

## Morphological and molecular diagnosis of *Hypoderma spp.* in Mosul city, Iraq

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### Abstract

Hypodermosis is a distinctive ectoparasitic disease infesting cattle; *Hypoderma bovis* and *Hypoderma lineatum* are the most common causes of this myiasis. In this study 78 larvae were collected from infected cattle by extraction in the Educational Veterinary Hospital, from Kokjali and Bazwaya flocks and from the skin of slaughtered in Mosul abattoirs for the period from October 2020 to March 2021. Morphological identification by using stereomicroscope depending on patterns of spinulation of the 10<sup>th</sup> abdominal segment and peritremes structure of L3 classified as *H. bovis* and *H. lineatum*. Molecular technique by traditional PCR applied on 16 L3 of the genus *Hypoderma* revealed that the reaction product was 500 bp by amplification of mt CO1 gene while the results of PCR-RFLP using restriction TaqI enzyme for differentiation between the two species indicated reaction products 300bp for *H. bovis* and 200bp for *H. lineatum* respectively. The results of molecular analysis by PCR and PCR-RFLP proved the existence of these two species of *Hypoderma* in Mosul.

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### Introduction

Cattle are exposed to different ecto-parasites as ticks (1) and endo-parasites as blood protozoa (2), tissue protozoa such as *Neospora caninum* and *Toxoplasma gondii* (3,4) infections. One of the most common ectoparasitic infestation is cattle grub also known as cattle hypodermosis resulted from larvae of *H. bovis*, and *H. lineatum* (5).

The warble fly infestation (WFI) or Hypodermosis, causes warbles in the subcutaneous of infested animals which considered the distinctive feature for this disease, this myiasis is present worldwide and has negative effects on cattle production (5,6).

*H. bovis* and *H. lineatum* mainly affect bovine, horses and human (7). The discrimination morphologically between the two species *H. bovis* and *H. lineatum* based on peritreme structure and on the order of spinulation located on the tenth segment of larvae 3 (8,9).

Recently, the cytochrome oxidase subunit 1 gene of the mitochondrial DNA (CO1-mtDNA) considered as the best

gene used in molecular detection and molecular phylogenetic analysis (5). Furthermore, molecular analysis by this gene is ideal for identifying 5 species of *Hypoderma*: *H. bovis*, *H. lineatum*, *H. diana*, *H. tarandi*, and *H. actaeon* (10).

*Hypoderma spp.* in Pakistan and Turkey were identified through CO1 mtDNA sequence analysis and PCR-RFLP (11,12).

It is essential for accurate identification between *H. bovis* and *H. lineatum* for proper treatment of the infested cattle, the discrimination of *H. bovis* from *H. lineatum* is significant because complications could occur during the use of drugs during migration of L1 which cause paralysis with *H. bovis* while esophagitis occur with *H. lineatum* (13).

The aim of this research is essentially using morphological and molecular differentiation between *Hypoderma spp.* in naturally infested bovine in Mosul city by morphological features and PCR-RFLP technique of gene CO1.

## Materials and methods

### Larvae collection

From October 2020 to March 2021, *Hypoderma spp.* Larvae 3 (L3) samples were collected by extraction from the back of naturally infested cattle coming to the Veterinary Hospital, from different flocks and from the skin of slaughtered cattle in Mosul city. The samples were washed in phosphate saline buffer several times and identified depending on morphological description such as shape, color, size and spinulation of the tenth segment as well as morphology of L3 spiracular plates under stereomicroscope (8-10,14) and then were preserved in ethanol 70% for PCR analysis.

### Extraction of DNA

DNA extraction done using tissue kit type Geneaid UKAS after drying the samples from 16 L3 of *Hypoderma spp.* following the manufacturer's instructions. Measuring the concentration and purification of DNA using biodrop instrument in the molecular laboratory of the Department of Biology, College of Sciences, Mosul University; by adding 1µl of DNA extracted from larvae samples and loaded into wells A<sub>260nm</sub>/ A<sub>280nm</sub>, DNA concentrations of all samples ranged between 50-150 ng/ µl and purification between 1.4 -1.7. Electrophoresis is processed on DNA samples extracted from larvae in 1% of agarose gel (Jena Bioscience, Germany).

### Polymerase Chain Reaction (PCR)

Concentration of all DNA extracted was controlled by diluting TE buffer to obtain the required concentration and it was 25 ng/ µl according to the following equation  $C1XV1=C2V2$ . The region of (COX1-mtDNA) amplified by using primer as used by (12) and then prepared the primer used in the PCR reaction supplied by Alpha DNA Montreal, Quebec H3C 0J7 as shown on (Table 1). Prepared DNA Master Reaction mixture. DNA reaction mixture was prepared according to the following (Table 2).

The negative control PCR sample contains all mixtures except DNA extracted from larvae samples. All tubes were centrifuged using microfuge with high speed 3-5 seconds to complete contents reaction mixture. Tube samples should be refrigerated during procedures. Reaction sample tubes were loaded into the thermocycler to perform the amplification reaction using the special program for each reaction as shown on (Table 3).

After the end of the reaction, 5 µl samples of each PCR tubes were loaded in the agarose wells 1% adding Ladder DNA (Biolaps) in one of the wells. Then samples were moved to electrophoresis with 50 volts 60-70 min. The gel was put in the ultraviolet instrument to see the dyed bands and to notice the amplification bands then photographed by a digital camera. Base pairs sizes were estimated according to bands locations by comparing them with DNA Ladder.

Table 1: Primer of Alpha DNA (12)

Primer	Primer sequences ( 5'-3')	bp
Hyp F	TACAGTTGGAATAGACGTTGATAC	500
Hyp R	TCCAATGCACTAATCTGCCATATTA	

Table 2: DNA reaction mixture

Contents	Total size (µl)
Master Mix 2X	10
DNA Template	4
Forward Primer	1
Reverse Primer	1
Distal water (deionized)	4

Table 3: Cycling conditions of PCR for amplification of *Hypoderma spp*

N	Stage	°C	Time (m)	n cycle
1	initial denaturation	95	5	1
2	denaturation	95	0.45	40
3	annealing	60	1.30	
4	extension	72	2	
5	final extension	72	7	1
6	cooling	4	4	1

### PCR-RFLP technique

5 µl PCR product is taken and added to it 0.5 µl of Restriction endonuclease enzyme Taq I the incubated at 37°C for 3h, then separated the restriction fragments agarose gel 1%, finally stained by red safe and photographed.

## Results

Third larvae of genus *Hypoderma* were classified morphologically by microscopic examination using stereomicroscope, identified L3 *H. lineatum* and L3 *H. bovis* (Table 4; Figure 1) show the presence or absence of dorsal spines on 10<sup>th</sup> abdominal segment as a distinctive feature in differentiating between the two species (Figure 2) illustrates the absence of dorsal spines in the 10<sup>th</sup> abdominal segment of *H. bovis*. The structure of the posterior peritreme in *H. bovis* is longer and narrower than *H. lineatum* which is considered another distinctive and reliable feature in distinguishing between the two species (Figures 3). The molecular discrimination using PCR technique by amplification of mt-CO1 gene of 16 larvae 3 of the genus *Hypoderma* revealed that the reaction product was 500 bp (Figure 4) whereas the results of molecular analyses using PCR-RFLP revealed 200 bp and 300 pb, bands of RFLP profile revealed 1,3,4,7 of *H. lineatum* and 2,5,8 of *H. bovis* respectively (Figure 5).

Table 4: Distinctive features of 10<sup>th</sup> of abdominal segment of L3of *Hypoderma* species

Species	Dorsal spines, tenth abdominal segment
<i>H. bovis</i>	Spines absent
<i>H. lineatum</i>	Spines present

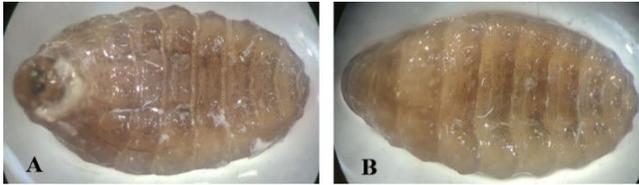


Figure 1: Larvae 3 of *H. bovis* (A. ventral, B. dorsal). 2.5x.

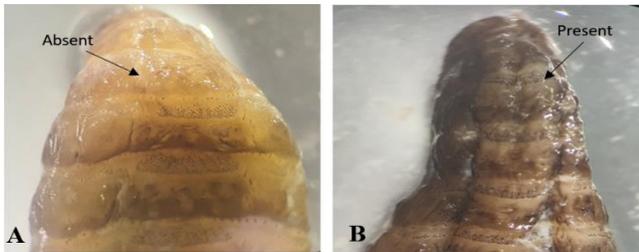


Figure 2: A- absence or B- Presence of dorsal spines in the 10<sup>th</sup> abdominal segment of *H. bovis*. 2.5x.

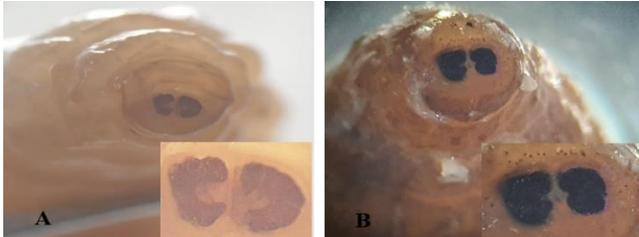


Figure 3: posterior peritreme (a. *Hypoderma bovis*, b. *Hypoderma lineatum*). 10x.

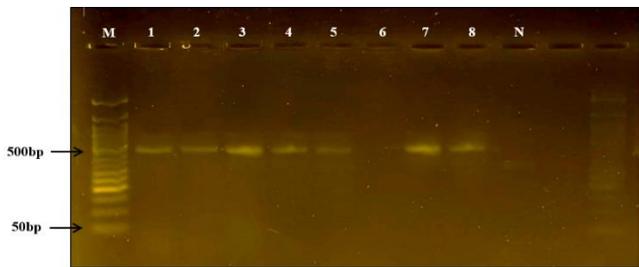


Figure 4: PCR Electrophoresis, CO1 mtDNA amplification carried out using specific primer: M: DNA marker 50bp 1, 2, 3, 4, 5, 7, 8 samples of L3 of *Hypoderma spp.* Positive 500bp, sample 6 Negative. N represents negative control.

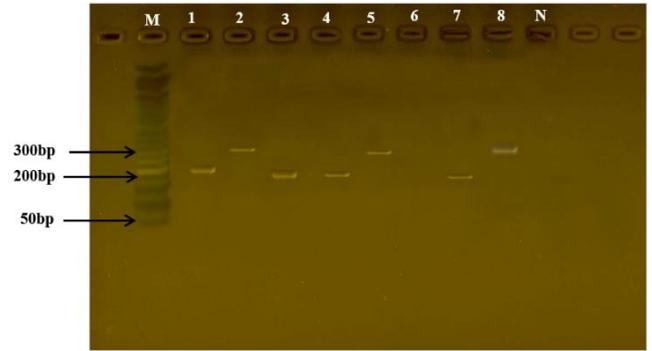


Figure 5: PCR-RFLP product digested with TaqI enzyme. M: DNA marker 50bp. 1,3,4,7 (200bp) *H. lineatum*, 2,5,8 (300bp) *H. bovis*.

### Discussion

The most important species cause hypodermosis in cattle are *H. bovis*, and *H. lineatum* (15). In this study morphological and molecular methods were applied to identify L3 *Hypoderma* species. Our results revealed that morphological identification L3 of both species is considered difficult because of debris and remains of the host cell, blood and puss covering and attaching the larvae make it difficult to recognize the spinulation pattern of the 10<sup>th</sup> abdominal segment even with ethanol preservation; furthermore, extraction of L3 could damage the peritremes which is also another important feature this was in conformity with Balkaya *et al.*, (11) who also added more reasons including: *Hypoderma spp.* share the same host on the back of infested cattle, variation between larvae samples collected from hosts and countries, the absence of recent unified morphological keys and finally dark or brown chitinous color of L3 make it uneasy to diagnose peritremes shapes which is considered as a distinctive feature for identification between species.

Since it was not reliable to identify morphologically between *H. bovis*, and *H. lineatum*, so current study highlighted usefulness and significance of molecular technique for accurate discrimination and confirmation between the two species in cattle especially when similar species parasitize in the same host and also considered a good tool for the study of the biology of the myasis specially in immature stages (5,11,16,17). The molecular identification by the PCR-RFLP and nucleotides sequences of the most variable region of gene CO1 mtDNA presented important data in the identification of *Hypoderma* species in China (17) in East Turkey (11) and in Portugal (18,19). and also used as goal gene in several molecular and phylogenetic researches for L3 (20).

The results of PCR-RFLP assay in this study by TaqI restriction enzyme allowed to differentiate between the species by CO1 amplicons and that the size of amplification

of bands is 200 bp of *H. lineatum* while 300 bp for *H. bovis*. The TaqI restriction enzyme is used to discriminate between *H. bovis* and *H. lineatum* on 438, and 250 bp bands for *H. bovis* and 488 and 200 bp for *H. lineatum* (17) and used *BfaI* and *HinfI* enzymes couldn't digest *H. bovis* and *H. lineatum*. The aim of differentiation between *Hypoderma* species is essential to accurately treat infected animals on proper time; furthermore, these species have different migrating patterns thus it is a major threat in drug administration when L1 of *H. bovis* presence causes paralysis in the hindquarter and L1 of *H. lineatum* causes blot the esophagus (21,22)

## Conclusion

This study concluded the morphological and molecular identification of *Hypoderma bovis* and *Hypoderma lineatum* depending on morphological characterization of L3 and by PCR-RFLP to confirm the existence of both species in Iraq, Mosul. The results of this research revealed that morphological differentiation is not sufficient for differentiation and difficult due to the fact that L3 of both species share the same host and localities in the back of the infested cattle. Using molecular analysis proved the existence of both species and it is very important in treatment because of the different migration patterns of L1 of both species.

## Acknowledgement

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## Conflict of interests

Researchers declare that they have no conflicts of interest regarding the publication of this research.

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يرقات من الحيوانات المصابة بطريقة استخراجها من التورمات الموجودة فيها ومن المستشفى التعليمي البيطري التعليمي ومن قطعان مختلفة في مناطق كوكجلي وبازوايا وكذلك من مجزرة الموصل وخلال الفترة من بداية تشرين الأول ٢٠٢٠ ولغاية نهاية آذار ٢٠٢١. تمت الدراسة الشكلية وباستخدام المجهر التشريحي بالاعتماد على نمط وجود أو عدم وجود الأشواك في القطعة العاشرة وعلى تركيب الصفائح التنفسية حيث تم تصنيف اليرقات الثالثة لدودة تحت الجلد البقرية والشمالية. كما أثبتت نتائج التقنية الجزيئية التقليدية التي طبقت على ١٦ يرقة ثالثة أن ناتج تفاعل التضخيم للجين  $mt CO_1$  كان بحجم ٥٠٠ كيلو دالتون بينما نتائج استخدام تقنية تعدد أطوال جزء الحصر - تفاعل السلسلة المتبلمرة بواسطة الإنزيم القاطع لغرض التفريق بين النوعين والتي أكدت وجود دودة تحت الجلد البقرية ودودة تحت الجلد الشمالية بواقع ناتج تفاعلي ٣٠٠ و ٢٠٠ زوجا قاعديا وعلى التوالي. أثبتت نتائج الدراسة باستخدام التقنية الجزيئية وجود نوعي دودة تحت الجلد في مدينة الموصل.

## التشخيص الشكلي والجزيئي لأنواع طفيلية *Hypoderma spp.* في مدينة الموصل، العراق

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### الخلاصة

داء النغف الجلدي هو من الأمراض الطفيلية الخارجية التي تخمج الأبقار يحدث بسبب دودة تحت الجلد البقرية ودودة تحت الجلد الشمالية وهي من أكثر الأنواع شيوعا لهذا النغف. في هذه الدراسة تم جمع ٧٨