Molecular Characterization and Chronobiology of Hypodermosis in Cattle Slaughtered in the Diyarbakir Province of Turkey

Diyarbakır İlinde Mezbahada Kesilen Sığırlarda Hypodermosisin Kronobiyolojisi ve Moleküler Karakterizasyonu

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ABSTRACT

Objective: The aim of present study was to investigate the chronobiology and identification of *Hypoderma* species in cattle slaughtered in the Diyarbakir Province of Turkey.
Methods: In total, 736 hides and subcutaneous tissue of slaughtered cattle were examined for the presence of second- and third-instar larvae in the slaughterhouse between November 2012 and May 2013. Third-instar larvae were collected from the slaughterhouses, and gDNA isolates were examined by PCR-RFLP analysis of the cytochrome c oxidase I (COI) gene of mt-DNA using *TaqI* enzyme.
Results: In total, 62 out of 736 cattle (8.42%) were found to be positive for *Hypoderma* larvae. A total of 328 (90 second- and 238 third-instar) *Hypoderma* larvae were detected in the hide and subcutaneous tissue of the back of infested cattle. All the 238 third-instar larvae (100%) were identified as *H. bovis* by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The mean number of *Hypoderma* larvae in each cow was 5.29% (62/736). In the examined cattle, second-instar larvae were observed starting from the second week of January and subcutaneous nodules were found until the last week of April.
Conclusion: *H. bovis* was the dominant species detected in the Diyarbakir Province.
Keywords: *Hypoderma bovis*, chronobiology, PCR-RFLP, cattle, Diyarbakir
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ÖZ

Amaç: Bu çalışmada Diyarbakır ilinde sığır hypodermosisine neden olan türler ve bu türlerin kronobiyolojisini araştırmak hedeflenmiştir.
Yöntemler: Kasım 2012-Mayıs 2013 tarihleri arasında mezbahada kesilen 736 sığırın deri ve subkutan dokuları ikinci ve üçüncü dönem larvaların varlığı yönünden muayene edilmiştir. Mezbahadan toplanan üçüncü dönem larvaların tür teşhislerini koymak için larvalardan elde edilen genomik DNA'larda (gDNA) cytochrome c oxidase I (COI) geni Restriksiyon Parçacık Uzunluk Polimorfizmi (PZR-RFLP) yöntemiyle *TaqI* enzimi kullanılarak incelenmiştir.
Bulgular: İncelenen 736 sığırın 62'sinin (%8,42) *Hypoderma* larvaları ile enfeste olduğu tespit edilmiştir. Enfekte sığırların deri ve sırt bölgesinin subkutan dokularından 328 (90 ikinci, 238 üçüncü dönem larva) adet larva toplanmıştır. Toplanan 238 (90 ikinci, 238 üçüncü dönem larva) adet üçüncü dönem larvanın PZR-RFLP ile incelenmesi sonucunda hepsinin *Hypodema bovis* olduğu belirlenmiştir. Hayvan başına ortalama larva sayısı 5,29 olarak hesaplanmıştır. İncelenen sığırlarda ikinci dönem larvalara Ocak ayının ikinci haftasında rastlanmaya başlanmış ve Nisan'ın son haftasına kadar subkutan nodüller tespit edilmiştir.
Sonuç: Bu çalışma ile Diyarbakır ilinde *Hypoderma bovis*'in yaygın bir tür olduğu moleküler olarak ortaya konulmuştur.
Anahtar kelimeler: *Hypoderma bovis*, kronobiyoloji, PZR-RFLP, Sığır, Diyarbakır
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INTRODUCTION

Bovine hypodermosis is a subcutaneous myiasis caused by the larvae of *Hypoderma bovis* (Linnaeus, 1758) and *H. lineatum* (De Villers, 1789). It is characterized by the presence of subcutaneous warbles in the dorsal and lumbar regions. It is widely distributed in the northern hemisphere of the world. Bovine hypodermosis causes significant losses in the countries' economies because of hide damage as well as a reduction in meat and milk yields (1-3). After mature females attach their eggs on the hair of cattle, larval development on the hosts endures for approximately a year. Following

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an 8–10-month-long travel period in the deep connective tissue of the hosts, the first-instar larvae show about 15 mm growth. These larvae move toward the mid-dorsal area of the host. Then, the larvae molt to the second instar and increase considerably in size. It takes 30–60 days to complete this phase (4). At this stage, characteristic raised skin nodules, called warbles, are formed. The larvae make perforations in the skin of animals, through which the animals can respire. The larvae molt into the third instar, and finally, pre-pupal stage in the warble. The larvae are approximately 30 mm long when mature and the pre-pupa exits the warble by the breathing aperture and falls to the ground. Climatic variations influencing the period of emergence of adult flies from the pupal stage have been reported in different geographical areas of the world (1, 5-7).

Bovine hypodermosis has a wide geographical distribution (8) and is common in 55 countries. The prevalence rate in provinces of Turkey was as follows: 31.9% in Kars (9), 26.3% in Elazig (10), 22.3% in Malatya (10), 38.6% in Sanliurfa (11), 5.3% in Afyonkarahisar (12), 28.6% in Erzurum (13), 3.56% in Thracia (14), 5.08% in Nigde (15), and 35.8% in Van (16).

In recent years, the cytochrome c oxidase I (CO1) gene of the mitochondrial DNA has been used as a target gene for a number of molecular phylogenetic and identification studies including the *Hypoderma* species (17). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and nucleotide sequences of fragments of the mitochondrial CO1 gene have provided valuable information on the identification of *Hypoderma* species (18). The aim of the present study was to investigate the chronobiology and identification of *Hypoderma* species in the Diyarbakır Province.

METHODS

Study period and study area

The study was conducted in the Diyarbakır province, located in south-eastern Anatolia, between November 2012 and May 2013. The province is located at an altitude of 670 m above sea level and its geographical coordinates are 37°55´ N longitude and 40°14´ E latitude. This city is warm and dry in the summer and cool in the winter. Annual average precipitation is 496 mm, average temperature is 15.8 ˚C, and the average humidity is 55% in the Diyarbakır Province.

Hide and subcutaneous tissue examination and larvae collection

A total of 736 hides and subcutaneous tissues on the back of slaughtered cattle were examined for the presence of second- (L_2) and third-instar (L_3) larvae in the slaughterhouse. The larvae found in the subcutaneous tissue and hides were collected, counted, and washed in physiological saline solution. They were fixed in 70% ethanol solution and larval stages were identified according to keys described by Zumpt (1). For this study, approval was obtained with 2011/19 decision number from of Experimental Animals Ethic Committee, University of Dicle on 11.05.2011.

Molecular analysis

Total genomic DNA (gDNA) was extracted from internal tissues of 238 third-instar larvae specimens with a commercial tissue DNA isolation kit (Thermo Fisher Scientific, Waltham, MA). Prior to gDNA isolation, ethanol-fixed larvae were washed five times with PBS and the internal tissues were digested overnight at 56 °C with 180 lysis buffer of the kit into which 20 µl Proteinase-K (20 mg/ml) was added. After digestion, the kit procedure was followed and the gDNA samples were stored at −20 °C until use.

The region of the mitochondrial CO1 gene was amplified by PCR using conserved specific primers previously described by Otranto et al. (18): (UEA7, 5'-TACAGTTGGAATAGACGTTGATAC-3' and UEA10, 5'-TCCAATGCACTAATCTGCCA TATTA-3') in a total volume of 50 µl containing 5 µl 10× PCR buffer, 5 µl 25mM $MgCl_2$, 250 µM each of dNTPs, 20 pmol of each primer, 5 µl of template gDNA, and 1.25 U of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA). The PCR conditions were 2 min at 95°C (initial denaturation), 40 cycles of 1 min at 95°C, 1 min at 52°C, 1 min at 72°C, and finally 7 min at 72°C (final extension). The PCR products were separated on agarose gel (1.5%) and stained with ethidium bromide.

Then, the PCR products were digested overnight with restriction endonuclease at 65°C, using buffers recommended by the manufacturer in a final 36 µl volume reaction mix containing 20 µl of PCR product, 1 µl *TaqI* enzyme (10 U/µl), 4 µl restriction buffer, and 11 µl distilled water. The restriction fragments were separated on 2% agarose gel, stained with ethidium bromide, and photographed. RFLP assays were repeated at least three times until restriction fragments were produced.

The CO1 sequences were automatically obtained using a 377 ABI PRISM system (Applied Biosystems, Foster City, CA.). Nucleotide sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov).

RESULTS

Hide and subcutaneous tissue examination

A total of 8.42% (62/736) examined cattle were infected with the *Hypoderma* larvae. In the examined hides and subcutaneous tissues, the second-instar larvae were observed starting from the second week of January and the subcutaneous nodules were found until the last week of April. A total of 328 larvae of *H. bovi*s were collected from the slaughterhouse. 90 of them were classified as L2 and 238 of them were classified as L3. The highest prevalence of infestation (23%) occurred in March. *H. bovis* larvae were not found in November and December of 2012 and May of 2013. The mean number of larvae per cattle was 5.29 (Table 1).

Molecular findings

In total, 238 gDNA isolates were examined by PCR-RFLP analysis of the CO1 region of mt-DNA using restriction endonuclease (*TaqI*). 668 bp amplicons in the mitochondrial COI gene regions were obtained from all isolates by PCR (Figure 1a). After digestion of the PCR products with the *TaqI* restriction enzyme, 432 and 256 bp bands in 236 samples and 432, 164, and 92 bp bands in two samples were obtained. RFLP patterns of isolates are presented in Figure 1b. All sequences obtained were 99% similar to the *H. bovis* sequence in the GenBank™ (Accession number AF497761). Analysis of the sequences demonstrated that different RFLP bands obtained originated from intraspecific polymorphism.

Table 1. Monthly prevalence and mean larval burden of hide examination in cattle slaughtered in the Diyarbakır Province

Months	Examined animals	Infested animals	Infestation (%)	Number of larvae	Larvae per animal	H. bovis L2 L3
November	84	0	0	0	0	0 0
December	98	0	0	0	0	0 0
January	122	7	5.73	16	2.28	14 2
February	103	13	12.62	153	11.76	56 97
March	113	26	23	104	4	20 84
April	92	16	17.39	55	3.43	0 55
May	124	0	0	0	0	0
Total	736	62	8.42	328	5.29	90 238

L2: The number of second-instar larvae
L3: The number of third-instar larvae

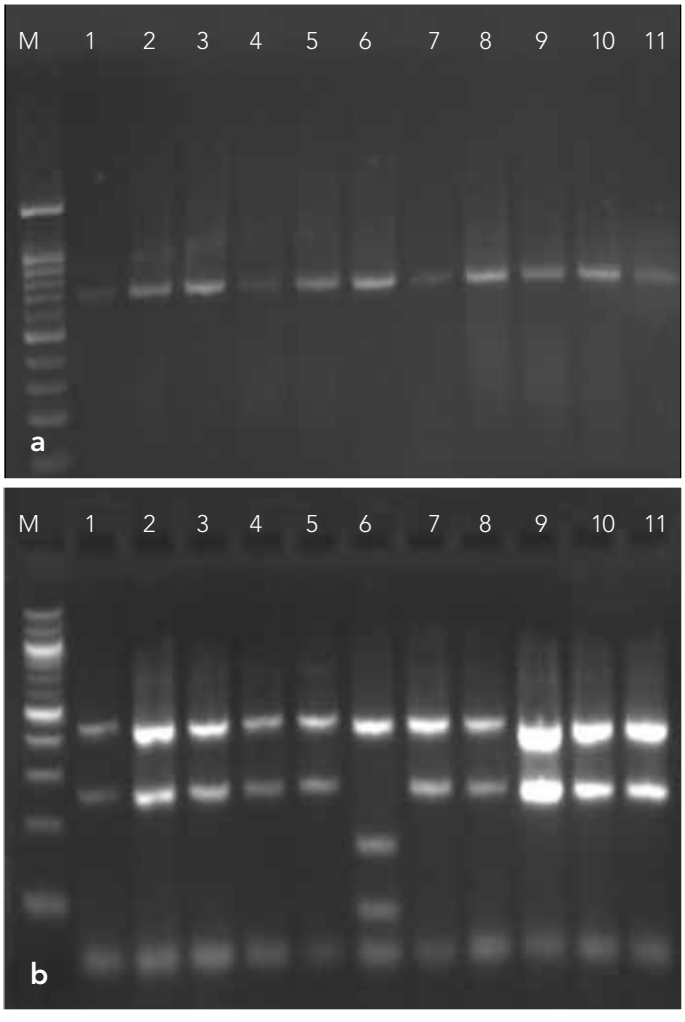

Figure 1. a, b. (a) PCR amplification of mitochondrial CO1 genes of Hypoderma species. M: Marker (100 bp); 1–11: *hypoderma bovis* (688 bp). (b) CO1-PCR products digested with TaqI enzyme. M: DNA marker (100 bp); 1–11: *hypoderma bovis* isolates. (sample 6 revealed different band profiles)

DISCUSSION

Hypodermosis is an important veterinary problem because of its worldwide prevalence and significant economic losses. Economic losses are due to decreasing of productivity potential and leather quality. The disease is caused by two parasitic fly species in cattle, *H. bovis* and *H. lineatum*. In this study, the infestation rate was found to be 8.42% in cattle slaughtered in the abattoirs of Diyarbakir. The prevalence of hypodermosis was lower than the results obtained by the studies conducted in Erzurum 28.6% (13), Kars 31.9% (9), Şanlıurfa 38.6% (11), and Van 35.85% (16). The prevalence of *Hypoderma* spp. larvae (8.42%) in this study was higher than the findings in Thrace (3.56%) (14), Niğde (5.08%) (15), and İstanbul (1.9%) (19).

The biological characteristics of the *Hypoderma* species differ from country to country and from region to region taking into account seasonal conditions. Therefore, to determine the most suitable time for the treatment of cattle with systemic insecticides, it is necessary to put forth the distribution of the dominant fly species and the months when large numbers of first-instar larvae in the esophagus or in the vertebral canal appears (1, 20, 21).

For the differential diagnosis of species, morphological features can be used. However, as the differential diagnosis of the third-instar larvae on the basis of morphological characteristics is difficult to perform, recently, molecular differentiation method seem to be more useful for species identification. Some studies have evaluated whether the differential diagnosis between these two species is possible with PCR-RFLP. These studies reported that the differential diagnosis between *H. bovis* and *H. lineatum* is possible with *TaqI* restriction enzyme analysis (13, 15, 22). We also used this restriction enzyme (*TaqI*) with PCR-RFLP. Otranta et al. (18) reported that, after the digestion of the PCR product with the restriction enzyme *TaqI*, the RFLP profile obtained from *H. bovis* revealed two fragments whereas *H. lineatum* was not digested. Simsek et al. (13) reported 438 and 250 bp bands for *H. bovis* and 488 and 200 bp bands for *H. lineatum* with the same enzyme using PCR-RFLP. Oğuz (22) conducted a similar study and reported that, after the digestion with the same enzyme, they observed the bands 288 bp and 400 bp for *H. lineatum* and the

bands 250 bp and 438 bp for *H. bovis*. In our study, after the digestion of PCR products with the *TaqI* restriction enzyme, we obtained two bands of approx. 432 and 256 bp in 236 samples and three bands containing 432, 164, and 92 bp in two samples. The results of sample sequence analysis showed that all samples revealed two different band profiles and were identified to be *H. bovis*. The analysis of the sequential results also revealed that there are intraspecific differences among the samples, as seen from the different profiles. In two samples, given the three band profiles, T nucleotide was replaced with C nucleotide on the 597 alignment position. The emerging nucleotide difference constitutes a digestion zone for the Taq1 enzyme. It is observed that Taq1 digestion enzyme, which seems to be useable in the differential diagnosis of species, may cause false results because of the intraspecific differences.

Because the animal owners did not give permission for the inspection of the carcass, L1 of *H. bovis* could not be obtained from the cattle that were slaughtered in the abattoir. But it was possible to collect L2 and L3 under the skin of infested animals. Consequently, the obtained findings of L2 and L3 enables the determination of the time they had spent in the cattle. Studies conducted in Europe showed the L2 and L3 of *H. bovis* were seen between December and May in Mediterranean areas, between May and June in Northern and Central parts, and between March and June in the Eastern parts of Europe (6). Karatepe and Karatepe (15) reported that L2 and L3 were seen starting in March and found until April, and the most suitable time for treating cattle with hypodermosis in Nigde province was between 15 October to 15 November. Based on these results, it was understood that *H. bovis* larvae reached L2 in the middle of January and L3 in February. An increase in the number of L2 and L3 was observed under the back skin of the cattle in February. So, it may mean that all the first-instar larvae that were moving in the cattle tissue turn into second-instar larvae between middle October-November, and they completely reached under the skin in middle January-March. Then, all the second-instar larvae that were under the skin would mostly turn into third-instar larvae in February and March. In April, the number of third-instar larvae that were under the skin decreased and in the following months no larvae could be seen in cattle.

CONCLUSION

Determination of the dominant species in the region and the most suitable time for treating cattle is important with regard to the possible eradication or prevention/control programs planned for implementation in the region.

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