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Sequence analyzing of Oestrus ovis in sheeps in Al-Diwaniyah province

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***Abstract*---**A total of 364 head sheep were examine from fourteen region in Al- Diwaniyah province 224 positive to infestation and larvae were collected and sent to the laboratory of veterinary medicine, from October 2021 to March 2022 for both sexes. The rate of *Oestrus ovis* infestation in sheep in the study areas was 61.53%. Al-Dighara abattoir had the highest infestation rate (82.75%) and Al-Salahia had the lowest infestation rate (17.64%). DNA extraction sequencing and phylogenetic analysis were used to identify *Oestrus ovis* larvae (16S rRNA) gene, the sequence was recoded in the National Center Biotechnology (NCBI) with accession numbers (ON059972, ON059973, ON059974, ON059975, ON059976, ON059977, and ON059978) Iraq for *O.ovis* larvae. The percentage of homology sequence identity between this gene and another global gene registered in NCBI was (99–100%) with Brazil and Bosnia and Herzegovina isolates, (97.17%) with USA isolates, and (96.17, 96.33, and 96.68%) with Brazil, Bosnia and Herzegovina, and USA isolates, respectively.

***Keywords*---**Oestrus ovis, PCR, 16S rRNA gene, sequence analyzing.

Introduction

Oestrus ovis (Diptera: Oestridae) is a significant parasite in sheep and infect the sinuses (Metwally *et al.*, 2021). Scientific Classification: Kingdom: Animalia, Phylum: Arthropoda, Class: Insecta, Order: Diptera, Family: Oestridae, Genus: Oestrus, Species: *O. Ovis* (Taylor *et al.*,2016). *Oestrus ovis* is a sheep species found on every continent where sheep are bred (Fogarty *et al* ., 2018). It is an obligate parasite that can be found all over the world, including Iraq particularly in the Baghdad (45.06)% (Alfalahy,2021), Saudi Arabia (5.5)% (Alahmed, 2000), France (33.2–65)% (Dorchies *et al.*, 2000; Jacquiet and Dorchies, 2002), Jordan

(58)% (Abo-Shehada *et al.*, 2000), and Turkey (40.3)% (Arslan *et al.*, 2009). However, few studies on *O. ovis* have been conducted in Iraq, particularly in the south (Alhayali *et al.*, 2022). A fly that can withstand extreme temperatures, humidity, photoperiod, and elevation changes can produce one to seven generations per year (Yilma and Genet, 2000). A female is the only creature capable of fertilizing, growing, and hatching *Oestrus ovis* eggs (Russell *et al.*, 2013).The larvae host-specific parasites of sheep, *Oestrus ovis* nasofrontal cavity larvae, also attack occasional hosts such as humans, dogs, swine, and goats (Giannetto *et al.*,1999). In sheep and other livestock, L1 and occasionally L2 can cause ocular, nasal, pharyngeal, and laryngeal myiasis; this is the human group most at risk (Pampiglione *et al.*, 1991).There were three instars or phases in the life cycle of the larvae known as L1 (the first), L2 (the second), and L3 (the third) (Sufyan *et al.*, 2014). In the larval stage, the fly spends between one and nine months of its life, depending on the climate and season (Cortinas& Jones, 2006). Warm, humid climates have the fastest generation times (Gracia *et al.*, 2019). In groups of two to twenty, the larvae are deposited on the nostrils of sheep and develop inside the host's nose while alimentation on the mucosal secretions (Russell *et al.*, 2013). At this stage of its life cycle, *Oestrus ovis* is parasitic (Allaie *et al.*, 2016).Other species of botflies, including those that live in sheep's nasal passages, have parasitic larvae because adult nasal botflies do not feed, they are not predators (Rolandsen *et al.*, 2021).

Aim of Study

The main purpose of the current study to analyse the gene sequence to compare the genetic diversity of *Oestrus ovis* with NCBI-BLAST deposited from other countries**.**

Materials and Methods

This study was design for traditional and molecular detection of *Oestrus ovis* in sheep. The morphological characteristics of the larvae were studied by measuring the length of each larva using a ruler under the dissecting microscope, the larvae were identified as described by (Abul-hab, 1970) and through its colors and the form of respiratory spiracles in the last segment of larvae (Hanan, 2018).

Collection of sample

Three hundred and sixty four head sheep were examined ,two hundred and twenty four infected from sheep were collected by weekly visit twice a week at Al-Diwaniyah province throughout the period from the 1/ 10/2021 to the 31/3/2022, in Al-Badir, Ghamass, Al-Sadir, Nfer, Eafk, Al-Salahia, Shaamia, Al-muhnawa, Al-Dighara , Al-shafii, sumer, Al-Shinafia, Al-sunni and Al-Hamza regions of Al- Diwaniyah province where it relied on gross observation and determined of infestation site in the animal's head and collected the larvae. For both sex (female 63 and male 161). All samples collected directly from sheep after slaughtering, the skin heads were removed and cut with a hand saw along their longitudinal axis (Allaie *et al.*,2016). The larvae were collected in clean plastic containers with alcohol 70% and marked with sample's numbers of the sex; Then these samples were transported in refrigerator bag to the parasitology laboratory

which belongs to the College of Veterinary Medicine, University of AL-Qadisiyah for laboratory examination (Macroscopic and Microscopic).

Molecular examination

Molecular technique for detection of larvae. The examined larvae of the various evolutionary stage are placed in ethanol an alcohol concentration of 70% later, in deep freeze under -20 C° for DNA extraction.

DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (Korea).

PCR Primers

The following primers targeting the 16S rRNA gene for *Oestrus ovis* was recruited (Hendawy *et al.*, 2012). Table (1)

Table (1): Primer of *16S rRNA* gene *Oestrus ovis*

Primers	Sequence	Product size	Target gene
For ward	CCGGTCTGAACTCAGATCACGT	548bp	16S rRNA
Reverse	CGCCTGTTTAACAAAAACAT		

Sequencing DNA and phylogenetic analysis

By selecting seven larvae from fifty positive PCR samples, DNA sequencing and phylogenetic analysis were used to identify *Oestrus ovis* larvae from Al- Diwaniyah province. The sequencing of the (16S rRNA) gene by the Korean company macrogen was then compared to that of other world strains. The Basic Local Alignment (BLAST) program and the Bio Edit program were used to direct the homology search at the (NCBI) online (http://www.ncbi.nlm.nih.gov). After that, all of the identified samples of *Oestrus ovis* was sent to NCBI-Gen Bank in order to get an accession number and use that number to build a phylogenetic tree.

Sequencing Amplicon and analysis

7 samples were selected from the positive PCR samples for DNA sequencing and phylogenic analysis of the detected pathogens. These were deposited in the gene bank with provided accession numbers and compared with other different world strains as following:

1. Amplicon sequencing and analysis: From the positive PCR samples, several samples were chosen for DNA sequencing and phylogenic analysis of the detected pathogens. These were deposited in the gene bank under the given accession numbers and compared to other world strains as follows:
2. The PCR products were shipped to the Macrogen Company in Korea in an ice bag by DHL for DNA sequencing using a sanger sequencing system.

3. After obtaining the sequences, they were submitted to NCBI-Gen Bank to obtain Gen Bank accession numbers.
4. The DNA sequencing analysis (phylogenetic tree analysis) was carried out with the help of Molecular Evolutionary Genetics Analysis version 10 (Mega x) and multiple sequence alignment analysis based on Clustal W alignment analysis.
5. Phylogenetic tree analysis was used to compare the identified species typing to NCBI-Blast known sequences.

Results

A total of 224 samples were collected and send to the laboratory of veterinary medicine, Al-Qadisiyah University, from both sexes during October (2021) to the March (2022).The results showed that, out of 364 samples, 224 (62) % samples were diagnosis as *Oestrus ovis* larvae, while 140 non infected Fig (1).

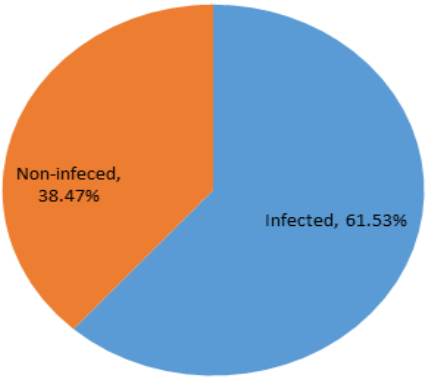


Figure (1): Total of infestation with *Oestrus ovis.*

Macroscopic and Microscopic examination

The body is milky in color and is made up of 12 body rings, each with a broad posterior end and a pointed anterior end. The leading edges of the body parts are covered in bundles of dark-colored spines. The rest of the myiasis-causing insect larvae. L1, L2, and L3 are the first two instars of the Old World Screw worm Fly's larval life cycle. The length and width of the larvae change as the larva grows older and more developed. The results showed the difference in their measurements and colors,1816 larvae of *Oestrus ovis* were detected by using dissecting microscope and ruler for 1816 larvae (10 of L1,1179 of L2 and 627 of L3). These larvae showed a variable length . The average length of the first larvae was 5.00 mm and white in color, while the second larvae distinguished by its large size comparison of the first larvae, where the mean of its measurements 13.26 mm and its color is yellowish or creamy. The third larvae were 20.35 mm and yellow when it is young and at the maturity it turns ,brown with black transverse band on the dorsal surface. Fig (2)

Many rows in ventral spins and prominent with the maturity of larval stages fig(2:a,b). The oral hooks sharply connect in cephalopharyngeal skeleton fig

(2:c).Closed dark (D)shaped, stigmal plates with radially arranged respiratory notes fig(2:d) in posterior end with central button without suture.

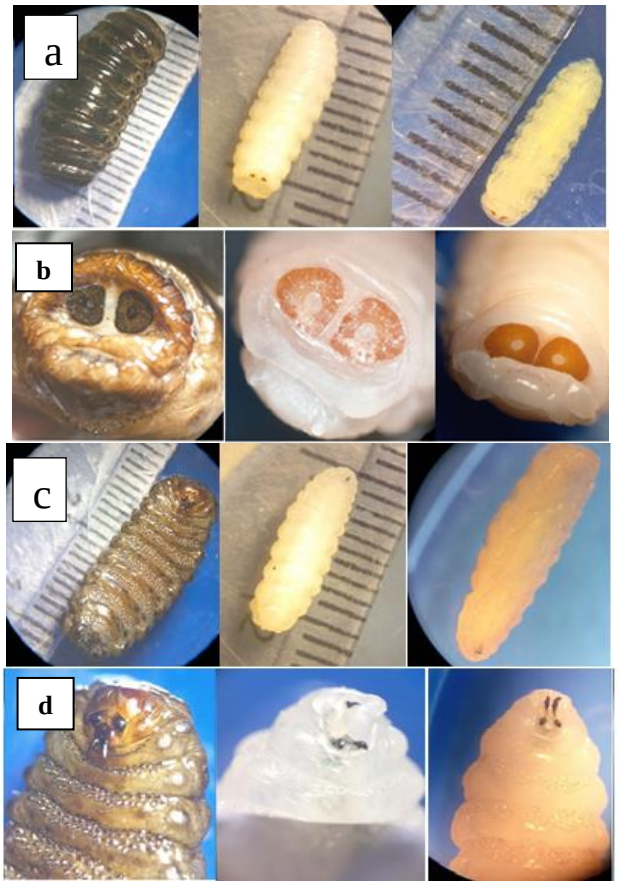


Figure (2): Microscopic examination for *Oestrus ovis* (a) ventral surface, (b) dorsal surface,(c)Posterior end, (d) anterior end

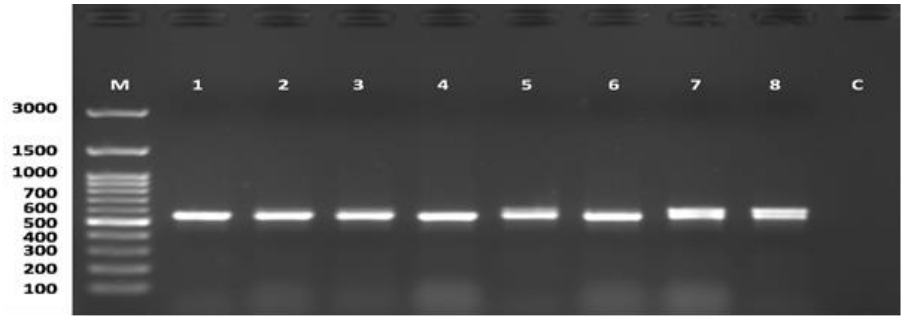
Infestation rate of *Oestrus ovis* in sheep according to the regions of study: The total infestation rate of the larval stage of nasal bot flies was 61.53%, where Al-dighara abattoir recorded the highest percentage rate of infestation (82.75%) and the lowest percentage was recorded in Al-Salahia (17.64%). Table (2)

Table (2): Infestation rate of *Oestrus ovis* in sheep according to the regions of study

Region	Examined No.	Infested No.	Percentage (%)
AL Badir	41	32	78.04
Ghamass	30	23	76.66
Al sadir	17	6	35.29
Nfer	20	7	35
Eafk	20	7	35
Al Salahia	17	3	17.64
Al shaamia	36	28	77.77

Al muhnawa	15	4	26.66
Al dighara	58	48	82.75
Al hamza	55	43	78.18
Al shafii	18	5	27.77
sumer	9	5	55.55
Shinafia	12	6	50
Al sunni	16	7	43.75
Total	364	224	61.53
Chi-square – X^2	21.280		
P-Value 0.068 (Non-Significant)			

In PCR amplification the figure (3) show the amplicons of *O.ovis* to 8 samples of larval stage to (16S rRNA) gene in 548bp ,in gel electrophoresis.



Figure(3): Gel electrophoresis image (1.5 % agarose) shows the amplicons of *Oestrus ovis* (1-8) (16S rRNA gene) (size= 548 bp). C is a control negative in which H2O was added instead of DNA. M is a molecular marker (Genedirex, Korea)

Molecular Study

In Table (3) appear the NCBI-BLAST Homology Sequence identity (%) between local *Oestrus ovis* sequences that were deposited in the gene bank under accession numbers (ON059972, ON059973, ON059974, ON059975, ON059976, ON059977, and ON059978) and NCBI-BLAST deposited sequences from other countries.

Table (3): the NCBI-BLAST Homology Sequence identity between local *Oestrus ovis* sequences were deposited in gene bank

Sequence name	Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	Gen bank Accession number	Country	Identity (%)
1	ON059972	*Oestrus ovis*	NC_059851	USA	97.19
2	ON059973		KR820848	Brazil	99.49
3	ON059974		MG755263	Bosnia and Herzegovina	99.48
4	ON059975		NC_059851	USA	97.19
5	ON059976		MG755263	Bosnia and	96.33

				Herzegovina	
6	ON059977		KR820848	Brazil	96.17
7	ON059978		NC_059851	USA	96.68

In Figure (4) appear phylogenetic tree analysis of *Oestrus ovis* (16S rRNA) gene of the currently identified sequences referred to as yellow circles. These were deposited in the global gene bank and can be seen as accession numbers. These were compared with the previously deposited sequence from the USA under accession number (NC_059851) referred to as the red circle. These were analyzed by Mega X.

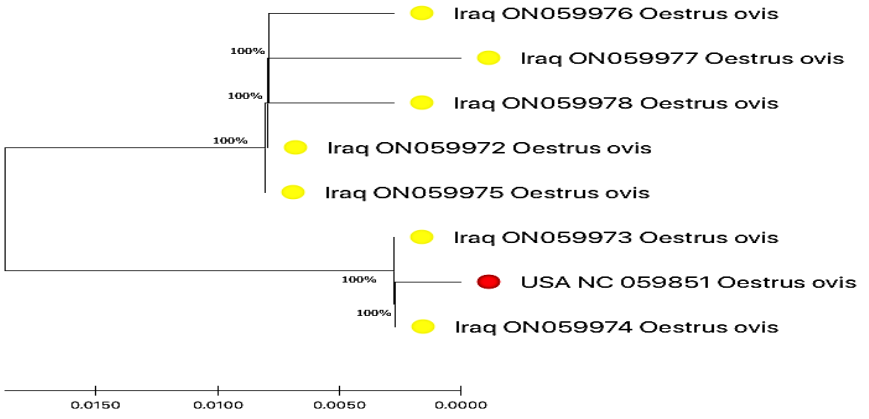


Figure (4): Phylogenetic tree analysis of *Oestrus ovis* (*16S rRNA*) gene

In Figure (5) appear multiple sequence alignment of the identified *Oestrus ovis* in comparison with homologues global sequence. Highlighting with four colours to indicate the similarity. This was analysed by Mega X.

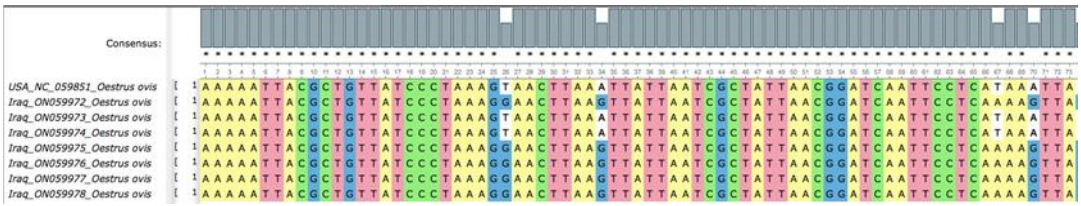


Figure (5): Multiple sequence alignment of the identified *Oestrus ovis* in comparison with homologues global sequence.

Discussion

As revealed by this study, Al-Diwaniyah province had the highest infestation rate at the Al-Dighara abattoir, at (82.75)%, while the lowest infestation rate was found in Al-Salahia, at (17.64)%, as revealed by this study. May be the most of samples from older one year or management of animals or seasons. Due to the low temperatures in the winter agree with (Alfalahy,2021). These results are somewhat similar to those (Al-Ubeid, 2018). As larvae matured, the sharply curved oral hooks attached to the cephalo-pharyngeal skeleton were also more apparent, as these outcomes were supported (Moya *et al.*, 2012). stigma plates with breathing pore distributed radially in a 'D' form, dark brown or black; the results were in agreement with the identification (Hazratian *et al.*, 2017).

The first study in Iraq used to detected *O.ovis* by conventional PCR use following primers targeting the (16S rRNA) gene with product size 548bp. There are many molecular studies have completed to identification *O.ovis* larvae by (28S rRNA) gene (Alfalahy,2021) or I(m COI) gene in Saudi Arabia (Metwally, 2021),(İpek and Altan, 2017) use semi-nested PCR for COX1 gene targeted to genetic markers CO1 and (28S rRNA) larvae from goats and sheep. The sequences of larvae of *O.ovis* have homology sequence identity with NCBI-BLAST with different country strain in USA, Brazil, Bosnia and Herzegovina.One of the *O. ovis* samples with the accession number KP974939.1 matched the sequence (99) % of the time. Data on Oestrid species is extremely limited. Also (Moreno *et al.*, 2015) found that *O. ovis* was parasitizing all of the host species studied, except for Iberian ibex (which is probably the focus of a different Oestrus species).(Stevens , 2003) found a (98)% homology with accession number AJ551428.1 in Italy: Apulia. When compared to other sequences, the sequences of larvae collected from local sheep were 99.9% identical except for ID MT875427.1. Both the 700 bp and 300 bp regions were amplified in eight and seven isolates, respectively. In total, 15 different local isolates were found to be genetically aligned with Kyrgyzstan, Italy, Spain, and Turkey in the PGS. Polymerase chain reaction (PCR) studies targeting amplification of the mitochondrial cytochrome oxidase subunit I (mt COI) gene (606 bp) fragment further confirmed the species identity of *O. ovis* larvae collected from the Abu-Arish area (Eastern Jazan, Saudi Arabia). On the basis of nucleotide pairs from *O. ovis* accessions retrieved from Gen Bank, accession KU921431 showed 97% similarity based on a phylogenetic analysis using the partial (mt COI) gene sequence. It has now been established that a molecular identification method for the *O. ovis* larvae of Saudi Arabia is a viable alternative to the more traditional morphological method. The sequence and *O. ovis* isolates with the accession number KP974939.1 had a (99)% match. Oestrid species' molecular data is extremely scarce. Similarly, a study by (Moreno *et al.*, 2015) found that *O.*

ovis parasitized all of the species of host animals except for the Iberian ibex, which is likely the focus of a different Oestrus species. Homology with accession number AJ551428.1 was 98% in Italy: Apulia (Stevens,2003).When compared to other sequences, the sequences of larvae collected from local sheep were 99.9% identical except for ID MT875427.1. Amplification was observed in 8 and 7 isolates, respectively, at 700 bp and 300 bp of both regions. In total, 15 different local isolates were found to be genetically aligned with Kyrgyzstan, Italy, Spain, and Turkey in the PGS. Further polymerase chain reaction (PCR) studies targeting the amplification of a partial fragment of the mitochondrial cytochrome oxidase subunit I gene (mt COI) further confirmed *O. ovis* is an infection-causing larvae from the Abu-Arish area (East Jazan), Saudi Arabia. *O.ovis* accessions from Gen Bank were found to be (97)% similar to the KU921431 (mt COI) gene sequence in a phylogenetic analysis. As a result, researchers in the Jazan region of Saudi Arabia have developed a method for molecularly identifying *O.ovis* larvae in place of the more traditional.

Conclusions

Prevalence nasal myasis by *Oestrus ovis* larvae in sheep in Al- Diwaniyah province and appear in March was the high rate infestation .March was a high average of larvae stage *O.ovis* per head .This the first study use 16S rRNA gene to diagnosis *O.ovis* in Iraq and sequencing with registered in Gen Bank.

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