

How to Cite:

Al-Atabi, A. C. S., & Khalaf, J. M. (2022). Clinical and molecular identification study of ovine respiratory syncytial virus in sheep, Iraq. *International Journal of Health Sciences*, 6(7), 8–16. <https://doi.org/10.53730/ijhs.v6n7.10644>

Clinical and molecular identification study of ovine respiratory syncytial virus in sheep, Iraq

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Abstract--Objectives: The goal of this study is to use polymerase chain reaction (RT-qPCR) and sequencing to assess ovine respiratory syncytial virus (ORSV) infection in Awassi sheep in the middle and south of Iraq (Najaf, Babylon, Wassit, and Dhi-Qar), followed by phylogenetic analyses. Methods: From December 2020 to November 2021, a total of 205 nasal swab samples were taken from sheep of various ages and health situations in Najaf, Babylon, Wassit, and Dhi-Qar, Iraq. During the study, the clinical examinations, and vital clinical signs of disease were recorded. RT-qPCR was used to detect nonstructural protein one (NS1) of ORSV, while nested RT-PCR was used to amplify the NS1 protein gene for sequencing, ORSV's NS1 gene (306-bp). PCR products were sequenced, and MEGA 6 analytic software was utilized. The quantitative findings were given as the mean standard error of the mean, the chi-square test (χ^2), and T test were used to determine the significance of the observed variations. Results: A total of 205 nasal swabs were taken from Awassi sheep and ORSV had a molecular prevalence of 23.41 % in sheep. The clinical signs of ORSV included fever, coughing, nasal and ocular discharge, dyspnea, abnormal respiratory sounds, rapid and shallow respiration, and no clinical signs. In some cases, there is a significant increase in the clinical signs of nasal and ocular discharge and abnormal respiratory sounds ($P \leq 0.05$). There are differences between governorates that are not statistically significant ($P > 0.05$). Phylogenetic analysis of the NS1 protein gene revealed that the local Iraqi isolate accession numbers (ON087834-ON087839) were closely related to Ovine respiratory syncytial virus NS1 from the United States strain L15452.1. Conclusion: This study provides the first molecular

record and phylogenic analysis of ORSV in sheep in the middle and south of Iraq, and it will be valuable for confirming clinical signs.

Keywords---ORSV, Phylogenetic analysis, syncytial virus, Iraq.

Introduction

One of the most common diseases in sheep flocks is small ruminant respiratory complex. It is caused by viruses, bacteria, and parasites as well as bad weather, moving animals, overcrowding, and stress, all of which make sheep more likely to get viral and bacterial infections.^[1]

In the sheep's respiratory system, they are found on a random basis and may be acute, chronic, or progressive. Adenovirus, parainfluenza type 3 virus, and respiratory syncytial virus are the most prevalent causes of pneumonia.^[2]

Animals of all ages and breeds can get it. Respiratory complex is a disease that affects sheep and cattle all over the world. It causes big economic losses through symptoms like slow growth, low weight gain, and expensive treatment.^[3,4]

RSV is one of the most widely disseminated viruses, having been isolated from herds of cows, sheep, goats, camels, and even wild animals in a variety of regions and quantities throughout the world. Also RSV is a common pathogen that causes upper and acute lower respiratory infections (ALRI) and clinical signs including rhinitis, tracheitis, bronchitis, and bronchiolitis. Pneumonia is the most frequent lower respiratory tract infection, and respiratory syncytial virus are the most prevalent causes of pneumonia.^[5,6]

RSV viruses are single-stranded, nonsegmented, negative sense RNA viruses that belong to the genus Pneumovirus in the family Paramyxoviridae and the viral name was related to its unique cytopathic action in growing syncytial giant cells in the lung tissues. Aerosols and direct contact are the primary mechanisms of transmission. RSV infection is characterized by cough, anorexia, fever, nasal and ocular discharge, and dyspnea.^[7,8]

Molecular tests such as RT-qPCR and Nested PCR and Serological assays such as the Virus Neutralization Test (VNT), ELISA, Immunofluorescent Antibody (IFA), and Hemagglutination Inhibition (HI) have been used to investigate the incidence of ruminant RSV and PI-3 agents, immunohistochemical (IHC), Direct Fluorescent Antibody Technique and electron microscopy methods are being employed to display comprehensive RSV.^[9]

RSV has been researched in Iraq in 2020, in cattle in Ninawa province, and there have been several studies on it in humans in 2018, but no adequate molecular investigation into the disease ORSV in Iraq or most Arab countries.^[10,11]

In Iraq, this is a first serological and molecular study for ORSV detection in sheep was conducted in the south and middle of the country. The current study aimed

to Investigate the presence of ORSV in sheep by using molecular PCR and evaluation of some clinical and epidemiological features of the disease.

Method

Animals and samples collection

Two hundred five Awassi sheep of different ages reared in different regions in four Iraqi provinces were studied here in. Some of the animals were suffering from nasal discharge, loss of appetite, cough, and dyspnea, while others appeared clinically healthy. Nasal swab samples were collected from each animal and then added to special transport media (RNA later) and then stored at -20°C.

Molecular detection of viral RNA in nasal secretion samples

RT-qPCR

Viral RNA was extracted from nasal swab samples by using the AccuZol™ Total RNA extraction kit (Bioneer, Korea) and performed according to company instructions. Then one pair of specific primers and probe were used in the amplification process of the conserved region of the NS1 gene of RSV. The sequences of primers and probes are listed in table 1. Reactions were conducted in 0.2 µl wells, in a total volume of 25 ml, using 10 ng of RNA and a RealMOD™ Probe 2X qRT-PCR mix (iNtRON, Korea). The wells were then positioned in a thermal cycler (Mx3005P Stratagene Real-Time system) following the steps of table 2 as below. The CT values were calculated and the accepted (positive) values were in a range of less than 30 °C.

Table1
The One step RT-qPCR primers and probes

Primer	Sequence 5'-3'		Amplicon
ORSV- nonstructural protein gene primer	F	TCACACACTTGCTAAATCAGTCA	130bp
	R	AGGCATTGTAGTGAAGTTGGC	
ORSV- nonstructural protein gene probe	FAM- TGATTTCTGTCCCACATCAGA -BHQ1		

Table2
RT-qPCR thermocycler conditions

Step	Condition	Cycle
Reverse transcription step	50°C	1
Initial denaturation	95 °C 10 min.	1
Denaturation	95 °C 15 sec.	45
Annealing and extension and scan	60.3 °C 30 sec.	

Sequencing and Phylogenetic analysis

To amplify the RNA extracted product, nested RT-PCR was used with the One-Step RT-PCR Premix Kit for cDNA synthesis and PCR (iNtRON, Korea). The RT-PCR mixture consisted of RNA template 5-100ng 5 μ L, PCR Forward primer (1 μ L), PCR Reverse primer 1 μ L, One Step RT-PCR premix 8 μ L, PCR water 5 μ L. Two pairs of primers were shown in Table 3. were used. The reverse transcription was carried out at 50 °C for 30 minutes, followed by a 5 minute denaturation step at 95 °C. The amplification was done in 35 cycles under the following conditions: 30s at 95 °C, 30 s at 57 °C, and 60 s at 72 °C. The reaction was completed by final elongation at 72 °C for 5 min. These steps were performed in the first and second PCR reactions and then the nested RT-PCR products were analyzed by agarose gel electrophoresis. Then 3 μ l of ethidium bromide stain were added into the agarose gel solution and RT-PCR products were visualized by using a UV transilluminator.

PCR products from 6 positive samples selected and submitted were sent to Macrogen Company in Korea for DNA sequencing by (AB DNA sequencing system). The DNA sequencing analysis was conducted by using the phylogenetic tree UPGMA method (MEGA 6.0 version), multiple alignment analysis based on Clustal W alignment analysis, and NCBI-BLAST for homology sequence identity.

Statistical analysis

The data for this study were analyzed using version 23.0 of SPSS's statistical package for the social sciences (Chicago, Illinois, USA). The qualitative information was expressed as a frequency and a percentage. The quantitative results were represented as mean \pm standard error for mean, Chi-square test (X²) and T test were used to assess the significance of group differences.

Therefore, the p-value was considered significant as follows: P \leq 0.05 was considered statistically significant, was deemed extremely significant, and P0.05 was deemed non-significant.^[12]

Table3
the Nested PCR primers

Primer	Sequence 5'-3'		Amplicon
ORSV- nonstructural protein gene First PCR primer	F	TGGGTAGTGAAACATTGAGTGT	459bp
	R	AGCATTGGTCCATTTGGTTTATCT	
ORSV- nonstructural protein gene Nested PCR primer	F	AACTCACACACTTGCTAAATCAGT	306bp
	R	AGGATTTAGTTCAAACCTAGAAGATCA	

Results

The results of the RT-qPCR revealed that in the four Iraqi provinces, the total prevalence of ORSV infection was 23.41% (48 out of 205). The molecular percentages of ORV positive samples for each place are shown in table 4. There was no significant difference between all Iraqi provinces, and also, all animals were clinically examined. The recurrence of characteristic clinical signs was determined and shown in table 5 and there is a statistically significant difference ($P \leq 0.05$) in clinical signs. All positive samples confirmed by the presence of NS gene (bands of the expected size: 306 bp segments corresponding to the universal ladder in size 100 -1500 bp were considered positive) Figure 1.

Sequencing and Phylogenetic Tree Analysis

The NS gene partial sequences of the six isolates identified in this study were found to be identical to ORSV. The accession numbers of these sequences were deposited in the GenBank databases under the accession numbers ON087834 - ON087839. Also, the DNA partial analysis of insertion sequence NS1 (306 bp) of the local Ovine respiratory syncytial virus isolates (ORSV-IQK.No.1- ORSV-IQK.No.6) and also showed close relatedness to the NCBI-BLAST Ovine respiratory syncytial virus (strain WSU) isolate (L15452.1) at total genetic changes (0.08-0.02%) as shown in figure 2.

Table 4
Infection rates of ORSV by RT-qPCR in Najaf, Babylon, Wassit, and Dhi-Qar Iraq

provinces	Result		Total	X2	p-value
	positive	Negative			
Najaf	15	45	60	0.557 ^a	0.906 NS
	25 %	75.0%			
Babylon	12	48	60		
	20 %	80 %			
Wassit	11	34	45		
Dhi-Qar	9	31	40		
Total	48	157	205		
	23.4%	76.6%			

NS: Non-significant difference ($P \geq 0.05$)

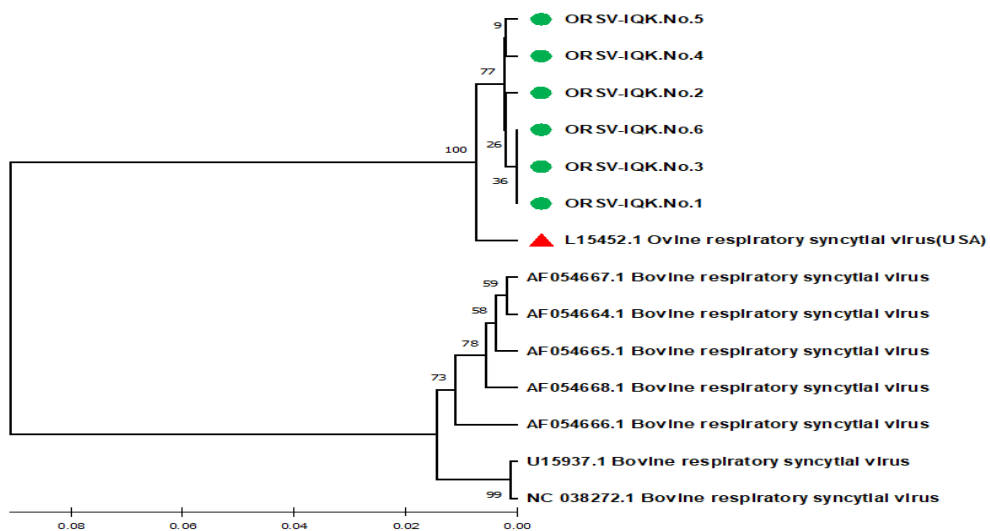
Table 5
Clinical signs of RSV in Sheep according to RT-qPCR results

Clinical signs	Number	Percentage	p-value
Fever	5/48		p-value= 0.03 $X^2=17.95^a$
Coughing	12/48	25%	
Nasal and ocular discharge	19/48	39.6%	
Dyspnea	12/48	25%	
Abnormal respiratory sound	21/48	43.8%	
Rapid and shallow respiration	10/48	20.8%	
No clinical signs	5/48	10.4%	

S: significant difference ($P \leq 0.05$).



Figure 1: Agarose gel electrophoresis image that showed the RT-Nested PCR product analysis of ORSV-nonstructural protein gene, where ladder (1500-100bp), lane (1-16) showed some positive ORSV nasal swab samples for nonstructural



protein gene at (306bp) RT-Nested PCR product size.

Figure 2: Phylogenetic tree analysis based on nonstructural protein 1 (NS1) gene partial sequence in local Ovine respiratory syncytial virus isolates that used for genetic confirmative detection and genetic relationship identification

Discussion

RT-qPCR is one of the most important tools in medical and veterinary diagnosis because it provides a rapid, specific, and sensitive method for detection ORSV and it was 100 times more sensitive than conventional PCR.^[13,14]

In the current study, the NS gene-based on RT-qPCR found ORSV in 23.41% of the tested samples, showing that the virus was circulating in the study area and lower than the results of a study conducted in Mexican sheep in 2017, reported that 38.4 % were positive for RSV by PCR.^[8] and another similar study conducted in Turkey in 1996 less than the results of who mentioned that the infection rate of ORSV was 35.77 %.^[15] Furthermore, the prevalence of ORSV infection was lower than in the Marmara area of Turkey, where it reached 50 % according to immunohistochemical methods.^[16]

Many clinical manifestations of ORSV were observed in this study and these clinical signs are similar to studies of natural and experimental infections. In study in experimental infections in Japan in 1995, that mild clinical symptoms included coughing, serous nasal discharge, and lethargy.^[17] And agreement with the result of another study conducted in Turkey, (2017).^[8] Furthermore, the clinical symptoms of infection with ORSV in bighorn sheep of USA include cough, anorexia, pyrexia, nasal and ocular discharge, and dyspnea.^[18,19]

Also, the DNA partial analysis of insertion sequence NS1 (306 bp) of local ovine respiratory syncytial virus isolates (IQK.No.1 to ORSV-IQK.No.6) and NCBI-Blast related country ORSV isolates relationships showed that the local ORSV isolates were genetically related to ORSV isolates in USA in previous studies.^[20]

The phylogenetic tree for the local isolate of ORSV revealed a high degree of genetic linkage between isolates, indicating that the geographical location, as well as the importation of animals from different parts of the world, plays a significant role in the re-distribution of genetic ties between isolates in different geographical regions.^[21,22]

Conclusion

Respiratory syncytial virus is one of the most important pathogens that are accompanied by respiratory signs to varying degrees, as it was recorded in middle and southern of Iraq, and this study first investigation of ORSV disease in sheep in middle and southern, Iraq.

Availability of data and material

The data used in this study are available and will be provided by the corresponding author on a reasonable request.

Competing interests

There is no conflicts of interest many of the authors concerning the publishing this manuscript.

Funding statement

None.

Authors' Contributions

All authors contributed equally.

Acknowledgments

We acknowledge Baghdad University's College of Veterinary Medicine, Department of Internal and Preventive Medicine. We also appreciate the assistance and cooperation of the animal owners during the handling of the animals and sample collection, as well as the entire veterinary staff working in the laboratory of the veterinary teaching hospital.

Conflict of interest

The authors declare that there is no conflict of interest.

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