptible to infection 12.5% than lambs 0% and old adult sheep 0% ,but

gender of sheep not affected significantly on JSRV infection rate among examined sheep table (2).

Table (2): Relationship between pulmonary adenocarcinoma (APO) infection rates and age and gender of examined sheep

Age									Gender					
< 2 yrs			2-5 yrs			>5yrs			Male			Female		
No.	+ve RT-PCR	%	No.	+ve RT-PCR	%	No.	+ve RT-PCR	%	No.	+ve RT-PCR	%	No.	+ve RT-PCR	%
70	-	- b	80	10	12.5 a	30	-	- b	60	3	5 a	120	7	5.8 a

The different small letters refers to significant variations at (p≤ 0.05)

Discussion:

In the present study that focused on detection of JSRV in Iraqi Awassi sheep which recoded the JSRV infection as first time, and that is corresponding to the global and widely incidence of the disease supporting by many reports as Griffiths and his group (11) , (1) and (12) about detection of disease in most sheep-rearing countries of world except Australia and Newzeland (13). The occurrence of the disease (OPA) in Iraq is very likely and the possibility of transmission of disease from neighboring countries, especially Iran, which recorded the disease for the first time in it at 1972 (14) and via the long land border between the two countries, which allows easy transfer of sheep for grazing or trade. The JSRV –RT PCR result in Awassi sheep 5.5% was closely to the global range of infection that generally around 2-5% (1), also our findings is supporting by Lovett about infection rate in UK as 5.6% (15).Whereas, some researchers recorded high infection rate in sheep like 23.5% (16), 18% in Iran (17), 8% by (18), while Cameron and others found APO infection rate is less than 0.05%in UK (19). The samples obtained from slaughterhouse (lung, lung fluid, mediastinal lymph nodes) overt higher infection rate 11.6% than that from field (blood samples) 2.5% ,these results were in agreement with report by De Las Heras and others (20) due to highly affinity of virus into lung tissues and the mediastinal lymph nodes. Therefore, as the slaughterhouse can receive animals from different and faraway areas in contrast field which reflect limited area with (16). While study in Iran (21) revealed infection in field blood 18.75% higher than lung samples infection 13.75% as well as Voigt and his team could not be detect JSRV in 488 samples from abattoir (9). In spite the APO infection were recorded in all samples in present study in similar findings by (22) because the chronicity, carcinogenic character and long lifespan of disease which allow to reach the agent to most these tissues, but we found higher infection rate in lung fluid than in other samples. Which matched with (23) whom suggest that Jaagsiekte virus was found in high concentration in pulmonary fluid more than other organs and that increase positivity results of infection in lung fluid. In addition to infection rate recorded in mediastinal lymph nodes higher than in lung tissues was agreement with report by (22) of easily detection of JSRV in lymphoid organs than in lungs. Infection of lung tissues 10% in present study was higher than it found by many researchers as in Fars-Iran 0.22% (24), 0.9% in UK (25), 2.57% in Tabriz-Iran (17), 8% in India (18).The discrepancy between our results and others by many researchers either in incidence infection rates or infection rates in organs is very likely possibility due to differences in types, numbers of examined samples and season of sampling as well as kind of test that be used. Examiner experience and age and animal breed , so system of animal rearing, All these factors play important roll on infection rates among different studies. In relationship between age of examined sheep in field and infection rate, all infected sheep was aged 2-5 years and that supporting by similar resultants in many separated studies. Which in general concluded the APO is age –dependent disease and the infection could

be occurred in all sheep aged ,but the more susceptible ages were adults and in old adult was more like findings by (26), (27),(28) and (22), so Sonawane in his study (18) found lamb with two month age was infected with range 1\322 which lesser than high infection in adult 15\106 and the report of most naturally APO infection appeared at age 2-4 years and rarely occurred at less than 7-9 months age lambs (6,26). The adult sheep are more susceptible to Apo infection due to long lifespan of disease with incubation period range 6 months-3years, so it transmitted vertically and carcinogenic nature of disease need long time to metastasis between organs until disease signs would be appeared. According to present study, there was no effect of gender on OPA infection in sheep and that supported by (2,6) reports that confirm both animal sex are susceptible when exposure to infection source. Therefore, the system of sheep husbandry and period in which animals stayed in close contact especially in cooled months could play important role in prevalence of OPA infection than gender of sheep and other susceptible animals like goats.

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