Iraqi Journal of Veterinary Sciences

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Molecular study of *Oestrus ovis* larvae infesting in sheep in Baghdad city, Iraq

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Article information

Article history:
Received August 05, 2022
Accepted December 14, 2022
Available online December 14, 2022

Keywords:
Oestrus ovis
PCR
Sheep

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Abstract

The aim of this study is to detect the 28S (rRNA) gene sequences of *Oestrus ovis* larvae by conventional polymerase chain reaction and to compare their genetic relatedness utilizing phylogenetic analysis. Fifty larvae were collected from sheep for DNA extraction after slaughtering during the period from the beginning of February until the end of April 2019 in Baghdad city. PCR product appeared as the band size 950 bp. Ten of the product PCR were selected for sequence analysis to obtain the partial nucleotides 28S (rRNA) gene. After that the sequence were recorded in National Center for Biotechnology Information (NCBI) with ID NO. (MT875427, MT875428, MT875429, MT875430, MT875431, MT875432, MT875433, MT875434, MT875435, MT875436) for *O. ovis* larvae. Then, compared these accession number with another global registered in NCBI by using phylogenetic tree examination which show NCBI-BLAST homology sequence identity between them, and these results were confirmed 99% identity with Spain and Brazil isolates and 98% with Italy.

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Introduction

The sheep bot fly, also known by its scientific name as *Oestrus ovis* (*Diptera*: *Oestridae*) is a universal, incumbent parasite that resides in ruminants; primarily sheep and goats and occasionally in some species of wildlife (1) and humans (2). Belongs to phylum: Arthropoda, class: insecta, order: Diptera, suborder: Cyclorrhpha Series: schizophora, Section: Calypterae, Super family: Oestroidea, family: Oestridae, Genus: *Oestrus*, Species: *O. ovis* (3). Among the most common clinical signs that occur in infested animals are Sneezing and nasal discharges, myiases hinder animal's health and causes significant loss to the economies of livestock industry in both emerging and industrial countries such as decrease in milk production, decreased fertility, quality and weight gain (4). Diagnosis of nasal myiasis depend on morphological characterization and molecular techniques help to diagnose and classify a broad variety of species like myiasis-causing flies (5). Molecular identification may be used on samples that have been obsolete, active or preserved. It can be applied effectively to the identification of all larval stage (6). The aim of this study to detection and molecular diagnosis of *O. ovis* larvae in sheep in Baghdad city.

Materials and methods

Fifty larvae were collected directly from sheep after slaughtering during the period from the beginning of February until the end of April 2019, in Baghdad city. The skin heads were removed and cut with a hand saw along their longitudinal axis (7). The larvae were collected in clean plastic containers with normal saline; Then these samples were transported in refrigerator bag to the parasitology laboratory which belongs to the College of Veterinary Medicine, University of Baghdad for laboratory examination.

Ethical approve

Ethical approval was granted through the local committee of animal care at the college of Veterinary Medicine with the University of Baghdad no1882 dated at 28/4/2019.

Laboratory examination

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 through its colors and the form of respiratory spiracles in the last segment of larvae (3).

Molecular technique for detection of larvae

Fifty of the examined larvae of the various evolutionary stage are placed in ethyl-alcohol 70% later, in deep freeze under -20ºC for DNA extraction (8).

Extraction of larval DNA

Fifty specimens of larvae were collected from infested sheep in different regions of Baghdad city. Genomic DNA was extracted from the preserved specimen by using kit of G spin DNA extraction, intron biotechnology cat no.17045, and was don according to company prescript.

Conventional polymerase chain reaction

The conventional PCR technique was achieved for identification of *O. ovis* larvae based on 28S (rRNA) gene for all genomic DNA samples extracted from larvae samples.

The primers

The primers were lyophilized, dissolved in the free ddH2O to provide a total concentration of 100 pmol/µl as a stock solution and preserve it at -20 °C to make a concentration of 10 pmol/µl as work primer suspended.10 µl of stock solution in 90 µl of the free ddH2O water to achieve a total volume 100 µl, was examined by Integrated DNA Technologies company (IDT) at Canada (Table 1).

PCR product preparation

PCR master mix was prepared by using *Maxime* PCR pre-Mix Kit (*i*-Taq) according to manufacturer's instructions. After then, These PCRmaster mix constituents transferred into thermocycler (T100 Thermal cycler BioRad, USA).

PCR Thermal Cycler Settings

Conventional PCR thermal cycler setting were done by using Multi Gene OptiMax Gradient Thermal Cycler. As shown (Table 2). The optimum condition for initial denaturation and annealing after having carried out multiple experiments to establish this condition by changing temperatures to get the Gradient PCR for all samples, also the concentration of DNA was changed 1.5-2 µl to obtain the optimal condition, the gradient annealing was 50, 52, 54, 56, 58, and 60.

Table 1: The specific primer 28S rRNA of gene

Primer	Sequence	Temperature (ºC)	Size (bp)	Reference
F	5'- GAG AGT TMA ASA GTACGT GAA AC- 3'	52.3	950	(9)
R	5'-TCG GAR GGAACCAGCTACTA_ 3'	56		(10)

Table 2: The optimum condition for gene detection

No.	Phase	tm (ºC)	Time	No. of cycle
1	Initial denaturation	95ºC	5 min.	1 cycle
2	Denaturation -2	95ºC	45 sec	
3	Annealing	58ºC	45 sec	35 cycles
4	Extension-1	72ºC	45 sec	
5	Extension -2	72ºC	7 min.	1 cycle

Sequencing DNA and phylogenetic analysis

Ten larvae were select from fifty positive PCR sample for DNA sequencing and phylogenetic analysis for detection of *O. ovis* larvae from Baghdad city, and were compared with the other different world strain Sequencing of 28S (r RNA) gene was completed by macrogen company in Korea, homology search was directed using (BLAST) program Basic Local Alignment which is accessible at the NCBI at online [available at] and Bio Edit program. Then, all larvae of *O. ovis* diagnosed were submitted into NCBI-GenBank to obtain accession number to designed the phylogenetic tree.

Results

Gross examination

Grossly observation performed to determine of infestation site in the animal's head and the number of larvae (Figures 1 and 2).

Figure 1 : Shows the parasitic larvael stages of nasal bot fly.

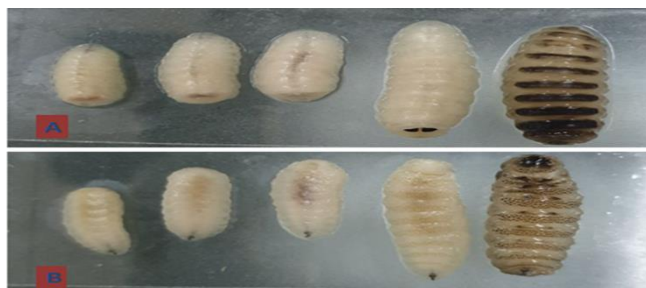

Figure 2 : Dorsal surface (A) and ventral surface (B) of the larval stage of nasal bot fly.

Genomic DNA extraction of *O. ovis* larvae

After process of DNA extraction from the larvae, which were checked by electrophoresis to determine DNA pieces (Figure 3).

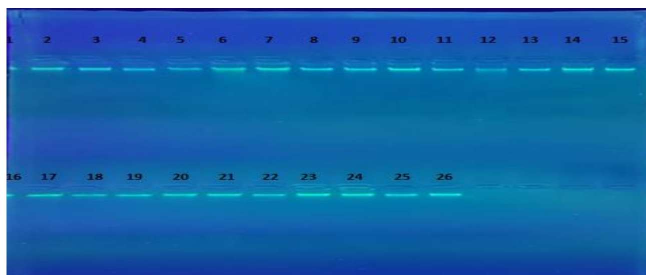

Figure 3: Gel electrophoresis of genomic DNA extraction from insect, 2% agarose gel at 5 vol /cm for 1:15 hours.

Sequence analysis

The PCR product analysis by agarose gel electrophoresis 2%, that stain with Red safe Nucleic acid staining solution at 70 voltand 65 AM for 1:5 hours. The posative DNA band were 950 bp as shown in the (Figure 4).

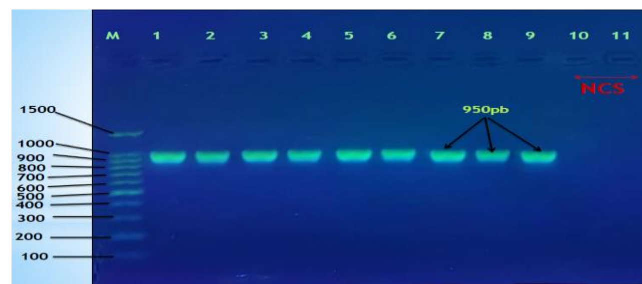

Figure 4: PCR product the band size 950 bp. The product was electrophoresis on 2% Agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100). NCS: negative control sample.

Sequence analysis

Ten sample of PCR products were taken from fifty PCR sample were chosen randomly performed sequenced by forward and reverse primers. The sequnce were employed in NCBI gen bank database, sample No. 1 (MT875427), sample No. 2 (MT875428), sample No. 3 (MT875429), sample No. 4 (MT875430), sample No. 5 (MT875431), sample No. 6 (MT875432), sample No. 7 (MT875433), sample No. 8 (MT875434), sample No. 9 (MT875435) and sample No. 10 (MT875436). These sequnce were analyzed by BLASTA-NCBI program to determine the converging sequences recorded in gen bank. The sequences of all sample confirmation by using *28SrRNA* of the nasal myiasis larvae in sheep of Iraq, it belong to the *O.ovis* larvae.All sequence were related to *O. ovis* (KP974939.1) isolate, with identity 99% (Table 3), and registered in NCBI.

Table 3: Represent type of polymorphism of *28S ribosomal RNA* gene from *Oestrus ovis* isolate

No. sample	Substitution	Location	Nucleotide	ID to compare	ID registry	Score	Identities	Source
	Transversion	735	T>G					
1	Transition	826	A>G	KP974939.1	MT875427	799	99%	*Oestrus ovis*
	Transition	836	T>C					
2	Transition	756	C>T	KP974939.1	MT875428	808	99%	
3	Transition	756	C>T	KP974939.1	MT875429	808	99%	
4	Transition	756	C>T	KP974939.1	MT875430	808	99%	
5	Transition	756	C>T	KP974939.1	MT875431	808	99%	
6	Transition	756	C>T	KP974939.1	MT875432	808	99%	
7	Transition	756	C>T	KP974939.1	MT875433	808	99%	
8	Transition	756	C>T	KP974939.1	MT875434	808	99%	
9	Transition	756	C>T	KP974939.1	MT875435	808	99%	
10	Transition	756	C>T	KP974939.1	MT875436	808	99%	

Phylogenetic tree of *O. ovis* larvae

Phylogenetic tree analysis established on 28S (rRNA) *gene* partial sequence of *Oestrus ovis* isolates from sheep in Baghdad city by using MEGA6 and BLAST-NCBI. The results showed that 99% homology with *O. ovis* ID KP974931.1, KP974932.1, KP974933.1, KP974934.1, KP974935.1, KP974936.1, KP974937.1, KP974938.1 KP974939.1, KP974940.1 Spain isolate, 99% homology

with *O. ovis* ID:KR820885.1 Brazil isolate and 98% homology with Italy: Apulia ID: AJ551428.1 as in the (Table 4 and Figure 5).

Table 4: Compatibility grade% between iraqi *O. ovis* sheep isolates and *O. ovis* Submitted NCBI-BLASTA for another conutries with accesion number.

Accession	Country	Source	Compatibility
KR820885.1	Brazil	*O. ovis*	99%
KP974939.1	Spain	*O. ovis*	99%
KP974933.1	Spain	*O. ovis*	99%
KP974931.1	Spain	*O. ovis*	99%
KP974932.1	Spain	*O. ovis*	99%
KP974935.1	Spain	*O. ovis*	99%
KP974934.1	Spain	*O. ovis*	99%
KP974940.1	Spain	*O. ovis*	99%
KP974938.1	Spain	*O. ovis*	99%
KP974937.1	Spain	*O. ovis*	99%
KP974936.1	Spain	*O. ovis*	99%
AJ551428.1	Italy	*O. ovis*	98%

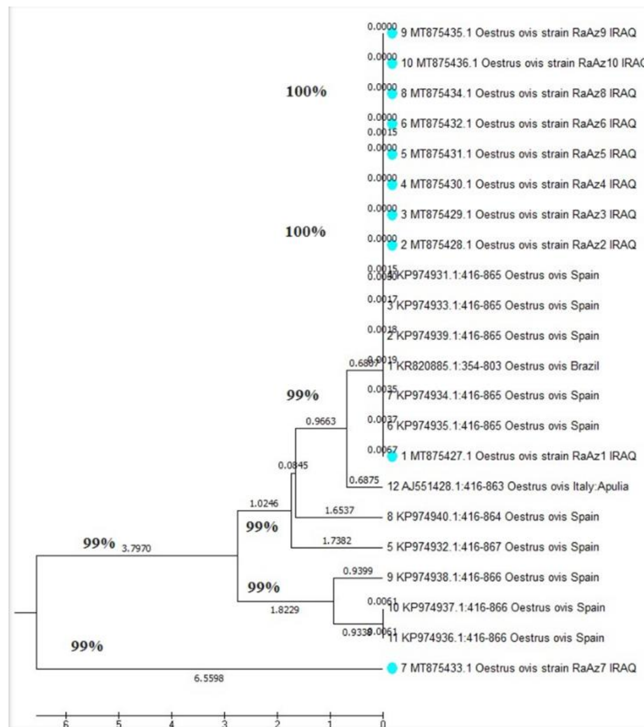

Figure 5: Neighbor joining tree *O. ovis* 28S ribosomal RNA gene.

Discussion

50 larvae of *O. ovis* were detected by using dissecting microscope and ruler, these larvae showed a variable length (mm). These results are somewhat similar to Al-Ubeid (3), Several rows of ventral spines were more prominent with maturity of larvae and the sharply curved oral hooks connected the cephalo-pharyngeal skeleton these results agreed with Moya *et al*. (11). D shaped, dark brown or black colored, stigma plates with radially arranged respiratory holes; the results were in accordance with that stated in identification (8,12). It was used conventional polymerase chain reaction technique was used specific forward and reverse primer 28S (rRNA) to detected *O. ovis* larvae product size 950 bp. There are several molecular studies have been completed to identification *O. ovis* larvae (8) of mitochondrial cytochrome oxidase subunit I (mtCOI) gene in Jazan region, Saudi Arabia, Stevens and Wallman (13) for *28S rRNA* gene**,** Ipek and Altan (14) by semi -nested PCR for COX 1 gene**,** Dowton *et al*. (9) two variable genetic markers COI and 28S (rRNA) had been targeted for the identification of larvae collected from domestic sheep and goats. The sequences of the larvae collected from local sheep was correspondence 100%, except of MT875427.1 and MT875433.1, where the similarity was 99%with other samples The sequence matched mainly *O. ovis* isolate under the accession number KP974939.1 with a query cover of 99%. Molecular data from oestrid species are very scarce, in this study, *28s rRNA* gene used with sequencing and phylogenetic analysis, our sequence was clustered with *O. ovis* isolate reference sequence. Similarly, a study was carried out by Dowton *et al*. (9) who, indicated that *O. ovis* was parasitizing all analyzed host species except the Iberian ibex, which is probably the focus of a different *Oestrus* species. homology with accession number AJ551428.1 was 98% in Italy by Stevens (15).

Conclusion

Compared current accession number obtained in this study with another global registered in NCBI by using phylogenetic tree examination which show NCBI-BLAST homology sequence identity between them, and these results were confirmed 99% identity with Spain and Brazil isolates and 98% with Italy.

Acknowledgements

The authors are grateful to the College of Veterinary Medicine, University of Baghdad, for the assistance and cooperation.

Conflict of interest

Authors declared that there is no conflict of interests.

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دراسة جزيئية ليرقات ذبابة نغف انف الأغنام المصابة في مدينة بغداد، العراق

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فرع الطفيليات، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

طفيلي نغف الاغنام من الطفيليات المشتركة يصيب أنواعًا مختلفة من الحيوانات، ويعتبر هذا المرض أحد الأمراض المهمة التي تصيب صناعة المجترات الصغيرة في العراق ويتسبب في خسائر اقتصادية ضارة أحيانًا يمكن للإنسان أن يصبح مجموعة عرضية للنغف. الهدف من هذه الدراسة هو الكشف عن تسلسل الجين, 28S (rRNA) ليرقات طفيلي نغف انف الأغنام ومقارنة ارتباطها الوراثي باستخدام تحليل النشوء والتطور .واختيرت ٥٠ عينة من اليرقات عشوائيا واجري لها استخلاص المادة الوراثية (الحامض النووي). تم ظهور نواتج التفاعل المتسلسل بشكل حزمة(٩٥٠) زوج قاعدي .بعد ذلك تم اخذ ١٠ نماذج من نواتج تفاعل سلسلة البلمرة لإجراء عملية تحليل التتابع الوراثي للمورث(28S (rRNA وتم تسجيل هذه العينات في بنك الجينات بالأرقام: ID : MT875427; ID : MT875428; ID : MT875429; ID : MT875430; ID : MT875431; ID : MT875432; ID : MT875433; ID : MT875434; ID : MT875435; ID : MT875436. كما تم مقارنة الارقام المدخل التي تم الحصول عليها مع أرقام المسجلة عالميا لإظهار التشابه فيما بينها وسجلت اعلى نسبة تشابه مع العينات المعزولة من إسبانيا والبرازيل ٩٩% في حين سجلت نسبة ٩٨% مع إيطاليا.