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Original Article

Prevalence and molecular identification of hypodermosis from slaughtered cattle in Sulaymaniyah province, Iraq

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Abstract

Introduction: Hypodermosis is a subcutaneous infestation in cattle that is caused by larvae of *Hypoderma* spp. and it is an economically important disease in the cattle industry. This study aimed to find the prevalence rate of hypodermosis and identify variations in the *COX1* gene among isolates present in Sulaymaniyah, in the Kurdistan region of Iraq.

Methodology: The study was conducted in a Sulaymaniyah slaughterhouse from March to July 2021. The carcasses of 867 cattle were carefully checked before and after skinning them to record the presence of boils containing the larvae of *Hypoderma* spp. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using *TaqI* enzyme, and sequencing of the *COX1* gene were used for diagnosis and molecular characterization of *Hypoderma* spp.

Results: The rate of infestation with *Hypoderma bovis* was 1.61% and the highest rate (3.57%) was detected in April. The disease was significantly ($p < 0.05$) higher in local breeds at 2.79%. PCR-RFLP confirmed that all recorded species were *H. bovis*. The result was further confirmed by Sanger sequencing of the *COX1* gene of the isolated species. Comparison of the sequences of the conserved *COX1* gene of the parasite led to identification of six different haplotypes in the research area. Two of the haplotypes were previously recorded internationally, while four new haplotypes associated with four novel mutations were recorded for the first time in the study region.

Conclusions: Based on these results we can conclude that *H. bovis* is a widespread species in the research region.

Key words: Hypodermosis; cattle; PCR-RFLP; *COX1* gene; Sulaymaniyah; Iraq.

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Introduction

Hypodermosis is a major parasitic disease in cattle, and it is an economic threat to global livestock. The disease is also known as warble fly infestation (WFI) because it is an external parasite that grows mainly in subcutaneous tissues and produces a bump called boil [1,2]. The larvae of *Hypoderma* cause a subcutaneous infection in wild and domestic ruminants. The hosts include different species of ruminants: deer, sheep, goat, buffalo, and cattle. There are seven species of *Hypoderma*. Among them, *Hypoderma bovis* and *Hypoderma lineatum* are the most common species affecting cattle [3].

WFI is an endemic disease in many countries. It has been reported in Africa, Canada, and Europe [1]. Differences in diagnostic methods in between countries have led to different estimations of the prevalence rates of the disease in different countries. The rates of infestation in Belgium, Spain, and Italy were 43%, 79%, and 85%, respectively [4–6]. The disease was also detected in the Middle East a long time ago. The rate of WFI in Iraq and Libya were 23% and 14.1%, respectively [7,8]. WFI is endemic in Asia and the Middle East. In recent studies, 47.3% of cattle in Turkey [9] and 28.6% in east Turkey [10] were infested with *Hypoderma* larvae. In Iraq, 40.3% of the tested cattle were serologically positive for hypodermosis [11]. In Pakistan, 18.4% of the cattle stock was found infested with the larvae of *Hypoderma* spp. [12].

Gross identification of the parasite can recognize the disease, but it cannot identify the species of *Hypoderma*. Molecular biological methods can be used to identify the species. *Cytochrome oxidase I* (*COX1*), is a highly conserved mitochondrial gene and has proven to be useful in identifying species of *Hypoderma* [13]. Polymorphisms in the *COX1* gene sequence may be used to develop a phylogenetic tree of *Hypoderma* species and to identify single nucleotide polymorphisms (SNP) associated with the *Hypoderma* species present in different geographical areas [13].

Recently, the gene was used to develop molecular diagnostic methods for species identification. This included the identification of restriction fragment length polymorphisms (RFLP) by using specific restriction enzymes such as *Taq1* and *Haelll* [14,15].

The prevalence of WFI, including its molecular characterization, is not well studied in the Kurdistan region of Iraq, especially in Sulaymaniyah province. Therefore, this study was aimed to determine the prevalence of WFI from slaughtered cattle in the Sulaymaniyah abattoir. In addition, the study aimed to identify the species of *Hypoderma* by morphological description, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, and nucleotide sequences analysis of a specific region of *COX1* gene.

Methodology

Study area, sampling, and morphological analysis

A total of 867 cattle carcasses of different genders (730 males and 137 females), ages (120 young and 747 adult), and breeds (430 local and 437 imported) were examined from March to July 2021, at the Modern Sulaymaniyah abattoir in the Sulaymaniyah province, Kurdistan Region, north-east Iraq, for the presence of *Hypoderma* spp. larvae in skins and subcutaneous tissues. The province is located between 35°04'–36°30′ latitude and 44°50′–46°16′ longitude. The region is characterized by seasonal rainfall from October to May. The farmers in this region have insufficient knowledge of the ruminants' husbandry and control of ectoparasites.

A total of 14 larvae were collected for morphological identification. They were physiologically saline-washed and preserved in 70% ethanol for the molecular biology analysis. The larvae were measured, identified, and categorized into the stages according to the key developed by Zumpt [16]. Based on Zumpt's key, the peritreme structure was the basis for the morphological identification of the third instar larvae.

Molecular biology analysis

DNA was extracted from all the *Hypoderma* larvae collected using a commercial DNA extraction kit (EasyPure™ Genomic DNA Kit, Trnas Gen Biotech Co., Beijing, China) according to the manufacturer's protocol. The concentration of the pure extracted DNA was measured by using Genova Nano Spectrophotometer (Jenway, Staffordshire, UK).

The region of the mitochondrial *COX1* gene was amplified by PCR using specific primers: UEA7, 5′-TACAGTTGGAATAGACGTTGATAC-3′ and UEA10, 5′-TCCAATGCACTAATCTGCCA TATTA-3′. The proofreading enzyme f-Pfu DNA polymerase was used for the PCR amplification of the gene (SBS Genetech Co., Beijing, China) under the conditions previously described by Otranto *et al.* [17]. The amplicons were verified by gel electrophoresis on 2% agarose gels (TBE, 0.5%) and stained with GoodView™ Nucleic Acid Stain (SBS Genetech Co., Beijing, China).

All PCR products (14 amplicons) were subjected to purification and sequencing using an upstream primer. Purification of extracted DNA fragments was performed from agarose gel by using SiMax™ PCR Products/Agarose Gel Purification Kit (SBS Genetech Co., Beijing, China). All purified DNA were partially sequenced by Sanger sequencing method (Macrogen Inc., Seoul, South Korea).

ClustalW multiple sequence alignment algorithm was used for the purpose of editing and alignment of the gene sequences. Thereafter, all sequences were deposited to the National Center for Biotechnology Information (NCBI) with accession numbers OR267287–OR267292.

The basic local alignment search tool (BLAST) algorithms were used to identify similarities between the *Hypoderma* larvae *COX1* gene sequence from this study and all previously published sequences available in the NCBI database. A phylogenic tree was constructed on the basis of comparison and alignment of the of coding genes in the *H. bovis* mitochondrial genome with the reference sequences of other *Hypoderma* species using the neighbour-joining approach (Table 1). The genetic distances were calculated using Kimura's two parameter model and the robustness of the tree topology was measured by using the bootstrap value of 1,000 replicates of the data sets

Table 1. Sequences of *H. bovis* from NCBI Gene Bank used for phylogenetic comparison.

Accession number	Country	Host	Reference
KT600279; KT600284; KT600292	China	Cattle	Fu *et al.* [18]
OM438133	China	Human	Jian and Han, 2022; unpublished
EU181164	China	Cattle	Guan *et al.,* 2016; unpublished
KF926088	Turkey	Cattle	Kaya and Acici, 2014; unpublished
GU984817; GU984818	Turkey	Cattle	Balkaya *et al.* [10]
MN120664	Iran	Cattle	Gholizadeh *et al.,* 2019; unpublished

Table 2. Monthly occurrence of hypodermosis in slaughtered cattle in Sulaymaniyah province, Iraq.

Month (2021)	No. examined	No. (%) infected	χ^2 (*p* value)
March	182	2 (1.09)	0.30
April	168	6 (3.57)	
May	171	2 (1.16)	
June	167	2 (1.19)	
July	179	2 (1.11)	
Total	867	14 (1.61)	

available. MEGA 11 was used for phylogenetic analyses [19].

In order to differentiate the species of *Hypoderma*, the *COX1* amplicons were digested overnight with restriction endonuclease (*TaqI*-v2) at 65 °C, using buffers recommended by the manufacturer (Biolabs Inc., New England, UK) in a final reaction mix volume of 50 µL, containing 20 µL of PCR product, 10 U enzyme (*TaqI*-v2, 10 U/µL), 5 µL restriction buffer, and 24 µL distilled water. The restriction fragments were separated in 4% agarose gels and stained with GoodView™ Nucleic Acid Stain (SBS Genetech Co., Beijing, China).

Statistical analysis

The data was analyzed using Chi-square (χ^2) test. Values of $p < 0.05$ were considered statistically significant.

Results

Rate of infestation

This study was carried out to indicate the epidemiology of this parasitic infection in the study area due to the importance of hypodermosis in the cattle industry. For this purpose, a total of 867 slaughtered cattle were inspected grossly for the presence of warble fly larvae in the subcutaneous tissue. Based on morphological characters of the third instar larvae of the parasite, 14 cattle (1.61%) were identified to be infested with *H. bovis*.

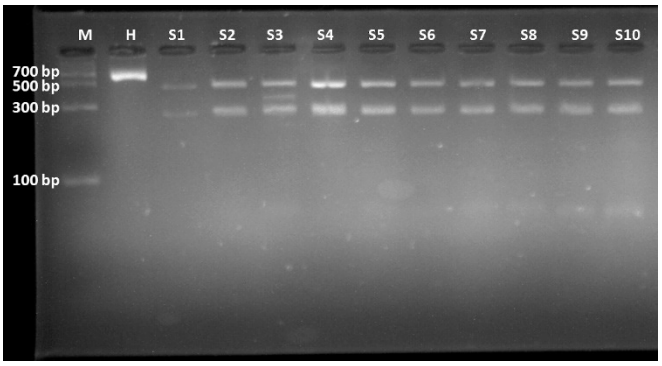
The study was performed in different months to indicate the rate of infection in different periods of time starting from March to July 2021. The highest rate of infestation was recorded in April (3.57%), and the lowest rate was recorded in March (1.09%; Table 2).

Risk factors

To further understand the epidemiology of the infection in the study area, the risk factors related to the disease were investigated. For this purpose, the rate of infection was studied according to the gender, age, and breed (Table 3).

It was found that the breed of cattle had a significant effect on the risk of infection. The rate of infection was significantly higher in local breeds (2.79%) than in imported breeds (0.45%; $p < 0.05$). The rate of infection was non-significantly higher in male cattle (1.78%) than in females (0.72%; $p = 0.37$). In addition, the rate was non-significantly higher in adult cattle (> 1 yrs; 1.74%) than in cattle that were less than one year old (0.83%; $p = 0.47$).

Figure 1. Hypoderma spp. isolates were identified using the PCR-RFLP analysis with TaqI restriction enzyme.



The COX1 gene was PCR amplified and then digested by enzyme TaqI to yield two different patterns of DNA: two bands in the case of H. bovis. M: marker (100 bp); H: amplified PCR product of *Hypoderma* spp. without TaqI enzyme (688 bp); S1-S10: PCR products digested with TaqI enzyme which produced two bands (250 bp, 438 bp) and were identified as *H. bovis*. PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

Table 3. Risk factors for the occurrence of hypodermosis in slaughtered cattle in Sulaymaniyah province, Iraq.

Overall	No. examined	No. (%) infected	χ^2 (*p* value)
	867	14 (1.61)	
Gender			
Male	730	13 (1.78)	0.37
Female	137	1 (0.72)	
Age			
Young (< 1year)	120	1 (0.83)	0.47
Adult (> 1year)	747	13 (1.74)	
Breed			
Local breed	430	12 (2.79)	< 0.05
Imported breed	437	2 (0.45)	

Gene sequence analysis

The PCR-RFLP method was used to identify the *Hypoderma* species. The *COX1* gene was amplified and the 688 bp PCR amplified product was digested by *TaqI* enzyme. The enzyme digestion resulted in DNA fragments of 2-3 sizes which were analyzed to identify the species of *Hypoderma* (*H. bovis* and *H. lineatum*) (Figure 1). In the case of *H. bovis*, the enzyme digestion products were 250 bp and 438 bp.; while the 488 and 200 bp products were characteristic of *H. lineatum* . Surprisingly, all the isolated and recorded species in Sulaymaniyah region were *H. bovis*, and *H. lineatum* was not recorded in our area of study.

The DNA of *H. bovis* isolated from this study were sent for Sangar sequencing.

The PCR amplified *COX1* gene product (688 bp) from the 14 samples were gel purified and sequenced. The analysis and alignment of the sequences showed that *H. bovis* contained six different haplotypes, containing various mutations.

The sequences from the 14 samples were compared with a reference sequence of *H. lineatum COX1* gene NC_013932.1 [20] by multiple sequence alignment to confirm that none of the sequences were *H. lineatum* (data not shown).

ClustalW multiple sequence alignment showed that six different haplotypes of isolated *H. bovis* larvae were obtained (Figure 2). Two of the haplotypes were previously recorded in China and Turkey, and were located in the same clade (clade 43) of the phylogenetic tree (Figure 3); while the other four haplotypes had not been previously identified. Among the new local haplotypes, three contained one novel mutation, and one contained three different mutations (clade 41, 55).

Discussion

WFI is a parasitic disease that is economically important in the cattle industry. Two distinct species of *Hypoderma, H. bovis* and *H. lineatum*, are primarily responsible for WFI. The larvae of this insect live in subcutaneous tissue of cattle and cause destruction of this tissue in the infested area because it lives on living tissue. This parasitic disease is endemic in most countries of the world. The disease is well studied in different countries and even in developed countries [2]; but there had been no previous study in Kurdistan region of Iraq. Therefore, our study aimed to indicate the rate of infection in the area and to characterize the parasite using partial sequencing of *COX1* gene; and this was the first such study in the area.

In the current study, the overall rate of WFI was 1.61%, which was considered a lower infestation rate compared to other studies that were conducted in Baghdad (10.34%) by Mallah and Rahif [21] and in Babylon (22.21%) by Aaiz *et al.* [22]. The cold environment of the study area may be a contributing factor to the low occurrence of WFI; however, it also demonstrates the effectiveness of control measures implemented by the Modern Sulaymaniyah abattoir. The rate of WFI in the present study differs from studies that were based on clinical examination in Pakistan (18.40%), Iran (1.18%), and Turkey (16.90%) [12,23,24]. These differences in prevalence rates may be due to differences in the environmental factors that

Figure 2. Multiple sequence alignments of *H.* bovis in cattle isolates in Sulaymaniyah, Iraq.

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Suli-1 (OR267287) CCTGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
Suli-2 (OR267288) CCCGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
Suli-4 (OR267290) CCTGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--GTTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
KF926088 (turkey) CCTGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
GU984818 (turkey) CCTGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
Suli-3 (OR267289) CCTGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
KT600292  (China) CCTGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
KT600284  (China) CCTGCAACTT--AAATGTTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
Suli-6 (OR267292) CCTGCAACTT--AAATATTAAACTATTAAAAAGCCAATTTATC--ATTATATTTTTAGGGGTAAA--TTATCCCGATGCTT
MN120664   (Iran) CCTGCAACTT--AAATATTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
KT600279  (China) CCTGCAACTT--AAATATTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
OM438133  (China) CCTGCAACTT--AAATATTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
GU984817 (Turkey) CCTGCAACTT--AAATATTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
EU181164  (China) CCTGCAACTT--AAATATTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
Suli-5 (OR267291) CCTGCAACTT--AAATATTAAACTATTAAAAAGCCAATTTGTC--ATTATATTTTTAGGAGTAAA--TTATCCAGATGCTT
                  ** *******--****.**********************.**--.*************.******--******.*******
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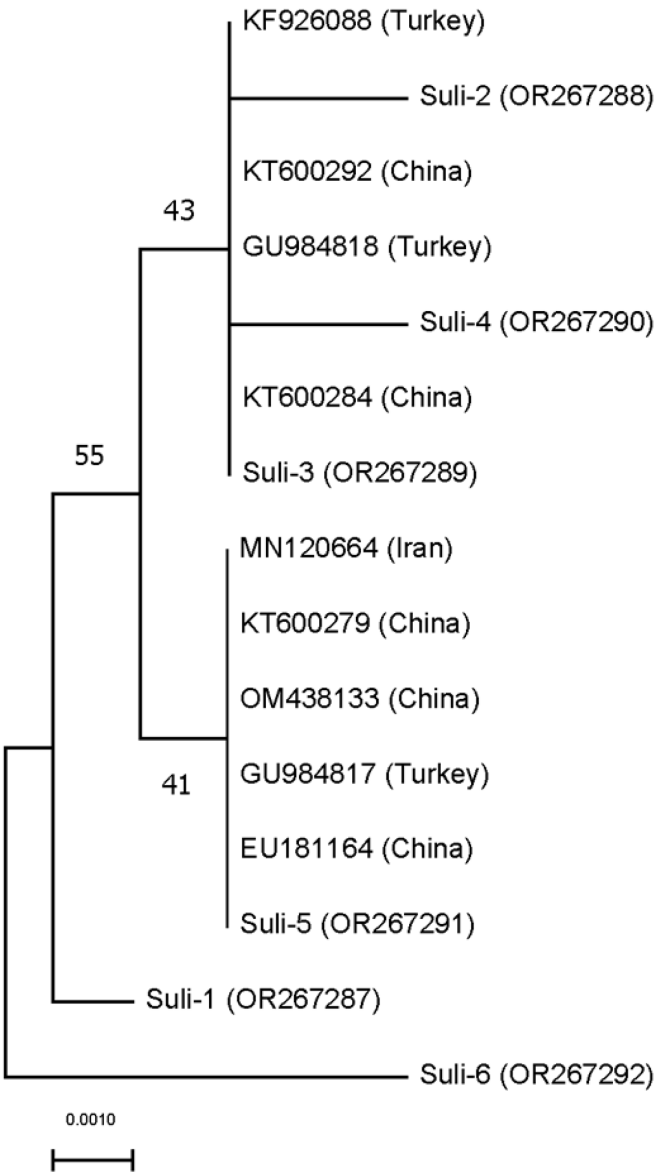
The highlighted yellow and green single nucleotide polymorphisms are mutations that were found in the study area. They had been previously identified in other countries. The sequence alignment was carried out using ClustalW multiple sequence alignment algorithm with the partial mitochondrial COX1 gene sequences. Nucleotide sequences recorded in the current study were labelled as Suli-1(OR267287)–Suli-6(OR267292) with accession nos. OR267287–OR267292.

impact the growth of warble flies, such as topography of the land, seasonal conditions, humidity, temperature, rainfall, and wind velocity [25]. In addition, host specificity, breeds, husbandry, and pesticide usage may reduce occurrence of WFI [26]. In our study, highest rate of infestation was in April and the lowest in March. Contrary to our findings, in another study, the infestation rate was highest in March and lowest in June [22], because of the significant spatial diversity in fly biology.

Figure 3. Phylogenetic tree of *H.* bovis in cattle isolates studied in Sulaymaniyah, Iraq, calculated by neighbor- joining (NJ) method from the partial mitochondrial COX1 gene sequences.



The strains were located in three clades: 41, 43, and 55. The scale bar represents 0.1% difference between nucleotide sequences.

We also analyzed the effect of risk factors including gender, age, and breed. We found that the breed of cattle had the most significant effect on the infestation rate. The rate of infection was significantly higher in local breeds than in imported breeds (2.79% versus 0.45%, $p < 0.05$). This indicates that the disease was endemic to the study area. The rate of infection was higher in male cattle (1.78%) than in female cattle (0.72%). This finding is in accordance with the findings of Khan *et al*. [26] and Yadav *et al*. [27]; however, it differs from the findings of Aaiz *et al.* [22]. Khan *et al.* suggested that the grazing system in the study region and the thicker skin in males may be the reason for higher occurrence of WFI in males than in females [26].

Our results revealed that infection was more common in adult cattle (> 1 year old, 1.74%) than it is in younger cattle (< 1 year old, 0.83%). This finding may be related to management practices, such as the fact that most young animals are kept in pens while adults are allowed to graze, which exposes the adults to more infestation in the field than the housed young animals. This finding agrees with that of Aaiz *et al.* [22] and Taşçi *et al.* [24], but differs from that of Khan *et al.* [26].

Morphological characteristics can be employed for species differential diagnosis. However, due to the difficulty of performing morphological characteristics-based differential diagnosis of third instar larvae, molecular differentiation methods were more effective for species identification. Several investigations have assessed if PCR-RFLP can be used to differentiate between species, and concluded that *TaqI* restriction enzyme analysis can be used to distinguish between *H. bovis* and *H. lineatum* [10,28,29]. In addition, PCR-RFLP analysis can be done using *TaqI* restriction enzyme [29].

Studies have performed molecular identification of *Hypoderma* spp. in China, east Turkey, Portugal, and Iraq by the PCR-RFLP and nucleotide sequences of the most variable regions of the *COX1* and mt-DNA [10,11,30,31]. Additionally, *COX1* was the target gene in a number of molecular and phylogenetic studies of larvae [13].

Otranto *et al.* utilized the *TaqI* restriction enzyme to distinguish between *H. bovis* and *H. lineatum.* They reported that the 438 and 250 bp products were characteristic of *H. bovis*, and the 488 and 200 bp products were characteristic of *H. lineatum* [30]. We used the PCR-RFLP method to identify the *Hypoderma* species: *H. bovis* and *H. lineatum* in samples collected from our study area. The *COX1* gene was amplified by PCR and then digested with the *TaqI* enzyme.

Following enzyme digestion, two DNA fragments are obtained in the case of *H. bovis*, and only one fragment was obtained in the case of *H. lineatum*. All the isolated samples of larvae found in our study area were *H. bovis*. Therefore, we can conclude that *H. bovis* is endemic in Sulaymaniyah region of Iraq. Balkaya *et al*. [10] and Ahmed *et al*. [32] reported similar findings in Turkey; and Otranto *et al.* [14], reported similar observations in China.

Sanger sequencing of the *COX1* gene in our samples was used to further confirm the PCR-RFLP results; and it was confirmed that all the samples collected were *H. bovis*. Next, we used multiple sequence alignment and a phylogenetic tree to look for sequence variations and the presence of single nucleotide polymorphisms. Six distinct haplotypes were identified in the region. Four of the haplotypes included novel mutations, which were found solely in the research area; while the other two were previously recognized and reported in China and Turkey.

In our study, these novel mutations resulted in new haplotypes that were previously not reported from any country [10,18]. However, the sequences from our study had the highest similarity, ranging from 99.34 to 99.78%, with haplotypes isolated from Iran (accession no: MN120664), Turkey (accession nos: GU984817-GU984818, KF926088), and China (accession nos: KT600279, KT600284, KT600292, EU181164, OM438133). These results demonstrate that the samples from different geographic areas may vary genetically. Previous studies have reported that *H. bovis* exhibits substantial genetic diversity and extensive population genetic divergence both within and between populations [18]. Otranto *et al.* reported similar observation, noting that intraspecific polymorphisms were found at six nucleotide locations [17]. The phylogenetic tree revealed that all the isolates of *H. bovis* identified in this study were clustered in different clades (41, 43, and 55), along with isolates from Iran, Turkey, and China.

Conclusions

Hypodermosis was endemic among the cattle in Sulaymaniyah, Iraq. The rate of infestation was low at 1.61% and was caused by only one species, *H. bovis*. Although the disease was detected at different times of the year, the rate was highest in April. PCR-RFLP and Sanger sequencing were used to confirm that all isolated species were *H. bovis*. Six different haplotypes were found in the area. Two of the haplotypes were previously recorded in Turkey and China, and four other haplotypes were specific to our study area. Four novel mutations were found among local haplotypes. PCR-RFLP is preferred over sequencing for species identification. However, due to the high levels of intraspecific genetic diversity and mutations in *Hypoderma* spp., sequencing is the most recommended method because PCR-RFLP can only identify a single base change at a particular restriction site. This study identified novel opportunities for research on *Hypoderma* spp. from various hosts and geographical regions around the world.

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Animal rights and ethics

The authors declare that the samples were taken from animals according to the specifications of the ethical committee of the College of Veterinary Medicine, and the regulations of the slaughterhouse.

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