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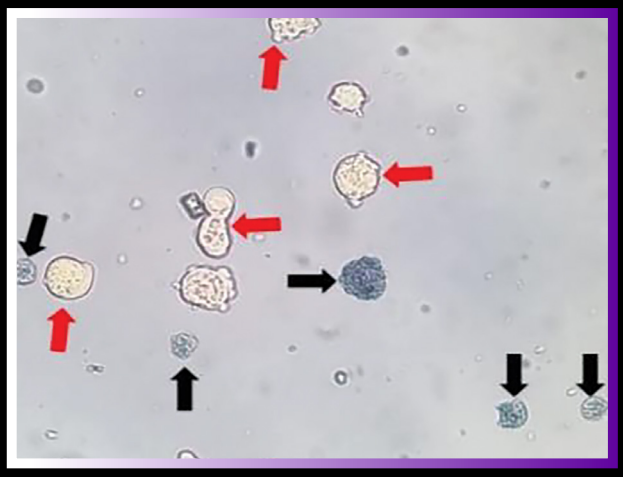
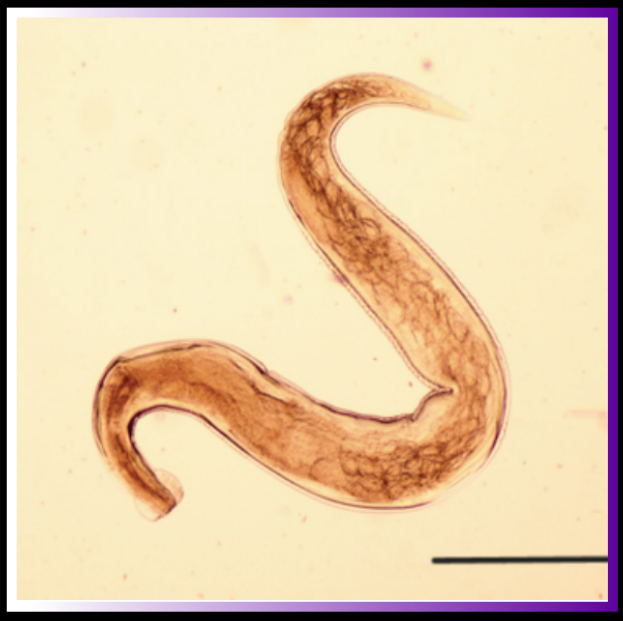
### Original Investigations / Özgün Araştırmalar







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2025 yılının dördüncü sayısını ikisi yurt dışından olmak üzere 7 özgün araştırma makalesi ve 1 olgu sunumu ile çıkarmaktayız. Özgün araştırmalar arasında; mikrobiyoloji kaboratuvarlarında bulunan besiyerlerinin *Leishmania* kültüründeki performansları ile yine *Leishmania*'da ilaç hassasiyetlerinin belirlenmesi için bir dik yöntemi tanımlanmıştır. Yabani kemirgen türlerinde ve laboratuvar farelerinde görülebilen parazitlerin bildirildiği iki çalışmaya yer verilmiştir. *Demodeks* blefaritinin tedavisinde yeni bir deneyim ve Endonezya'da uyuz farkındalığı konusunda bir anket çalışması da bu sayıya alınmıştır. Olgu sunumunda da bir köpekte görülen *Dirofilaria immitis* olgusuna yer verilmiştir.

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**Contact**

**Editorial Office**

**Editor in Chief:** Yusuf Özbel, MD, Prof

**Address:** Ege Üniversitesi Tıp Fakültesi, Parazitoloji Anabilim Dalı, 35100 Bornova-İzmir, Türkiye

**Phone:** +90 232 390 47 24 / +90 232 373 00 08

**Fax:** +90 232 388 13 47

**E-mail:** yusuf.ozbel@ege.edu.tr / yusuf.ozbel@gmail.com

# CONTENTS /İÇİNDEKİLER

## ORIGINAL INVESTIGATIONS / ÖZGÜN ARAŞTIRMALAR



## OLGU SUNUMU / CASE REPORT



## 2025 İNDEKS / 2025 INDEX



Türkiye PARAZİTOLOJİ Dergisi

Turkiye Parazitol Derg 2025;49(4):132-7
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# Evaluation of Alternative Culture Media for Cost-effective and Reliable *in vitro* Cultivation of *Leishmania*

## *Leishmania'nın Maliyeti Düşük ve Güvenilir in vitro Üretimi için Alternatif Kültür Besiyerlerinin Değerlendirilmesi*

Yener Özel[1], İbrahim Çavuş[2], Gülhan Vardar Ünlü[1], Mehmet Ünlü[1], Ahmet Özbilgin[2]

[1]Balıkesir University Faculty of Medicine, Department of Medical Microbiology, Balıkesir, Türkiye
[2]Manisa Celal Bayar University Faculty of Medicine, Department of Medical Parasitology, Manisa, Türkiye

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## ABSTRACT

**Objective:** This study aimed to evaluate the efficiency of commonly available culture media in routine microbiology laboratories for the cultivation of *Leishmania tropica* (*L. tropica*) promastigotes.

**Methods:** Various media including yeast extract agar, tryptic soy broth, sabouraud dextrose agar, brucella agar, and Columbia agar were tested. A total of sixteen media were prepared: eight blood-free (BY1-BY8) and eight supplemented with erythrocyte suspension blood-containing medium (KBY1-KBY8). Each medium was inoculated with *L. tropica* promastigotes at a concentration of $10^5$ promastigotes/mL and incubated for 12 days. Daily promastigote counts were performed to monitor growth.

**Results:** Among the tested media, BY7, BY8, KBY7, and KBY8 showed the most favorable growth patterns. In BY7 and BY8, the promastigote count increased from $10^3$/mL on day 1 to $10^4$/mL by day 5. BY7 supported continuous growth, reaching $10^7$ promastigotes/mL by day 8 and maintaining this level until day 12. BY8 peaked at $10^5$/mL on day 8 but declined to $10^3$/mL by day 12. KBY7 and KBY8 both demonstrated rapid growth, reaching $10^7$/mL by day 8 and sustaining this level through the end of incubation.

**Conclusion:** The presence of Columbia agar in BY7, BY8, KBY7, and KBY8 media significantly enhanced *L. tropica* promastigote proliferation. Due to its low cost, ease of preparation, and availability in routine laboratories, Columbia agar is proposed as a practical and effective alternative to the conventional Novy-McNeal-Nicolle medium for promastigote culture.

**Keywords:** *Leishmania tropica*, Columbia agar, Novy-McNeal-Nicolle, promastigote

## ÖZ

**Amaç:** Bu çalışmada rutin mikrobiyoloji laboratuvarlarında bulunabilecek besiyerlerinin *Leishmania tropica* (*L. tropica* promastigotlarının üretilmesindeki etkinliğinin test edilmesi amaçlanmıştır.

**Yöntemler:** Bu çalışmada rutin mikrobiyoloji laboratuvarında kullanılan yeast extract agar, triptik soy broth, saboroud dextroz agar, brucella agar ve Columbia agar denenmiştir. Sekiz adet kansız (BY1-BY8), sekiz adet kanlı (KBY1-KBY8) besiyeri oluşturulmuştur. *L. tropica* promastigotları 105 promastigot/mL konsantrasyonda tüm besiyerlerine inoküle edilmiş ve 12 gün boyunca inkübe edilmiştir. Besiyerlerindeki promastigot sayısı günlük olarak sayılmıştır.

**Bulgular:** Novy-McNeal-Nicolle (NNN) besiyeri ile karşılaştırıldığında, *L. tropica* promastigotlarının üremesinde en iyi performansı BY7, BY8 ve KBY7 ve KBY8 besiyerleri göstermiştir. BY7 ve BY8 besiyerlerinde 1. gün promastigot sayısı $10^3$ promastigot/mL iken 5. günde $10^4$ promastigot/mL'ye ulaşmıştır. BY7 besiyerinde 8. günde $10^7$ promastigot sayılmış ve 12. güne kadar bu şekilde devam etmiştir. BY8 besiyerinde ise 8. günde $10^5$ promastigot sayılırken sonraki günlerde azalarak 12. günde $10^3$ promastigot/mL'ye düşmüştür. KBY7 ve KBY8 besiyerlerinin her ikisinde de 1. günde promastigot sayısı $10^3$ promastigot/mL iken 8. günde $10^7$ promastigot/mL'ye çıkmış ve 12. güne kadar bu şekilde devam etmiştir.

**Sonuç:** *L. tropica*'nın üretilmesinde en iyi performansı içeriğinde Columbia agar bulunan BY7, BY8 ve KBY7, KBY8 besiyerleri göstermiştir. Columbia agar, ucuz, hazırlanması kolay ve rutin mikrobiyoloji laboratuvarlarında kolayca temin edilebilir olması nedeniyle bu tür uygulamalar için güçlü bir alternatif olarak öne çıkmaktadır. Bu nedenle, promastigotların kültüründe konvansiyonel NNN besiyerine pratik ve etkili bir alternatif olarak önerilebilir.

**Anahtar Kelimeler:** *Leishmania tropica*, Columbia agar, Novy-McNeal-Nicolle, promastigot

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**Address for Correspondence/Yazar Adresi:** Asst. Prof. Yener Özel, Balıkesir University Faculty of Medicine, Department of Medical Microbiology, Balıkesir, Türkiye
**E-mail/E-Posta:** yener_ozel@hotmail.com **ORCID ID:** orcid.org/0000-0001-6618-8251



Türkiye PARAZİTOLOJİ Dergisi

## INTRODUCTION

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the genus *Leishmania*, transmitted to humans through the bite of infected female phlebotomine sandflies. The World Health Organization estimates 700,000 to 1 million new cases annually, with 350 million people at risk (1). Classified as a neglected tropical disease (NTD), it disproportionately affects impoverished populations in developing countries and ranked second among all NTDs in 2017 in terms of disability-adjusted life years (DALYs), contributing to a global burden of 774,000 DALYs (2). The disease presents in three clinical forms: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis, with CL being the most frequently encountered form (3).

A wide range of diagnostic methods is employed for leishmaniasis. Microscopy remains the gold standard, owing to its high specificity. However, its sensitivity can be limited by factors such as insufficient sample volume, low parasite burden, smear preparation errors, and lack of technical expertise among laboratory personnel. In the case of VL, bone marrow biopsies are commonly used, since splenic biopsies—though highly sensitive—pose a significant risk of severe hemorrhage (4,5). For CL, microscopic examination of parasites in skin lesions is a routine method, with reported sensitivity ranging between 42% and 70% (6).

Immunodiagnostic methods that detect *Leishmania* antigens or antibodies still play a fundamental role in diagnosis. Among antibody-based tests, immunochromatographic test (ICT) and enzyme-linked immunosorbent assay are widely used, employing antigens such as crude soluble antigen, recombinant K39 (rK39), and synthetic peptides in both commercial kits and in-house assays. The performance of these tests varies depending on the type and purity of the antigen, with rK39 ICT being a rapid and reliable method. However, the high cost of these tests and their limited applicability in all laboratories constitute a significant limitation in the diagnostic process (7,8).

Molecular methods, such as direct DNA extraction from clinical samples, are also widely employed to detect *Leishmania* infection and determine strain types. However, PCR-based sequencing techniques can struggle to differentiate between closely related species, particularly when parasite DNA levels in the sample are low (9,10). Recently, emerging technologies have started to complement traditional diagnostic methods. Promising innovations include nanoparticles, nanobiosensors, and novel point-of-care platforms such as breath analysis, portable PCR, nanotechnology-assisted rapid tests, next-generation sequencing, immunoinformatics, and artificial intelligence-based diagnostic systems (7).

Although clinical findings contribute to diagnosis, definitive identification of leishmaniasis relies on the direct detection of the parasite in clinical specimens and/or the cultivation of promastigotes using appropriate culture techniques. These methods are considered diagnostic gold standards, making culture media critical to the process. In 1904, Novy, McNeal, and Nicolle successfully cultured the promastigote form in a biphasic medium, which they named Novy-McNeal-Nicolle (NNN). This medium remains in widespread use today (11).

Culturing the parasite not only enhances diagnostic precision but also yields clinical isolates that facilitate advanced genetic studies-such as analyses of parasite evolution, lineage tracing, hybridization events, and identification of drug resistance genes. Expanding the collection of clinical isolates thus represents a valuable resource for *Leishmania* research.

This study aimed to achieve rapid and efficient cultivation of *Leishmania tropica (L. tropica)* promastigotes using culture media that are readily available in routine microbiology laboratories.

## METHODS

### Ethical Approval

No human or animal materials were used in this study. The experiments were conducted using parasite strains preserved in liquid nitrogen. Therefore, ethical approval is not required.

### Parasite Isolate

For the evaluation of the modified media, the *L. tropica* strain MHOM/TR/2012/CBCL-LT, obtained from the Parasitology Bank of the Manisa Celal Bayar University Faculty of Medicine was used.

### Preparation of NNN Medium

1.4 g of agar and 0.6 g of sodium chloride were dissolved in 90 mL of distilled water and sterilized in an autoclave (Hirayama HG-80, Japan) at 121 °C for 15 minutes. After cooling to 50-55 °C, 10 mL of defibrinated rabbit blood and 0.2 mL of penicillin/streptomycin solution were added to the medium. Then, 4 mL of the prepared medium was poured into sterile screw-cap tubes, which were placed at a 10° angle to allow solidification of the medium. The tubes were stored at +4 °C until use.

### Preparation of Roswell Park Memorial Institute-1640 (RPMI-1640) Stock Liquid Medium

Commercially obtained RPMI-1640 was supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% gentamicin to prepare the stock liquid medium. The medium was stored at +4 °C until use.

### Preparation of Modified Culture Medium

To formulate the solid phase of the modified media, culture media commonly available in routine microbiology laboratories were used, including yeast extract agar (113116, Merck), tryptic soy broth (TSB) (100525, Merck), sabouraud dextrose agar (SDA) with 2% glucose (110413, Merck), brucella agar (110490, Merck), and columbia agar (100214, Merck). Each medium was prepared in slant tubes following the manufacturer's instructions.

A total of sixteen media formulations were created:

Eight blood-free modified media (BY1-BY8)

Eight blood-containing modified media (KBY1-KBY8)

Human erythrocyte suspension was added to the blood-containing media. For the liquid phase, either normal saline or RPMI-1640 was used. None of the media contained FCS. To prevent bacterial contamination, 1% penicillin/streptomycin and 1% gentamicin were included in all media formulations.

### Inoculation into Modefied and NNN Medium

The strain retrieved from liquid nitrogen was thawed in a water bath at 37 °C (Memmert WBN-10, Germany) and inoculated into NNN medium for growth. The tubes were then incubated

at 26 °C (Miprolab MSI-120, Türkiye). Upon observation of promastigote proliferation, the culture was transferred to RPMI-1640 liquid medium for further analysis. To obtain a sufficient number of promastigotes, fresh RPMI-1640 medium was added to the culture flasks every 2-3 days. *L. tropica* promastigotes were cultured until the logarithmic growth phase was reached with invert misroscope (Olympus CKX-41, Japan). Subsequently, the parasites were adjusted to a concentration of $10^5$ promastigotes/mL for inoculation into the test media. The modified culture media were incubated at 26 °C for 12 days.

Promastigotes in all tested media were counted daily using a Thoma hemocytometer under 40× magnification with a light microscope (Zeiss Primo Star, Germany). The results were compared with those obtained from the reference NNN medium. All experiments were repeated three times on independent days. All aseptic procedures included in the experimental design of the study were carried out in biosafety level II cabinets (Miprolab, Türkiye) while wearing personal protective equipment. The detailed compositions of the prepared media are provided in Table 1.

### Statistical Analysis

All statistical analyses were performed using Python 3.10 with the pandas, numpy, scipy, and matplotlib libraries. The growth data of *L. tropica* promastigotes were collected over 12 consecutive days for each culture medium. The Shapiro-Wilk test was used to assess the normality of data distribution. As the data did not follow a normal distribution, non-parametric tests were employed. The Kruskal-Wallis test was initially used to assess overall differences among groups. For selected media (BY7, BY8, KBY7, and KBY8), pairwise comparisons with the standard NNN medium were conducted using the Wilcoxon signed-rank test. P-values less than 0.05 were considered statistically significant. Graphical representations of promastigote growth were plotted on a logarithmic scale, and the most successful media were visually emphasized using distinct colors and symbols.

## RESULTS

No promastigote growth was observed in BY1, BY2, KBY1, and KBY2 media, and no viable promastigotes were detected in these media even as early as day 2. While no growth occurred in BY4 medium, in BY3 medium, $10^2$ promastigotes/mL were counted on day 4, and $10^3$ promastigotes/mL on day 5. After day 5, the promastigote count gradually declined, and by day 12, no live promastigotes were observed. In KBY3 and KBY4 media, the initial promastigote concentration of $10^2$/mL on day 1 peaked at $10^5$/mL on day 7, then gradually declined to $10^3$/mL and $10^1$/mL, respectively, by day 12. In BY5 medium, the initial count of $10^2$ promastigotes/mL on day 1 increased to $10^5$/mL by day 6 and remained stable at this level until day 12. In BY6 medium, the promastigote count increased from $10^2$/mL on day 1 to $10^3$/mL on day 2, then progressively declined, with no promastigotes detected by day 9. In KBY5 and KBY6 media, the promastigote count started at $10^3$/mL on day 1, peaked at $10^7$/mL and $10^5$/mL, respectively, by day 8, and then declined to $10^4$/mL (KBY5) and $10^2$/mL (KBY6) by day 12.

In BY7 and BY8 media, the initial count of $10^3$ promastigotes/mL on day 1 increased to $10^4$/mL by day 5. In BY7, the count reached $10^7$/mL by day 8 and remained stable until day 12 (p=0.801). In BY8, $10^5$ promastigotes/mL were observed on day 8, decreasing to $10^3$/mL by day 12 (p=0.798). In both KBY7 and KBY8 media, the promastigote count started at $10^3$/mL on day 1, rose to $10^7$/mL by day 8, and remained stable through day 12 (p=0.987 for both KBY7 and KBY8). In the control NNN medium, the initial promastigote concentration of $10^3$/mL on day 1 increased to $10^7$/mL by day 7 and then declined to $10^5$/mL by day 12. Promastigote counts in blood-containing and blood-free media over time are presented in Table 2 and Table 3, and their graphical representations are shown in Figure 1 and Figure 2.

Statistical comparison was performed using the Wilcoxon paired-samples test. No statistically significant difference was observed in promastigote counts between the modified media and the NNN medium (BY7: p=0.139, BY8: p=0.110, KBY7: p=0.110, KBY8: p=0.110, false discovery rate-adjusted). These results suggest that both blood-free (BY7, BY8) and blood-containing (KBY7, KBY8) formulations exhibited a comparable level of promastigote growth to the standard NNN medium. Furthermore, BY7 and KBY7 demonstrated the highest yield, reaching $10^7$ promastigotes/mL by day 8 and maintaining this level through day 12. Based on these findings, the modified culture media BY7-BY8 and KBY7-KBY8 can be proposed as reliable alternatives to the traditional NNN medium for the *in vitro* cultivation of *L. tropica* promastigotes.

## DISCUSSION

In the diagnosis of leishmaniasis, confirmation can be achieved either by demonstrating the parasite through various staining methods or by culturing promastigote forms. Culturing promastigotes not only supports clinical diagnosis but also enables the acquisition of a large number of parasites for scientific

**Table 1.** Ingredients of the prepared culture media

| Media | Solid phase | Liquid phase |
|---|---|---|
| BY1/KBY1 | Yeast extract agar + sabouraud dextrose agar with 2% glucose + tryptic soy broth | RPMI-1640 |
| BY2/KBY2 | Yeast extract agar + sabouraud dextrose agar with 2% glucose + tryptic soy broth | Normal saline |
| BY3/KBY3 | Sabouraud dextrose agar with 2% glucose | RPMI-1640 |
| BY4/KBY4 | Sabouraud dextrose Agar with 2% glucose | Normal saline |
| BY5/KBY5 | Brucella agar | RPMI-1640 |
| BY6/KBY6 | Brucella agar | Normal saline |
| BY7/KBY7 | Columbia agar | RPMI-1640 |
| BY8/KBY8 | Columbia agar | Normal saline |

BY1-BY8: Blood-free medium, KBY1-KBY8: Blood-containing medium, RPMI-1640: Roswell Park Memorial Institute-1640

**Table 2.** Number of promastigotes grown in blood-free media by day (promastigotes/mL)

| | BY1 | BY2 | BY3 | BY4 | BY5 | BY6 | BY7 | BY8 | NNN |
|---|---|---|---|---|---|---|---|---|---|
| Day 1 | $10^2$ | 0 | $10^2$ | 0 | $10^2$ | $10^2$ | $10^3$ | $10^3$ | $10^3$ |
| Day 2 | $10^1$ | 0 | $10^2$ | 0 | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ |
| Day 3 | 0 | 0 | $10^2$ | 0 | $10^3$ | $10^2$ | $10^3$ | $10^3$ | $10^3$ |
| Day 4 | 0 | 0 | $10^2$ | 0 | $10^4$ | $10^2$ | $10^4$ | $10^4$ | $10^4$ |
| Day 5 | 0 | 0 | $10^3$ | 0 | $10^4$ | $10^1$ | $10^4$ | $10^4$ | $10^4$ |
| Day 6 | 0 | 0 | $10^2$ | 0 | $10^5$ | $10^1$ | $10^5$ | $10^4$ | $10^5$ |
| Day 7 | 0 | 0 | $10^1$ | 0 | $10^5$ | $10^1$ | $10^6$ | $10^4$ | $10^6$ |
| Day 8 | 0 | 0 | $10^1$ | 0 | $10^5$ | $10^1$ | $10^7$ | $10^5$ | $10^7$ |
| Day 9 | 0 | 0 | $10^1$ | 0 | $10^5$ | 0 | $10^7$ | $10^5$ | $10^7$ |
| Day 10 | 0 | 0 | $10^1$ | 0 | $10^5$ | 0 | $10^7$ | $10^4$ | $10^7$ |
| Day 11 | 0 | 0 | 0 | 0 | $10^5$ | 0 | $10^7$ | $10^4$ | $10^6$ |
| Day 12 | 0 | 0 | 0 | 0 | $10^5$ | 0 | $10^7$ | $10^3$ | $10^5$ |

BY: Blood-free medium, NNN: Novy-McNeal-Nicolle medium

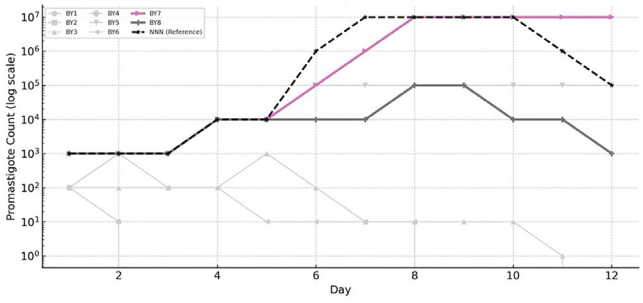


**Figure 1.** Growth curves of *Leishmania tropica* promastigotes in blood-free modified media over 12 days. Promastigote counts (promastigotes/mL) were monitored daily for each blood-free modified medium (BY1-BY8) and compared with the standard NNN medium. BY7 and BY8 demonstrated the highest proliferation, reaching $10^7$ promastigotes/mL by day 8, comparable to NNN medium performance. No growth was observed in BY1, BY2, and BY4 throughout the incubation period. Data represent mean values of three independent experiments

*BY1-BY8: Eight blood-free modified media, NNN: Novy-McNeal-Nicolle*

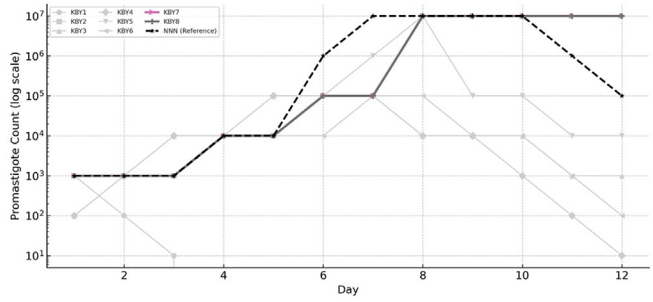


**Figure 2.** Growth curves of *Leishmania tropica* promastigotes in blood-containing modified media over 12 days. Promastigote counts (promastigotes/mL) were monitored daily for each blood-containing modified medium (KBY1-KBY8) and compared with the standard NNN medium. KBY7 and KBY8 media reached $10^7$ promastigotes/mL on day 8 and maintained this level through day 12, demonstrating growth performance comparable to NNN. No growth was observed in KBY1 and KBY2 media throughout the incubation period. Data represent the mean values of three independent experiments

*KBY1-KBY8: Eight blood-containing modified media, NNN: Novy-McNeal-Nicolle*

studies. These cultured parasites are essential for applications such as animal model development, vaccine research, elucidation of disease pathogenesis, evaluation of immune responses, development of diagnostic kits, screening of drug candidates, and assessment of therapeutic efficacy.

Over the years, various culture media have been developed for cultivating *Leishmania* species, primarily categorized as biphasic (semi-solid) and monophasic (liquid) types. Commonly used media, including RPMI-1640, Medium 199, brain heart infusion, NNN, and Schneider's Drosophila medium, often require supplementation with fetal bovine serum (FBS) or blood to support long-term proliferation of *Leishmania* promastigotes (12,13). FBS contains crucial components such as hormones, vitamins, growth factors, and carrier proteins, all of which are vital for the maintenance and propagation of cultured parasites (14).

Among these, the NNN medium remains the most widely used and accepted for both diagnostic and research purposes. While it is relatively easy to prepare, its reliance on rabbit blood and costly FBS limits its accessibility in routine microbiology laboratories. Therefore, recent years have seen a growing interest in developing cost-effective, serum-free, and autoclavable alternative media (15,16).

The use of easily accessible and cost-efficient media in routine microbiology laboratories can significantly improve the detection of rare leishmaniasis cases, especially in non-endemic regions. In this context, various studies in Türkiye have investigated the potential of alternative biphasic and liquid culture media. In 1997, Limoncu et al. (17) compared a liquid P-Y medium supplemented with 10% FBS (containing peptone and yeast extract) with NNN and RPMI-1640+10% FBS media, reporting comparable results. Similarly, Ozbilgin et al. (18) observed promastigotes in nutrient

**Table 3.** Number of promastigotes grown in blood-containing media by day (promastigotes/mL)

| | KBY1 | KBY2 | KBY3 | KBY4 | KBY5 | KBY6 | KBY7 | KBY8 | NNN |
|---|---|---|---|---|---|---|---|---|---|
| Day 1 | $10^3$ | 0 | $10^2$ | $10^2$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ |
| Day 2 | $10^2$ | 0 | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ |
| Day 3 | $10^1$ | 0 | $10^4$ | $10^4$ | $10^3$ | $10^3$ | $10^4$ | $10^4$ | $10^3$ |
| Day 4 | 0 | 0 | $10^4$ | $10^4$ | $10^4$ | $10^4$ | $10^4$ | $10^4$ | $10^4$ |
| Day 5 | 0 | 0 | $10^5$ | $10^5$ | $10^4$ | $10^4$ | $10^5$ | $10^5$ | $10^4$ |
| Day 6 | 0 | 0 | $10^5$ | $10^5$ | $10^5$ | $10^4$ | $10^5$ | $10^5$ | $10^5$ |
| Day 7 | 0 | 0 | $10^5$ | $10^5$ | $10^6$ | $10^5$ | $10^6$ | $10^6$ | $10^6$ |
| Day 8 | 0 | 0 | $10^4$ | $10^4$ | $10^7$ | $10^5$ | $10^7$ | $10^7$ | $10^7$ |
| Day 9 | 0 | 0 | $10^4$ | $10^4$ | $10^5$ | $10^4$ | $10^7$ | $10^7$ | $10^7$ |
| Day 10 | 0 | 0 | $10^4$ | $10^3$ | $10^5$ | $10^4$ | $10^7$ | $10^7$ | $10^7$ |
| Day 11 | 0 | 0 | $10^3$ | $10^2$ | $10^4$ | $10^3$ | $10^7$ | $10^7$ | $10^6$ |
| Day 12 | 0 | 0 | $10^3$ | $10^1$ | $10^4$ | $10^2$ | $10^7$ | $10^7$ | $10^5$ |

KBY: Blood-containing medium, NNN: Novy-McNeal-Nicolle medium

broth medium containing 20% FBS as early as day 1, whereas detection in NNN medium began on day 2. Yereli et al. (19) also successfully cultivated *L. infantum* and *L. tropica* promastigotes in nutrient broth enriched with 10% FBS, reaching $3\times10^6$ parasites/mL by day 5. However, the use of FBS in these media diminishes their cost advantage.

Internationally, several research groups have proposed alternative methods as well. Ali et al. (20) developed an egg-based biphasic medium free of FBS and blood, showing comparable proliferation to modified Tobie + FBS and Medium 199 + FBS. Kaddu and Nyamori (21) successfully cultured *L. donovani, L. major, L. adleri*, and a rodent *Leishmania* species in nutrient broth. Sadigursky and Brodskyn (22) reported similar promastigote proliferation in a serum-free liver infusion broth + tryptose (LIT) medium supplemented with 1% RPMI + Medium 199 (R9), compared to NNN, Warren medium, and LIT + 5% FBS. As also emphasized in a recent review, a major challenge in *Leishmania* culture remains the absence of a standardized, inexpensive, easy-to-prepare, and high-performance medium suitable for drug sensitivity testing (23).

In this study, the selected culture media are widely used in routine microbiology laboratories and serve different purposes in microbial cultivation. Yeast extract agar provides essential vitamins and nitrogen sources for the growth of various bacteria, yeasts, and molds. TSB supports the growth of both aerobic and anaerobic bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Bacteroides fragilis*. SDA, characterized by its high glucose content and slightly acidic pH, is ideal for the isolation of pathogenic fungi like *Candida albicans*. Brucella agar promotes the cultivation of fastidious and anaerobic microorganisms. Columbia agar, enriched with casein, heart, and meat peptones, provides a rich base for the growth of demanding bacteria such as *Streptococcus* and *Haemophilus* species (24).

In the our study, we evaluated the potential of routinely available microbiological media for the cultivation of *L. tropica* promastigotes. Compared to the NNN medium, the fastest and highest levels of proliferation were observed in Columbia agar-based BY7/BY8 and their blood-containing variants KBY7/KBY8. BY7 and BY8 media, which do not contain FBS or blood, achieved statistically comparable growth levels to NNN medium (p=0.139 for BY7, p=0.110 for BY8). This renders them advantageous in terms of cost and ease of preparation. Similarly, KBY7 and KBY8 media demonstrated comparable performance (p=0.110 for both). The use of human erythrocytes, which can be obtained from blood banks, instead of animal blood, adds practical utility. Additionally, promastigotes cultured in these media exhibited greater motility and typical fusiform morphology, suggesting preservation of their infective potential.

The enhanced growth observed in Columbia agar-based media (BY7, BY8, KBY7, KBY8) may be attributed to the balanced nutrient composition and favorable oxygen diffusion of the biphasic structure. Peptones, yeast extract, and maize starch provide essential amino acids, vitamins, and energy sources that support continuous promastigote metabolism, while the semi-solid matrix ensures optimal gas exchange similar to the sand fly midgut environment. In blood-containing variants, erythrocytes may further contribute to redox balance and iron availability, promoting sustained proliferation comparable to the NNN medium.

These culture media, which are easy and inexpensive to prepare and support high promastigote production, present a promising potential for anti-leishmanial drug screening when redesigned with viability-indicating colorimetric markers and supplemented with defined drug dilutions. Furthermore, the integration of such media into future automated analysis systems could facilitate the standardization of drug efficacy testing and enable the development of high-throughput screening platforms.

### Study Limitations

The most notable limitation of this study is the lack of evaluation of parasite growth directly from clinical specimens in the high-performing media. Future studies are planned to assess the diagnostic potential of these media using both animal models and clinical samples.

## CONCLUSION

Columbia agar (base) offers a highly supportive growth environment due to its rich composition of peptones derived

from casein, meat, and heart tissue, along with yeast extract and maize starch. Yeast extract serves as a vitamin source, while maize starch contributes as an energy source. These nutritional components likely contributed to the enhanced proliferation of *L. tropica* promastigotes observed in the BY7, BY8, KBY7, and KBY8 media. Columbia agar stands out as a strong alternative for such applications, as it is inexpensive, easy to prepare, and readily available in routine microbiology laboratories. Therefore, it can be recommended as a practical and effective substitute for conventional NNN medium in promastigote cultivation.

### *Ethics

**Ethics Committee Approval:** No human or animal materials were used in this study. The experiments were conducted using parasite strains preserved in liquid nitrogen. Therefore, ethical approval is not required.

**Informed Consent:** No human or animal materials were used in this study.

### Footnotes

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### *Authorship Contributions

Concept: Y.Ö., Design: Y.Ö., İ.Ç., G.V.Ü., M.Ü., A.Ö., Data Collection or Processing: Y.Ö., İ.Ç., G.V.Ü., M.Ü., A.Ö., Analysis or Interpretation: Y.Ö., İ.Ç., G.V.Ü., M.Ü., A.Ö., Literature Search: Y.Ö., İ.Ç., G.V.Ü., M.Ü., A.Ö., Writing: Y.Ö., İ.Ç.

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# An Innovative Method for Determining Amphotericin B Susceptibility in *Leishmania infantum* Isolates: A Modified Disk Elution Approach

## *Leishmania infantum İzolatlarında Amfoterisin B Duyarlılığının Belirlenmesinde Yenilikçi Bir Yöntem: Modifiye Disk Elüsyon Yaklaşımı*

Yener Özel[1], İbrahim Çavuş[2], Ahmet Özbilgin[2]

[1]Balıkesir University Faculty of Medicine, Department of Medical Microbiology, Balıkesir, Türkiye
[2]Manisa Celal Bayar University Faculty of Medicine, Department of Medical Parasitology, Manisa, Türkiye

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## ABSTRACT

**Objective:** Due to the potential development of resistance to amphotericin B (AmpB), a widely used drug in leishmaniasis treatment, monitoring drug susceptibility has become increasingly important. This study aimed to evaluate the applicability of a modified version of the disk elution method—originally developed for detecting colistin resistance in bacteria—for the first time in determining AmpB susceptibility in *Leishmania infantum* (*L. infantum)* isolates.

**Methods:** The minimum parasiticidal concentration (MPC) of AmpB against *L. infantum* was determined using the broth microdilution method. Additionally, the disk elution method was modified for use with *Leishmania*. Disks impregnated with AmpB were placed into indicator-containing culture tubes, and parasite viability was visually assessed based on a color shift from purple to yellow. The MPC was recorded as the lowest concentration at which complete parasite death occurred.

**Results:** In both methods, AmpB exhibited complete parasiticidal activity at concentrations of ≥0.5 µg/mL. Statistical comparison using the Mann-Whitney U test revealed no significant difference between the two methods at 48 and 72 hours ($p>0.05$).

**Conclusion:** The findings indicate that the modified disk elution method provides comparable reliability to the standard broth microdilution technique. Its low cost, ease of implementation, and visual interpretability make it a promising alternative for drug susceptibility testing, especially in resource-limited laboratories or field settings. Moreover, the use of commercially prepared AmpB disks could facilitate standardization and broader adoption. This study introduces an innovative approach that may simplify routine drug susceptibility screening in Leishmania isolates and support wider surveillance of anti-leishmanial resistance.

**Keywords:** Modified disk elution method, *Leishmania infantum*, amphotericin B, leishmaniasis

## ÖZ

**Amaç:** Leishmaniasis tedavisinde yaygın olarak kullanılan amfoterisin B'ye (AmpB) karşı direnç gelişim potansiyeli, ilaç duyarlılığının izlenmesini gerekli kılmaktadır. Ancak *Leishmania* suşlarında ilaç duyarlılığını değerlendiren mevcut yöntemler yüksek maliyet, teknik zorluklar ve cihaz bağımlılığı gibi sınırlılıklar taşımaktadır. Bu çalışmada, mikrobiyoloji alanında son yıllarda önem kazanan ve özellikle dirençli bakterilerdeki kolistin direncinin doğru ve pratik şekilde belirlenmesini sağlayan disk elüsyon yönteminin modifiye edilerek, ilk kez *Leishmania infantum* (*L. infantum)* izolatına karşı AmpB'nin ilaç duyarlılığının belirlenmesinde denenmesi amaçlanmıştır.

**Yöntemler:** AmpB'nin *L. infantum* suşuna karşı etkinliğini ifade eden minimum parazitisit konsantastrasyon (MPK) değeri sıvı mikrodilüsyon yöntemi ile belirlenmiştir. Disk elüsyon yöntemi *Leishmania* izolatına karşı AmpB'nin etkinliği saptamak için modifiye edilmiştir. AmpB emdirilmiş diskler indikatör içeren besiyerlerine eklenmiş ve parazit canlılığı renk değişimi (mor→sarı) ile görsel olarak değerlendirilerek MPK değeri belirlenmiştir.

**Bulgular:** Her iki yöntemde de AmpB, 0,5 µg/mL ve üzerindeki konsantrasyonlarda tüm parazitleri öldürmüştür. Mann-Whitney U testi ile yapılan istatistiksel analizde 48. ve 72. saat sonuçları arasında anlamlı fark saptanmamıştır ($p>0.05$).

**Sonuç:** Çalışmanın sonuçları, modifiye disk elüsyon yönteminin klasik mikrodilüsyonla karşılaştırılabilir düzeyde güvenilir olduğunu ortaya koymuştur. Yöntemin düşük maliyetli, kolay uygulanabilir ve görsel olarak yorumlanabilir oluşu, alt yapısı sınırlı

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**Address for Correspondence/Yazar Adresi:** Asst. Prof. Yener Özel, Balıkesir University Faculty of Medicine, Department of Medical Microbiology, Balıkesir, Türkiye
**E-mail/E-Posta:** yener_ozel@hotmail.com **ORCID ID:** orcid.org/0000-0001-6618-8251



Türkiye PARAZİTOLOJİ Dergisi

laboratuvarlarda veya saha koşullarında ilaç duyarlılık testleri için alternatif bir seçenek sunmaktadır. Ayrıca, ticari olarak önceden hazırlanmış AmpB diskleri ile yöntemin standardizasyonu sağlanarak yaygın kullanımı mümkün olabilir. Bu yönüyle çalışmamız, *Leishmania* türlerinde rutin ilaç duyarlılık taramalarının basitleştirilmesine katkı sağlayacak yenilikçi bir yaklaşım sunmaktadır.
**Anahtar Kelimeler:** Modifiye disk elüsyon yöntemi, *Leishmania infantum*, amfoterisin B, layşmanyaz

## INTRODUCTION

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the genus *Leishmania*. It presents in three main clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). CL is the most common form, with an estimated annual incidence of 600,000 to 1 million new cases (1). Typically, each *Leishmania* species is associated with a specific clinical manifestation. Old world CL is caused by *Leishmania tropica* or *Leishmania major*, while VL is attributed to *Leishmania donovani (L. donovani)* and *Leishmania infantum (L. infantum)* (2).

Chemotherapy remains the cornerstone of leishmaniasis management and control. The choice of treatment depends on various factors, including the form of the disease, comorbid conditions, parasite species, and geographic location. Pentavalent antimonials (SbV), amphotericin B (AmpB), paromomycin, miltefosine, pentamidine, and sitamaquine are the drugs currently used in treatment regimens (3).

Drug resistance, high treatment costs, and reduced drug efficacy are significant public health concerns of modern times, leading to serious economic losses associated with treatment failures and relapses, increased disease transmission risk, and prolonged hospital stays. This issue also poses a substantial social burden, particularly in low-resource settings of developing countries, where limited access to treatment options contributes to increased morbidity and mortality, and negatively affects the quality of life of patients and their families (4).

In many regions, antimonials remain the primary drugs used for the treatment of leishmaniasis. However, antimony resistance—particularly in the Indian subcontinent—has necessitated the use of alternative therapies. Currently, parenteral paromomycin, AmpB, and oral miltefosine are widely used. AmpB is highly effective; however, when administered in its free deoxycholate form, it is associated with toxicities such as fever, nausea, vomiting, chills, hypertension or hypotension, and hypoxia. These adverse effects have been largely mitigated with the development of its liposomal formulation (5,6).

One of the major challenges in determining drug resistance in *Leishmania* species is the lack of a universally accepted standard testing method. This hinders comparability between studies, weakens the correlation with clinical outcomes, and leads to inconsistencies in identifying resistant isolates (7). Significant limitations in the evaluation of anti-leishmanial drug resistance often arise from methodological variability, including differences in the parasite stage used, incubation duration, and analytical techniques (8).

One of the most commonly used methods in drug susceptibility studies of *Leishmania* species is the microdilution assay. This method is preferred primarily because it allows for the quantitative measurement of parasite viability or metabolic activity under *in vitro* conditions. Various *in vitro* assays are employed to evaluate *Leishmania* susceptibility; viability assays based on microdilution, such as MTT, resazurin, or CellTiter-Glo tests, have been widely adopted (9).

However, many laboratories lack the infrastructure required to perform drug susceptibility testing on isolated *Leishmania* strains. This process is costly, labor-intensive, and demands experienced personnel. Currently, drug resistance in leishmaniasis cases is often inferred based on treatment failure or relapse. Nevertheless, it is also essential to evaluate drug resistance directly on parasite strains. In this study, a modified version of the disk elution method—recently gaining attention in the field of microbiology for its accurate and practical detection of colistin resistance in resistant bacteria—was applied, for the first time, to assess the susceptibility of *(L. infantum)* isolates to AmpB.

## METHODS

### Ethical Approval

This study did not involve the use of any human or animal materials. All experiments were conducted using parasite strains preserved in liquid nitrogen. Therefore, ethical approval was not required.

### *Leishmania* Strain

In this study, the *L. infantum* reference strain (MHOM/TN/1980/IPT-1) was obtained from the Parasite Bank of the Faculty of Medicine at Manisa Celal Bayar University. The strain obtained from the parasite bank was genotyped using primers and probes specific to the *ITS1* and *hsp70* gene regions.

### Preparation of Novy-McNeal-Nicolle (NNN) Medium

A mixture was prepared by dissolving 1.4 g of agar and 0.6 g of sodium chloride in a total volume of 90 mL of distilled water. The mixture was sterilized by autoclaving at 121 °C for 15 minutes. After sterilization, the medium was cooled to 50-55 °C, followed by the addition of 10 mL defibrinated rabbit blood and 0.2 mL penicillin/streptomycin solution. The resulting mixture was dispensed into sterile screw-cap tubes at 4 mL per tube and placed at an approximate 10° angle to allow the agar phase to solidify in a slanted position. The prepared media were stored at +4 °C until use (10).

### Preparation of Roswell Park Memorial Institute-1640 (RPMI-1640) Stock Liquid Medium

RPMI-1640 medium was obtained from commercial sources and supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% gentamicin to prepare the stock liquid medium. The prepared medium was stored at +4 °C until use (10).

### Inoculation of *Leishmania* Strain into NNN Medium

Prior to use, NNN media were brought to room temperature, and 1 mL of RPMI-1640 stock liquid medium was added to each tube. *L. infantum* promastigotes removed from liquid nitrogen were thawed in a water bath and transferred into the NNN media

using a sterile glass Pasteur pipette. The inoculated media were incubated at 26 °C and maintained in an incubator for 5 days. At the end of the incubation period, samples were taken from the liquid phase of the medium, and smears were prepared directly on microscope slides. Promastigote proliferation and density were evaluated under a light microscope using a 40x objective lens.

### Propagation of *Leishmania* Strain in Liquid Medium

Promastigotes that had proliferated sufficiently in NNN medium were passaged into RPMI-1640 stock liquid medium for use in drug susceptibility tests and incubated at 26 °C. To achieve optimal cell density, fresh medium was added to the flasks containing RPMI-1640 with promastigotes every 2-3 days. In the final step, the parasite suspension was adjusted to a concentration of $10^7$ promastigotes/mL for use in drug testing.

### Preparation of AmpB Stock Solution

The pure and analytical form of AmpB (B22TS01051) was obtained from BOC Sciences (United States of America). The appropriate solvent/diluent type and potency of AmpB were determined, and stock concentrations were calculated in accordance with European Committee on Antimicrobial Susceptibility Testing guidelines. A stock solution of AmpB at a concentration of 1000 µg/mL was prepared by weighing the compound on an analytical balance. The prepared stock solution was aliquoted into 1 mL volumes and stored at -20 °C until testing.

### Broth Microdilution Method

The anti-leishmanial activity of AmpB was determined *in vitro* using the broth microdilution method (11). For the drug susceptibility tests, a sterile, flat-bottomed 96-well cell culture plate was used. Each well designated for AmpB testing was filled with 100 µL of RPMI-1640 stock liquid medium (supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% gentamicin). In the first well, 100 µL of AmpB stock solution was added and subjected to serial dilutions. After completing the serial dilutions, 100 µL of the parasite suspension, adjusted to a concentration of $10^7$ promastigotes/mL, was added to all wells except the negative control. The plate was sealed with its lid and wrapped with parafilm, then incubated at 26 °C. At 48 and 72 hours of incubation, the minimum parasiticidal concentration (MPC) values of the compound were determined by examining the wells under an inverted microscope. Wells in which promastigote motility was completely absent and morphological integrity was lost were considered dead. To confirm parasite viability, samples from wells considered dead were re-inoculated into NNN medium. The lowest drug concentration that completely eliminated all parasites in the wells was defined as the MPC.

### Modified Disk Elution Method

The disk elution method described by Simner et al. (12) was modified for use against the *L. infantum* strain. For the procedure, 5 mL of RPMI-1640 stock liquid medium was dispensed into each of nine sterile, screw-cap glass tubes. To enable visual assessment of parasite viability and determination of the MPC, 500 µL of bromothymol blue was added to each tube as a viability indicator. As the number and viability of parasites increase, the color of the bromothymol blue-containing medium changes from purple to yellow. In a sterile U-bottom 96-well microplate, 100 µL of RPMI-1640 medium (without indicator) was added to eight wells in a single row. To the first well of the row, 100 µL of AmpB stock solution (1000 µg/mL) was added, and a serial dilution was performed to prepare drug concentrations ranging from 500 to 3.9 µg/mL. From these diluted solutions, 20 µL was carefully absorbed onto sterile 6 mm diameter blank paper disks (each disk has a maximum absorption capacity of 20 µL). As a result, the AmpB concentrations in the seven disks ranged from 10 to 0.15 µg/mL. These disks were then numbered from 1 to 7 and transferred into tubes containing 5 mL of indicator-supplemented RPMI-1640 medium. The tubes were left at room temperature for 30 minutes, during which the final AmpB concentrations ranged from 2 to 0.031 µg/mL. Additionally, two more tubes containing the indicator-supplemented medium were prepared for use as positive and negative controls. The positive control contained medium plus promastigotes, while the negative control contained only medium. From the parasite suspension adjusted to $10^7$ promastigotes/mL in logarithmic phase, 100 µL was added to all tubes except the negative control. Tubes were capped and incubated at 26 °C for 48 and 72 hours. At the end of the incubation period, parasite viability in each tube was assessed by comparing color changes to the positive and negative controls. The lowest drug concentration that resulted in no color change and complete parasite death was considered the MPC. To verify parasite viability, microscope slides were prepared from each tube and examined under a light microscope. Tubes with no observed color change and confirmed parasite death were further subcultured onto NNN medium for confirmation. The preparation algorithm of the disk elution method is presented in detail in Figure 1.

### Statistical Analysis

In this study, the cell viability data obtained from both the broth microdilution and disk elution methods were analyzed to compare the anti-leishmanial efficacy of AmpB against *L. infantum*. "Live" and "dead" outcomes were scored as 100% and 0%, respectively, and the mean viability percentages were calculated for each drug concentration and time point. Since the data did not meet the assumptions of parametric tests, the non-parametric Mann-Whitney U test was used to compare the two independent groups. Statistical analyses were performed using SPSS version 25.0. A p-value of less than 0.05 was considered statistically significant for all tests.

## RESULTS

### Anti-leishmanial Activity of AmpB by the Broth Microdilution Method

The anti-leishmanial activity of AmpB, as evaluated by the broth microdilution method, was assessed comparatively at 48 and 72 hours across different concentration levels. According to the results, complete promastigote death was observed at both time points in all samples treated with 2 µg/mL, 1 µg/mL, and 0.5 µg/mL concentrations of AmpB. These findings indicate that AmpB exhibits marked anti-leishmanial activity at higher concentrations. At a concentration of 0.25 µg/mL, all promastigotes remained viable at 48 hours, whereas complete parasite death was observed at 72 hours. This suggests a time-dependent increase in AmpB efficacy at this concentration. At lower concentrations—0.125 µg/mL, 0.0625 µg/mL, and 0.03125 µg/mL—promastigotes maintained viability at both 48 and 72 hours. No promastigote growth was observed in NNN media subcultured from wells

considered non-viable, confirming the accuracy of viability assessments. These results demonstrate that AmpB exhibits a dose-dependent effect under *in vitro* conditions and exerts rapid and potent anti-leishmanial activity at concentrations above 0.5 µg/mL (Table 1 and Figure 2).

## Anti-leishmanial Activity of AmpB by the Modified Disk Elution Method

The anti-leishmanial activity of AmpB assessed via the disk elution method was evaluated based on the observed viability of promastigotes at 48 and 72 hours. According to the findings, complete parasite death was observed at both time points in all samples treated with 2 µg/mL, 1 µg/mL, and 0.5 µg/mL concentrations. No promastigote growth was detected in NNN media subcultured from tubes considered non-viable, confirming the results. This indicates that AmpB exhibits strong leishmanicidal activity at higher concentrations. In contrast, at 0.25 µg/mL and lower concentrations (0.125, 0.0625, and 0.03125 µg/mL), all parasites remained viable at both 48 and 72 hours. These findings suggest that AmpB is ineffective below 0.25 µg/mL in this method, and the threshold for activity lies above this concentration. Overall, AmpB showed similar efficacy in the disk elution method as in the liquid microdilution method at higher concentrations; however, no time-dependent effect was observed at lower concentrations (Table 2 and Figure 3).

When comparing the cell viability data obtained from the broth microdilution and disk elution methods, both techniques yielded similar results in evaluating the anti-leishmanial activity of AmpB. "Live" and "dead" outcomes were scored as 100% and 0%, respectively, and mean viability rates were calculated for each concentration and time point.

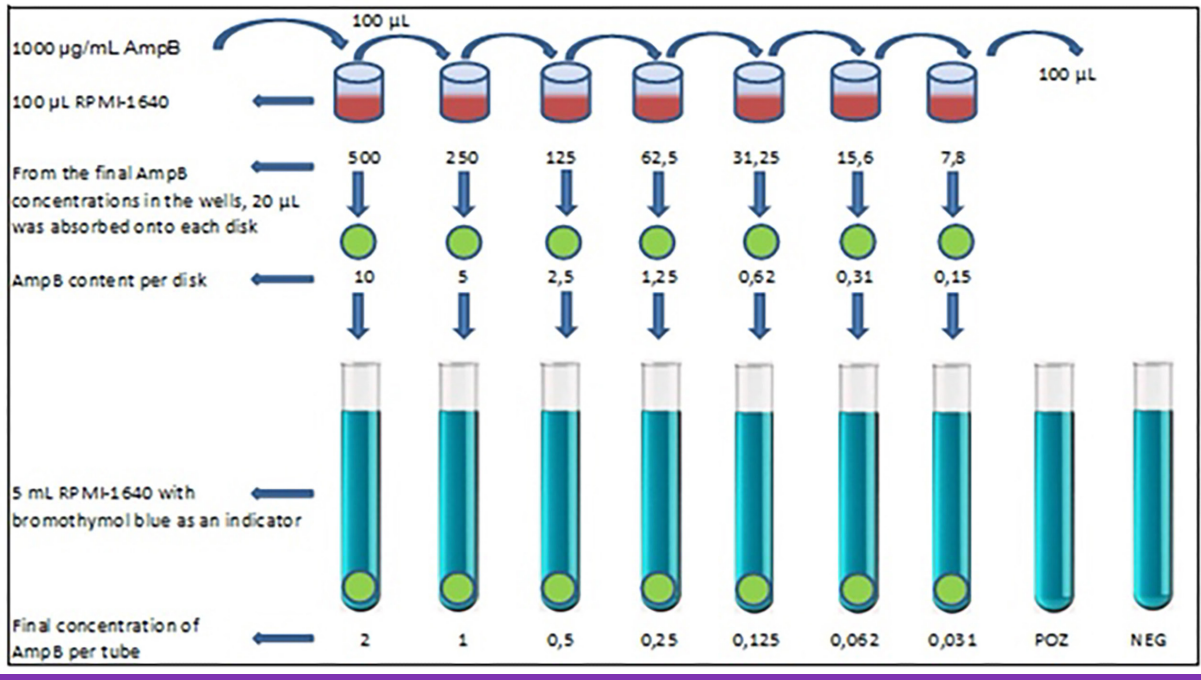


**Figure 1.** Schematic representation of the application process of AmpB into tubes using the modified disk elution method. Initially, serial dilutions were prepared from the 1000 µg/mL stock solution to obtain different AmpB concentrations. From each well, 20 µL of the AmpB solution was absorbed onto sterile disks, which were then transferred into tubes containing 5 mL of RPMI-1640 medium supplemented with bromothymol blue. Based on the concentration of AmpB in the disks, the final drug concentrations in the tubes were adjusted to 2,1,0.5,0.25,0.125, 0.062, and 0.031 µg/mL, respectively. Positive control and negative control tubes were included for comparison

*AmpB: Amphotericin B, RPMI-1640: Roswell Park Memorial Institute-1640*

**Table 1.** Anti-leishmanial activity of AmpB determined by the broth microdilution method

| Concentration (µg/mL) | 48 hours | | | 72 hours | | |
|---|---|---|---|---|---|---|
| 2 | Dead | Dead | Dead | Dead | Dead | Dead |
| 1 | Dead | Dead | Dead | Dead | Dead | Dead |
| 0.5 | Dead | Dead | Dead | Dead | Dead | Dead |
| 0.25 | Live | Live | Live | Dead | Dead | Dead |
| 0.125 | Live | Live | Live | Live | Live | Live |
| 0.0625 | Live | Live | Live | Live | Live | Live |
| 0.03125 | Live | Live | Live | Live | Live | Live |

AmpB: Amphotericin B

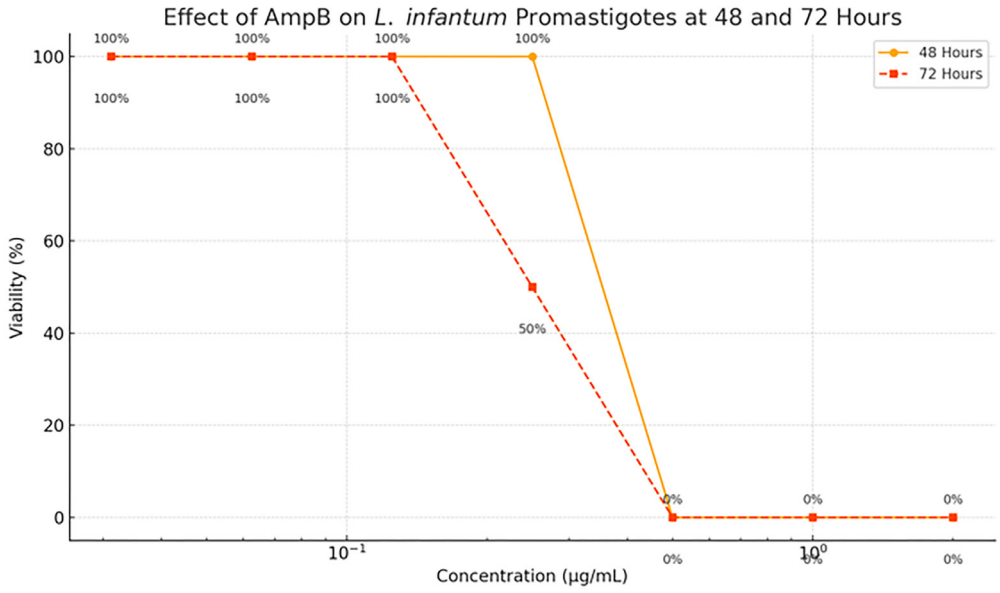


**Figure 2.** Line graph comparing the effects of different concentrations of AmpB on *L. infantum* promastigotes at 48 and 72 hours. The Y-axis represents parasite viability (%) and the X-axis shows AmpB concentrations (µg/mL) on a logarithmic scale. At both time points, 0% viability was observed at concentrations ≥0.5 µg/mL, while at 0.25 µg/mL, 50% viability was detected only at 72 hours

*AmpB: Amphotericin B, L. infantum: Leishmania infantum*

**Table 2.** Anti-leishmanial activity of AmpB determined by the disk elution method

| **Concentration (µg/mL)** | **48 hours** | | | **72 hours** | | |
|---|---|---|---|---|---|---|
| 2 | Dead | Dead | Dead | Dead | Dead | Dead |
| 1 | Dead | Dead | Dead | Dead | Dead | Dead |
| 0.5 | Dead | Dead | Dead | Dead | Dead | Dead |
| 0.25 | Live | Live | Live | Live | Live | Live |
| 0.125 | Live | Live | Live | Live | Live | Live |
| 0.0625 | Live | Live | Live | Live | Live | Live |
| 0.03125 | Live | Live | Live | Live | Live | Live |

AmpB: Amphotericin B

The differences between the two methods were analyzed using the Mann-Whitney U test. The analysis revealed no statistically significant difference between the broth microdilution and disk elution methods at 48 hours (U=18.0, p=1.000). Similarly, the data from 72 hours showed no significant difference between the two methods (U=15.0, p=0.640). These findings indicate that both methods are statistically consistent and comparable in determining the anti-leishmanial activity of AmpB.

Figures 4 and 5 show the color changes observed in the tubes containing AmpB using the modified disk elution method. The indicator present in the medium is purple, and it turns yellow when parasites are viable or begin to proliferate. This allows for easy visual differentiation between live and dead parasites at the end of the incubation period, enabling practical determination of the MPC.

## DISCUSSION

VL is the most severe form of the disease, caused by *L. donovani* in Asia and *L. infantum* in South America and the Mediterranean Basin. VL remains a significant public health issue in many countries, with most reported cases originating from Brazil, East Africa, and India, disproportionately affecting socioeconomically disadvantaged populations (13). In Türkiye, VL caused by *L. infantum* is endemic, with higher incidence rates reported in southeastern regions such as Şanlıurfa and Diyarbakır (14).

Leishmaniasis treatment remains limited to a few drugs, including SbV, which, despite low efficacy rates, continue to be the first-line option in some endemic areas. Miltefosine, an oral drug with a cure rate of approximately 70%, has been approved in Brazil since 2018 as an alternative treatment. Paromomycin has been recommended as a parenteral therapy for VL in Southeast Asia and East Africa, either as monotherapy or in combination with miltefosine or AmpB (15). Although AmpB demonstrates excellent *in vitro* activity against *Leishmania* species, its initial use was limited due to toxic side effects. However, liposomal formulations of AmpB later provided improved tissue penetration, effective dosing at lower concentrations, and reduced toxicity (16). AmpB is now being increasingly used in the treatment of leishmaniasis.

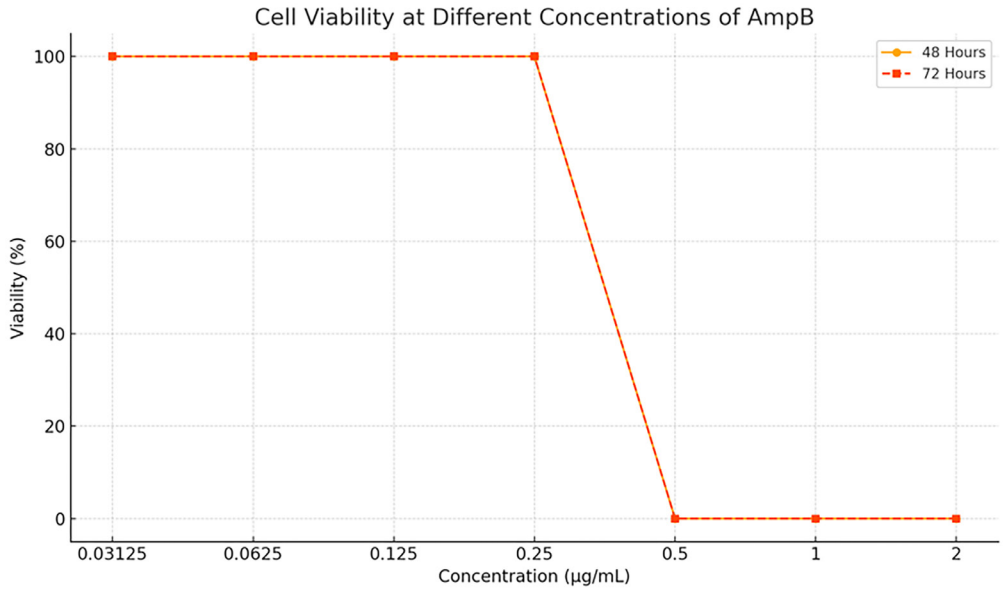


**Figure 3.** Line graph comparing the effects of different concentrations of AmpB on *L. infantum* promastigotes, as evaluated by the disk elution method, in terms of parasite viability (%) at 48 and 72 hours. The X-axis represents AmpB concentrations (µg/mL), and the Y-axis represents viability rates (%). At concentrations ≥0.5 µg/mL, 0% viability was observed at both time points, whereas complete viability was maintained at concentrations ≤0.25 µg/mL. The graph indicates that the efficacy threshold of the drug is ≥0.5 µg/mL
*AmpB: Amphotericin B, L. infantum: Leishmania infantum*

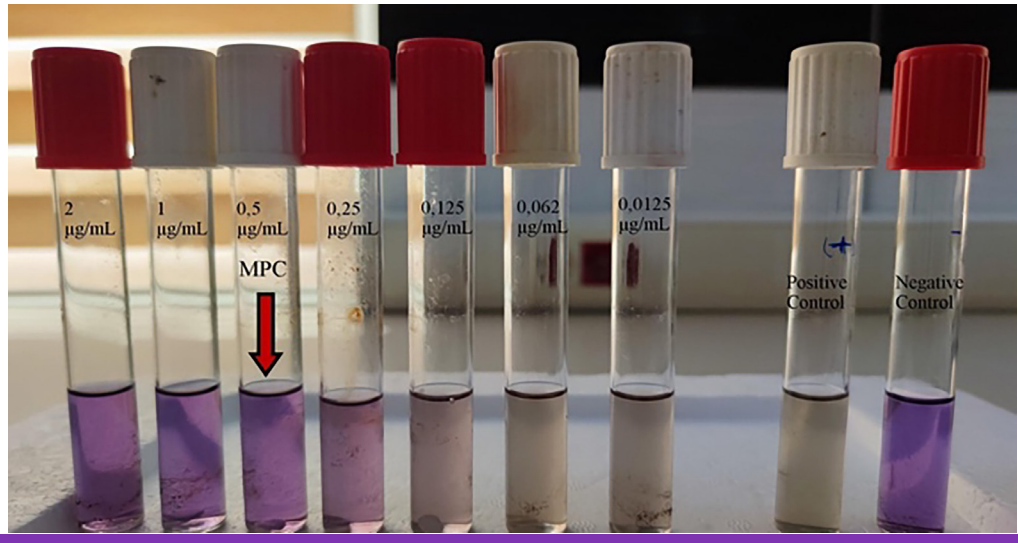


**Figure 4.** Appearance of the tubes prepared using the modified disk elution method at 48 hours. Each tube contains an AmpB disk at a different concentration. The indicator in the medium (normally purple) turns yellow in the presence of viable promastigotes. The third tube (indicated by the yellow arrow) shows the lowest drug concentration at which a color change is observed and is defined as the MPC
*AmpB: Amphotericin B, MPC: Minimum parasiticidal concentration*

While antimicrobial resistance is a persistent threat for all drugs, resistance to AmpB, although rare in the field, remains a concern since it is used not only for recurrent VL cases but also in treating fungal infections and other invasive diseases (17). Therefore, routine monitoring of AmpB susceptibility in *Leishmania* strains isolated from VL patients is essential. However, in Türkiye, drug susceptibility testing in *Leishmania* is not commonly performed due to costly infrastructure, complex procedures, and the need for trained personnel.

Currently, several *in vitro* systems are employed for drug susceptibility testing of *Leishmania* strains, including agar dilution, broth microdilution, flow cytometry, reporter gene-based assays, enzymatic detection methods, $H^3$-thymidine incorporation, and colorimetric systems such as resazurin-based Alamar Blue (18-22). Among these, the most widely used method is broth microdilution, where viability is typically assessed using MTT, XTT, CellTiter-Glo, or resazurin-based reagents. However, the high cost of these kits and the requirement for specialized

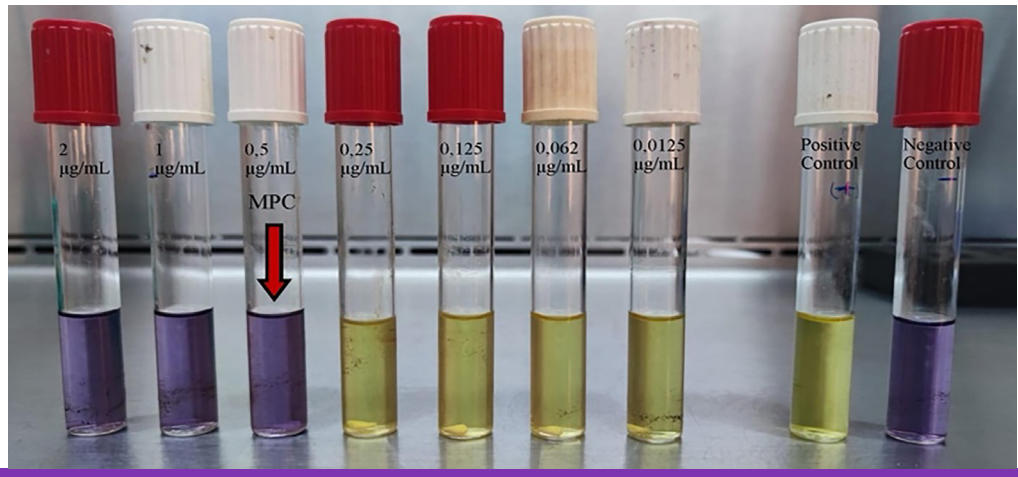


**Figure 5.** Appearance of the tubes prepared using the modified disk elution method at 72 hours. Each tube contains an AmpB disk at a different concentration, and the color of the indicator in the medium changes depending on parasite viability. A distinct yellow color is observed in tubes containing live parasites, whereas the purple color is retained in tubes with dead parasites. At this time point, color differences became more pronounced compared to 48 hours, making visual determination of the MPC easier

*MPC: Minimum parasiticidal concentration*

equipment (e.g., spectrophotometers or fluorometers) limit their widespread use. Thus, there is a growing need for drug susceptibility tests that are more affordable, practical, and less dependent on advanced infrastructure.

The modified disk elution method, unlike the conventional microdilution technique, offers the advantage of visual assessment of parasite viability. In this method, bromothymol blue enables straightforward visualization of promastigote viability through color change. The distinct purple-to-yellow shift observed at the end of incubation allows for practical determination of the MPC without the need for microscopic evaluation, making the method fast, user-friendly, and cost-effective.

In this study, the anti-leishmanial efficacy of AmpB against *L. infantum* was evaluated using two different *in vitro* methods, and the results were compared. No statistically significant difference was found in promastigote viability data between the conventional broth microdilution and modified disk elution methods at either 48 or 72 hours ($p>0.05$). In both methods, AmpB completely eliminated promastigotes at concentrations ≥0.5 µg/mL, while viability was maintained at lower concentrations. These findings suggest that the modified disk elution method provides results comparable to current standards. In a separate study, the commercially available Sensititre™ YeastOne™ YO9 platform—originally developed for antifungal susceptibility testing—was experimentally applied to assess AmpB efficacy against *Leishmania* strains. The minimum inhibitory concentration (MIC) of AmpB for *L. infantum* was found to be 0.22 µg/mL, consistent with the MIC values reported in the present study (23).

The disk elution method was originally used for determining bacterial resistance in antibiotic susceptibility testing (24,25). However, its adaptation for *Leishmania* species has been scarcely reported in the literature. This study is the first to demonstrate the feasibility of applying this method to *Leishmania* promastigotes, thereby addressing an important gap in the field. To date, no standardized colorimetric indicator system for visual assessment of *Leishmania* viability has been thoroughly described in the literature. Therefore, this study offers a methodologically novel approach and paves the way for a cost- and time-saving tool for clinical and field applications.

In this study, AmpB was manually applied onto sterile paper disks to implement the modified disk elution method. However, for field or routine laboratory use, standardization of this process is possible. The availability of pre-prepared, commercially standardized disks containing fixed amounts of AmpB would greatly simplify the procedure. These disks could be directly added to tubes containing bromothymol blue-supplemented medium, facilitating rapid preparation of drug-containing test tubes. This approach could enable drug susceptibility testing without the need for specialized laboratory equipment and may even be feasible under field conditions with limited infrastructure. It would offer a major advantage in standardizing and lowering the cost of field-based screening of *Leishmania* strains.

Nonetheless, the modified disk elution method has some limitations. First, accurate interpretation of color change requires observation under consistent lighting conditions during incubation. Additionally, the method only evaluates the promastigote stage and does not provide information about the amastigote form. Testing this method with different *Leishmania* species would allow for a more comprehensive evaluation. Moreover, for field applicability, it should be supported by stability studies and adaptations for automation. Future studies validating this method with other anti-leishmanial drugs and *Leishmania* species will be essential to support its integration into routine diagnostic and surveillance programs.

## CONCLUSION

This study demonstrated that the modified disk elution method can be used to assess AmpB susceptibility in *L. infantum* promastigotes and yields efficacy results comparable to those of the conventional method. Owing to its practicality and ease of interpretation, this method may serve as an alternative screening tool, particularly in laboratories with limited infrastructure.

## *Ethics

**Ethics Committee Approval:** This study did not involve the use of any human or animal materials. All experiments were conducted using parasite strains preserved in liquid nitrogen. Therefore, ethical approval was not required.

**Informed Consent:** This study did not involve the use of any human or animal materials.

## Footnotes

## Acknowledgments

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## *Authorship Contributions

Concept: Y.Ö., İ.Ç., Design: Y.Ö., İ.Ç., A.Ö., Data Collection or Processing: Y.Ö., İ.Ç., Analysis or Interpretation: Y.Ö., İ.Ç., A.Ö., Literature Search: Y.Ö., İ.Ç., A.Ö., Writing: Y.Ö., İ.Ç., A.Ö.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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# Investigation of the Antiparasitic Potential of Luteolin: *in vitro* Activity and Comparison with Standard Therapeutics

## *Luteolin'in Antiparaziter Potansiyelinin Araştırılması: in vitro Aktivitesi ve Standart Terapötiklerle Karşılaştırılması*

Evren Tileklioğlu[1], Elif Aydın[2]

[1]Aydın Adnan Menderes University Faculty of Medicine, Department of Parasitology, Aydın, Türkiye
[2]Kütahya Health Sciences University, Tavşanlı Vocational School of Health Services, Kütahya, Türkiye

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## ABSTRACT

**Objective:** Due to the limitations of current therapeutic approaches in treating parasitic diseases, there is a growing need for new and effective products, prompting interest in alternative approaches such as medicinal plants. Flavonoids, including luteolin, have shown promise in the treatment of many diseases due to their natural properties and pharmacological effects. This study aimed to investigate the *in vitro* activity of luteolin against *Acanthamoeba castellanii (A. castellanii), Entamoeba histolytica (E. histolytica),* and *Leishmania tropica (L. tropica).*

**Methods:** The reference parasite strains were tested for antiparasitic activity using luteolin concentrations ranging from 200 to 1.5 µg/mL. Positive controls included chlorhexidine, metronidazole, and glucantime, while dimethyl sulfoxide and parasite specific culture medium served as negative controls. Parasite mortality was assessed XTT [2,3-bis (2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide] and trypan blue dye exclusion assays. Minimum inhibitory concentration (MIC) and median lethal dose ($LD_{50}$) values were determined via non-linear regression analysis.

**Results:** Luteolin exhibited significant activity, with MIC values of 100 µg/mL for *A. castellanii* and *E. histolytica*, and 12.5 µg/mL for *L. tropica*. $LD_{50}$ analysis revealed effective concentrations of 3.125 µg/mL for *E. histolytica* and 1.5 µg/mL for *A. castellanii*, while *L. tropica* displayed an $LD_{50}$ below 1.5 µg/mL, indicating the highest sensitivity.

**Conclusion:** Luteolin demonstrated potent antiprotozoal effects *in vitro*, with *L. tropica* being the most susceptible, followed by *A. castellanii* and *E. histolytica*. Notably, luteolin's anti-leishmanial activity was comparable to glucantime. In conclusion, luteolin demonstrates significant potential as a broad-spectrum antiparasitic agent, and comprehensive *in vivo* studies are recommended to further evaluate its therapeutic efficacy.

**Keywords:** Luteolin, antiprotozoal activity, *A. castellanii*, *E. histolytica*, *L. tropica*

## ÖZ

**Amaç:** Paraziter hastalıkların tedavisinde kullanılan mevcut tedavi ajanların sınırlılıkları nedeniyle yeni ve etkili ürünlere ihtiyaç duyulmaktadır. Bu durum, tıbbi bitkiler gibi alternatif yaklaşımlara olan ilgiyi artırmaktadır. Flavonoid grubu içerisinde olan luteolin, doğal özellikleri ve farmakolojik etkileri sayesinde birçok hastalığın tedavisinde umut verici potansiyeli olduğu belirtilmiştir. Bu çalışmada, luteolinin *Acanthamoeba castellanii (A. castellanii), Entamoeba histolytica (E. histolytica),* ve *Leishmania tropica'ya (L. tropica)* karşı *in vitro* aktivitesinin araştırılması amaçlanmıştır.

**Yöntemler:** Çalışmada kullanılan referans parazit suşlarına, 200 µg/mL ile 1,5 µg/mL arasında değişen luteolin konsantrasyonları uygulanarak antiparazitik aktivite test edildi. Pozitif kontroller olarak klorheksidin, metronidazol ve glukantim, negatif kontroller olarak dimetil sülfoksit ve parazite özgü kültür ortamları kullanıldı. Parazit mortalitesi, XTT[2,3-bis (2-metoksi-4-nitro-5-sülfenil)-(2H)-tetrazolium-5-karboksanilid] ve trypan blue boya testleri ile değerlendirildi. Minimum inhibitör konsantrasyon (MIC) ve medyan ölüm dozu ($LD_{50}$) değerleri, doğrusal olmayan regresyon analizi ile belirlendi.

**Bulgular:** Luteolinin, *A. castellanii* ve *E. histolytica* için 100 µg/mL, *L. tropica* için 12,5 µg/mL MIC değerleri ile önemli bir etkinlik göstermiştir. $LD_{50}$ analizi, *E. histolytica* için 3,125 µg/mL ve *A. castellanii* için 1,5 µg/mL etkin konsantrasyonları ortaya koyarken, *L. tropica* 1,5 µg/mL'nin altında bir $LD_{50}$ değeri göstererek en yüksek duyarlılığı sergilemiştir.

**Sonuç:** Bu çalışma ile luteolinin *in vitro* ortamda güçlü antiprotozoal etkisi belirilenmiştir. Çalışmada en duyarlı parazit *L. tropica*, ardından *A. castellanii* ve *E. histolytica* olmuştur. Özellikle luteolinin anti-leishmanial aktivitesi, glukantim ile karşılaştırılabilir

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**Address for Correspondence/Yazar Adresi:** Asst. Evren Tileklioğlu, Aydın Adnan Menderes University Faculty of Medicine, Department of Parasitology, Aydın, Türkiye
**E-mail/E-Posta:** etileklioglu@gmail.com **ORCID ID:** orcid.org/0000-0003-2141-1311



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düzeydedir. Sonuç olarak luteolinin, geniş spektrumlu bir antiparazitik ajan olarak önemli potansiyel göstermektedir. Terapötik etkinliğinin değerlendirilmesi için kapsamlı *in vivo* çalışmaların yapılması önerilmektedir.
**Anahtar Kelimeler:** Luteolin, antiprotozoal aktivite, *A. castellanii*, *E. histolytica*, *L.tropica*

## INTRODUCTION

Millions of individuals, predominantly in low- and middle-income countries, are affected by parasitic infections, particularly those caused by protozoans such as *Entamoeba* spp., *Acanthamoeba* spp., and *Leishmania* spp., which contribute to significant morbidity and mortality globally (1-3).

Amoebiasis, caused by *Entamoeba histolytica* (*E. histolytica*), is a notable parasitic disease, leading to an estimated 55,500 deaths and 2.237 million disability-adjusted life years annually, as reported by the Global Burden of Disease 2010 Study (4). The prevalence of *E. histolytica* infection can reach up to 50% in some developing regions, including parts of South and Central America, Africa, and Asia. In Türkiye, the incidence varies regionally, with an average rate of 2.27% (1). Although the disease is often asymptomatic, it can present with symptoms ranging from diarrhea to dysentery, and in severe cases, it may disseminate to other organs such as the liver, lungs, and brain, leading to abscess formation. The standard pharmacological treatment includes nitroimidazoles like metronidazole (MTZ), but these drugs have limitations, including adverse effects such as neurotoxicity with prolonged use and the emergence of drug-resistant strains (5).

*Acanthamoeba castellanii (A. castellani)* is an opportunistic, free-living amoeba found in soil and water, associated with approximately 2.9 cases per million globally. It causes two primary clinical conditions: *Acanthamoeba* keratitis (AK) and granulomatous *Acanthamoeba* encephalitis. AK begins with the attachment of trophozoites to the corneal epithelium, potentially progressing to stromal invasion and vision loss or blindness (2). Current treatments for *Acanthamoeba* infections include 0.02% chlorhexidine and 0.02% polyhexamethylene biguanide, though prolonged use of these topical agents can result in corneal toxicity (6).

Leishmaniasis, caused by over 20 pathogenic *Leishmania* species, is transmitted by various species of female phlebotomine sandflies (3). The disease is endemic in more than 102 countries/regions bordering the Mediterranean and the Black Sea, including Türkiye, with thousands of new cases reported annually (7). Clinical manifestations vary based on the *Leishmania* species and the host's immune response. Treatment options include antimony (first line), amphotericin B (second line), imidazole's, miltefosine, paromomycin, and liposomal amphotericin B. However, these drugs are expensive, exhibit toxic side effects, and are often rendered ineffective by drug-resistant strains (8).

Considering the challenges associated with current treatments for these parasitic diseases, there is growing interest in exploring medicinal plants for alternative therapies. Flavonoids, a diverse group of polyphenolic compounds found in various plants, are gaining attention due to their pharmacological properties (9). With over 9,000 types identified, flavonoids are characterized by their C6-C3-C6 structural backbone and include several subgroups based on structural variations (10). Luteolin, a prominent flavonoid found in numerous plants used in traditional medicine, has demonstrated a range of biological effects, including anti-diabetic, anti-allergic, and anti-cancer activities. Despite extensive research on its antibacterial and antiviral properties, its antiparasitic activity remains underexplored (11-13). This study aims to evaluate the *in vitro* activity of luteolin against *A. castellanii*, *E. histolytica*, and *Leishmania tropica (L. tropica),* contributing to the search for novel therapeutic agents.

## METHODS

### Ethical Approval

No clinical material or data were used in this study. Therefore, ethics committee approval is not required.

### Preparation and Storage of Luteolin Stock Solution

Luteolin was purchased from Sigma-Aldrich (St. Louis, MO). A stock solution of one hundred millimolars in dimethyl sulfoxide (DMSO) was ready and held on at -20 °C.

### *In vitro* Cultures of *A. castellanii, E. histolytica* and *L. tropica*

Axenic cultures of *A. castellanii* trophozoites (ATCC 30010) were propagated in protease peptone-yeast extract-glucose medium. This medium contained 0.75% (w/v) protease peptone, 0.75% (w/v) yeast extract, and 1.5% (w/v) glucose, supplemented with penicillin G (500 U/mL) and streptomycin (50 µg/mL) to maintain sterility and promote optimal axenic growth. Cultures were maintained in 25 mL cell culture flasks (Sigma), refreshed weekly, and incubated at 30 °C. For cell harvesting, the culture medium was removed by centrifugation at 1500 rpm for 5 minutes, followed by three washes in phosphate-buffered saline to eliminate any remaining medium components. To detach the trophozoites adhering to the flask walls, the flasks were gently agitated on ice for 30 minutes.

The *E. histolytica* strain (ATCC 30459) was generously provided by Dr. Charles Graham Clark from the London School of Hygiene and Tropical Medicine. *E. histolytica* trophozoites were axenically grown in LYI (liver digest, yeast extract, iron) medium, which included 880.0 mL of LYI broth, 20.0 mL of a vitamin mixture, and 100.0 mL of heat-inactivated adult bovine serum. The medium was further supplemented with penicillin G (500 U/mL) and streptomycin (50 µg/mL). To ensure continuous growth and viability, trophozoites were routinely subculture into screw-capped test tubes containing 7 mL of LYI medium.

*L. tropica* promastigotes (ATCC 50129) were cultured at 26 °C in RPMI-1640 medium (Sigma), supplemented with 10% heat-inactivated fetal bovine serum sourced from Cegrogen, Stadtallendorf, Germany. This enriched medium provided the necessary nutrients for promastigote growth and development. The cultures were maintained in 25 mL flasks, ensuring optimal conditions to support the promastigotes' stationary phase proliferation.

### *In vitro* Antiparasitic Test of Luteolin

*A. castellanii*, *E. histolytica*, and *L. tropica* were seeded into 96-well microtiter plates (Greiner, Germany), with luteolin applied in serial concentrations ranging from 200 µg/mL to 1.5 µg/mL. For

this, the trophozoite densities of *A. castellanii* and *E. histolytica* were adjusted to $5\times10^4$ cells/mL and $1\times10^6$ cells/mL, respectively, and *L. tropica* promastigotes were standardized to $1\times10^5$ cells/mL. The trophozoites of *A. castellanii* and *E. histolytica* were given 20 minutes to adhere to the wells, a process that was confirmed under a Leica inverted microscope (Leica, Wetzlar, Germany). The plates were then incubated at 30 °C for 24 hours for *A. castellanii*, at 26 °C for 72 hours for *E. histolytica*, and at 37 °C for 48 hours for *L. tropica*.

Two distinct assays were employed to assess the antiprotozoal activity of luteolin *in vitro*. The anti-leishmanial effect was evaluated using the XTT cell proliferation kit from Roche Molecular Biochemicals (Mannheim, Germany), following the manufacturer's instructions (14). A viability assay was conducted for *A. castellanii* and *E. histolytica*, which involved staining the cells with 0.1% trypan blue [(TB) 0.4%] at a 1:1 ratio. Live cells remained unstained, while alive cells were stained, and both were counted using a hemocytometer (15). The percentage of parasite mortality was calculated using the formula: % mortality = (negative control-test sample) × 100/negative control.

Parasite mortality was determined as 100% when no motile parasites were observed. Minimum inhibitory concentration (MIC) and $LD_{50}$ values were determined, with MIC representing the lowest concentration that fully inhibited parasite growth and $LD_{50}$ representing the dose required to kill 50% of the parasites. To verify trophozoite and promastigote viability, the samples were reinoculated into fresh media and monitored over 24, 48, and 72 hours for regrowth. $LD_{50}$ values were calculated by fitting a non-linear sigmoidal dose-response curve (four-parameter logistic regression) to the mortality data using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). The 95% confidence intervals (CI) were determined from the regression model. Analyses were performed separately for each protozoan species.

Each experiment included appropriate controls. Negative controls consisted of DMSO (final concentration <1%), which was used as the solvent for luteolin, and parasite specific culture medium appropriate for each organism without luteolin. For *A. castellanii*, the negative control medium was PYG, for *E. histolytica*, LYI medium; and for *L. tropica*, RPMI-1640. These media served as parasite maintenance controls to ensure that any observed mortality was due solely to the treatment and not to culture conditions. The positive controls consisted of MTZ (Specia Rhone Poulenc Rorer, Paris, France) for *E. histolytica*, chlorhexidine (Sigma) for *A. castellanii*, and N-methyl meglumine (Glucantime™, Rhone Poulenc, France) for *L. tropica*. All assays were conducted three times in triplicate to ensure reliability and reproducibility of the results.

### Statistical Analysis

The mean, degrees of freedom, and t-value (t) were calculated. Data on the antiprotozoal activity were analysed for statistical significance by using the two-tailed Student's t-test for unpaired samples. A p-value of 0.05 or less was considered indicative of a statistically significant difference. $LD_{50}$ regression was conducted using GraphPad 10 (GraphPad Software, San Diego, CA, USA). $LD_{50}$ values were determined by fitting the data to a non-linear regression model (four-parameter logistic, variable slope). The goodness-of-fit ($R^2$) and 95% CIs for each $LD_{50}$ estimate were calculated to assess model reliability. Dose response curves were plotted for each parasite species to visualize differences in susceptibility. All experiments were performed in triplicate and repeated three independent times, and the results are presented as mean ± standard error.

## RESULTS

According to the *Acanthamoeba* TB dye exclusion assays, mortality in the negative control group was minimal, with a maximum value of 8%, while significantly higher mortality rates were recorded in both the luteolin and chlorhexidine groups, reaching 100%. The highest mortality was determined at concentrations of 100 µg/mL and 200 µg/mL for both luteolin and chlorhexidine, with 100% mortality. At the lowest concentration (1.5 µg/mL), the mortality rate decreased to 30% for luteolin, while it remained 98% for chlorhexidine. Overall, an increase in mortality rates was observed as the concentration increased (Figure 1a). The *in vitro* effect of luteolin on *Acanthamoeba* was determined to be significant and dose dependent. A statistical relationship was not found between luteolin and the positive control ($p=0.0172$; $t=2.7026$; $df=14$).

The TB dye exclusion assays demonstrated that luteolin achieved complete effectiveness (100% mortality) against *E. histolytica* trophozoites at concentrations of 100 µg/mL and 200 µg/mL. At 50 µg/mL, luteolin induced 98% mortality, while at lower concentrations, the parasites were affected but their viability remained higher compared to the positive control. The number of motile trophozoites increased as the drug concentrations decreased (Figure 1b). No statistical relationship was detected between luteolin and the positive control (MTZ) ($p=0.0953$; $t=1.7886$; $df=14$).

According to XTT analysis, luteolin induced 100% mortality at concentrations ranging from 200 to 12.5 µg/mL against the promastigote form of *L. tropica*. Mortality rates between 95% and 98% were determined at the three lowest concentrations (6.25, 3.125, and 1.5 µg/mL). No significant differences were detected between luteolin and glucantime ($p=0.1108$; $t=1.8249$; $df=7$).

The MIC of luteolin was determined as 100 µg/mL for *A. castellanii* and *E. histolytica*, while a value of 12.5 µg/mL was noted for *L. tropica*. Histogram graphs showing the *in vitro* activity of luteolin against *A. castellanii*, *E. histolytica*, and *L. tropica* are presented in Figures 2-4, respectively.

$LD_{50}$ regression analysis, performed using a non-linear dose-response model, revealed that luteolin exhibited the highest potency against *L. tropica*, with an $LD_{50}$ value below 1.5 µg/mL (95% CI: X-Y) (Figure 5). For *A. castellanii* and *E. histolytica*, the $LD_{50}$ values were calculated as 1.5 µg/mL (95% CI: X-Y) and 3.125 µg/mL (95% CI: X-Y), respectively (Figure 5a and 5b). The steep slope of the dose–response curve for *L. tropica* indicates a rapid decline in viability with small increases in concentration, suggesting greater susceptibility compared with the other protozoa. In contrast, *E. histolytica* demonstrated the least sensitivity to luteolin within the tested range, as reflected by the higher $LD_{50}$ value.

## DISCUSSION

The decreasing effectiveness of conventional therapeutic agents in treating parasitic diseases has led to delays and failures in treatment. Moreover, long-term use of these agents often

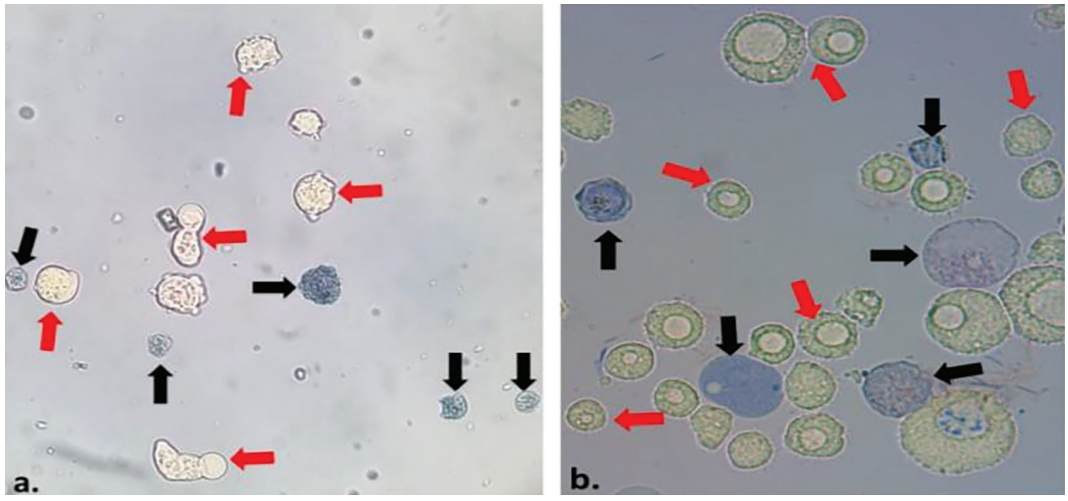


**Figure 1.** Trophozoites of *E. histolytica* **(a)** and *A. castellanii* **(b)** with trypan blue dye exclusion assays (40x). Alive (red arrow-unstained) and dead (black arrow-stained) cells

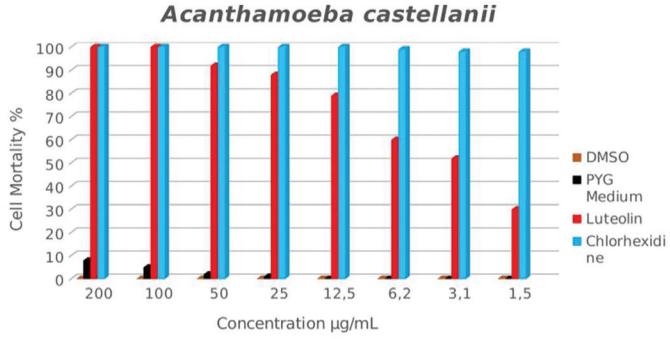


**Figure 2.** Mortality of luteolin against *A. castellanii* trophozoites

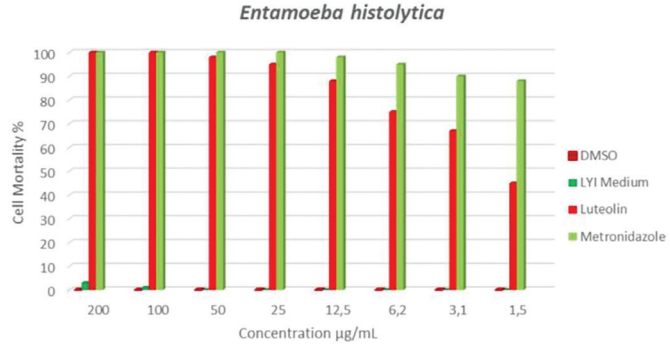


**Figure 3.** Mortality of luteolin aganist *E. histolytica* trophozoites

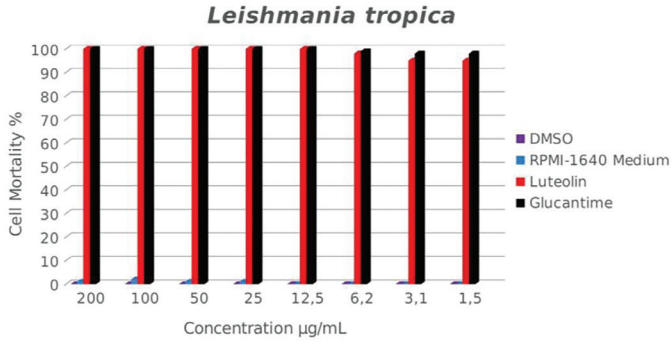


**Figure 4.** Mortality of luteolin aganist *L. tropica* promastigote

results in toxicity and adverse side effects (16). Accordingly, there has been a shift towards the discovery of natural medicinal compounds with antiparasitic properties. Medicinal plants, commonly used as raw materials across various sectors, including medicine and pharmacy, produce phytoalexins in response to microbial invasion. These compounds, derived from secondary metabolites, are mainly found in flavonoids (17). Many studies have demonstrated that flavonoids possess a wide array of biological activities, such as antioxidant, antimutagenic, antibacterial, antiangiogenic, anti-inflammatory, antiallergic, enzymatic regulatory, and anticancer effects (10,18,19).

Luteolin and its glycosides are significant flavonoids with demonstrated antimicrobial activity. Several studies have reported their effectiveness against bacteria, viruses, and fungi. However, studies on the antiparasitic activity of luteolin are limited. For instance, research has shown that flavonoids, when used alone or in combination, are effective against *Cryptosporidium parvum* and *Encephalitozoon intestinalis* (20). One study revealed that luteolin inhibits the transformation of *Plasmodium falciparum* rings into more advanced stages, without affecting the sensitivity to chloroquine or artemisinin (21). In addition, luteolin was shown to markedly inhibit the growth of *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*, starting from an initial parasitemia of 1%. Furthermore, at a dosage of 5 mg/kg, luteolin resulted in a 77.5% reduction in the growth of *Babesia microti* in BALB/c mice, indicating its potential applicability in the treatment of babesiosis. (22).

Although research on luteolin's effects against *Acanthamoeba* is scarce, it has been suggested that luteolin could play an important role in developing alternative treatments for *Acanthamoeba* infections. Luteolin has demonstrated cytotoxicity against mouse macrophages (J774A.1) and has been reported to induce

programmed cell death in *A.castellanii* (23). However, luteolin's effects appear to vary among species. For example, in a study evaluating the anti-amoebic activity of 18 flavonoids against *A. castellanii*, *A. polyphaga*, and *Naegleria fowleri* (*N. fowleri*), luteolin was highly effective against *N. fowleri* but less so against *A. castellanii*. Notably, *A. polyphaga* was found to be more sensitive to luteolin than *A. castellanii*, with the former exhibiting higher sensitivity index values (>11) and lower $IC_{50}$ values (<30 µM) (24). In the present study, the highest mortality rate of luteolin on *A. castellanii* was 100% at concentrations of 200 µg/mL and 100 µg/mL. The MIC and $LD_{50}$ values were determined to be 100 µg/mL and 3.125 µg/mL, respectively. However, luteolin's efficacy at lower concentrations was less pronounced compared to chlorhexidine, highlighting the need for further *in vitro* and *in vivo* studies to fully understand luteolin's effects on *Acanthamoeba* species.

Recent research has increasingly focused on the potential of medicinal plants as complementary or specific treatment strategies with amoebicidal properties against *Entamoeba* species, given the limitations of current therapeutic agents (25). Notably, natural compounds have been observed to induce morphological changes in amoebae, including chromatin condensation and cytoskeletal protein rearrangement (26). In one study, ten flavonoids and four iridoids were evaluated for their anti-amoebic activity and cytotoxicity against MT-4 cells. Among them, kaempferol ($IC_{50}$=10.3±2.3 µg/mL), apigenin ($IC_{50}$=12.7±4.3 µg/mL), and luteolin ($IC_{50}$=17.8±4.3 µg/mL) exhibited stronger activity than their glycoside counterparts (27). In the current study, luteolin demonstrated a potent effect against *E. histolytica* at higher concentrations, with 100% mortality at 200 and 100 µg/mL and 98% mortality at 50 µg/mL. The mortality effect decreased dose-dependently, with an MIC value of 100 µg/mL and an $LD_{50}$ of 1.5 µg/mL. Although MTZ proved more effective at lower concentrations, these results indicate that luteolin effectively eliminates half of the trophozoites at the lowest concentration tested.

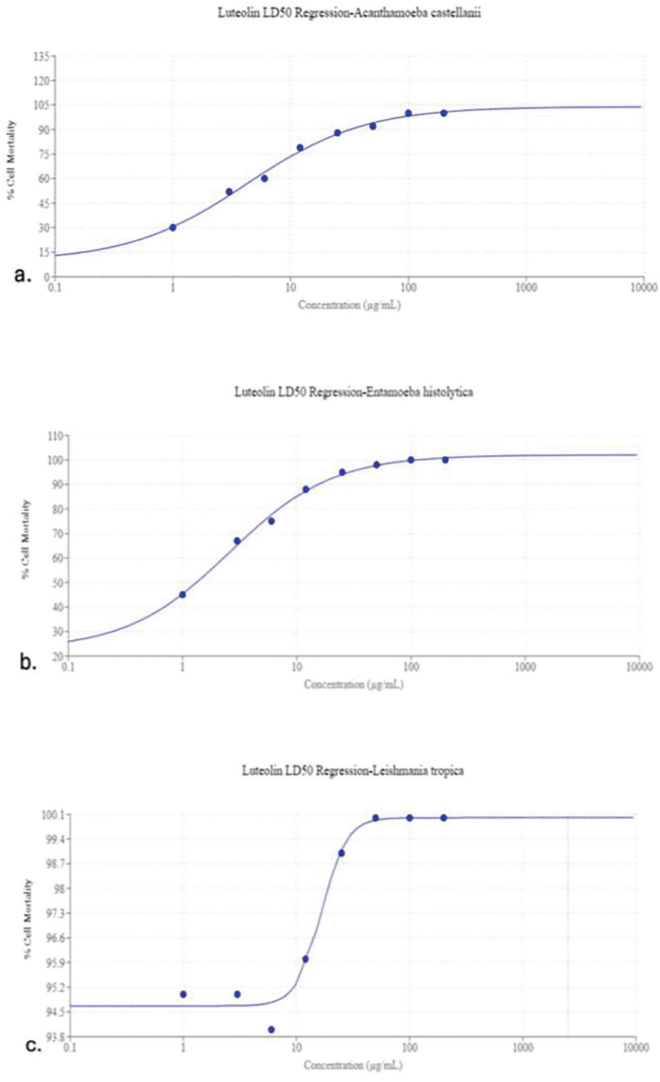


**Figure 5.** $LD_{50}$ regression analysis of luteolin aganist *A. castellanii* **(a)**, *E. histolytica* **(b)** and *L. tropica* **(c)**

Studies have also suggested that flavonoids could serve as food supplements in the treatment of leishmaniasis due to their low $IC_{50}$ values (28). In one study, it was noted that the side effects of quercetin and luteolin on cutaneous wounds caused by *Leishmania* species were less severe than those caused by meglumine (29). While quercetin exhibited non-specific effects on normal human T-cells, luteolin was found to be non-toxic and a strong candidate for anti-leishmanial drug development (30). Additionally, *in vivo* studies have demonstrated that luteolin has greater cytotoxicity against lymphocytes and is a more potent inhibitor of *L. tropica* amastigotes than luteolin-4'-O-β-D-glucopyranoside (12). Similarly, another study evaluated the cytotoxicity of 105 compounds on mammalian L6 cells and their antiparasitic activities, identifying fisetin, 3-hydroxyflavone, luteolin, and quercetin as the most potent anti-leishmanial agents, with $IC_{50}$ values of 0.6, 0.7, 0.8, and 1.0 µg/mL, respectively (31). In the present study, luteolin exhibited strong anti-leishmanial activity against *L. tropica*, comparable to glucantime. According to the XTT analysis, luteolin induced 100% mortality at concentrations ranging from 200 to 12.5 µg/mL, with mortality rates of 95-98% at the lower concentrations. These findings are consistent with glucantime's effect, which induced 100% mortality at concentrations of 200-12.5 µg/mL and 98-99% at the lower concentrations. No meaningful distinction was found between luteolin and glucantime (p=0.1108; t=1.8249; df=7). The MIC value of luteolin was 12.5 µg/mL, and its $LD_{50}$ value at even lower concentrations underscores its potential as an effective anti-leishmanial agent.

## CONCLUSION

This study highlights the significant antiparasitic potential of luteolin against *E. histoytica*, *A. cestallanii* and *L. tropica*. Luteolin demonstrated comparable effectiveness to established treatments like MTZ, chlorhexidine, and glucantime under *in vitro* conditions. The lack of significant statistical differences between luteolin and these standard treatments suggests that luteolin may possess similar antiparasitic properties. Furthermore, its effective performance, particularly against *L. tropica*, supports the potential for luteolin to be explored further as an anti-leishmanial agent. Our results may form the basis for future studies to evaluate the anti-parasitic activity of luteolin in potential therapeutic applications.

## *Ethics

**Ethics Committee Approval:** No clinical material or data were used in this study. Therefore, ethics committee approval is not required.

**Informed Consent:** Not required.

## Footnotes

## *Authorship Contributions

Surgical and Medical Practices: E.T., Concept: E.T., E.A., Design: E.T., E.A., Data Collection or Processing: E.T., E.A., Analysis or Interpretation: E.T., E.A., Literature Search: E.T., E.A., Writing: E.T., E.A.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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# First Morphological Identification of *Eimeria* spp. and *Cryptosporidium* spp. in Different Wild Rodent Species from Central and Northwest Iran

## *İran'ın Orta ve Kuzeybatı Bölgeleri'ndeki Farklı Yabani Kemirgen Türlerinde Eimeria spp. ve Cryptosporidium spp.'nin İlk Morfolojik Tanımlaması*

Bahram Rastad[1], Mousa Tavassoli[2], Bijan Esmaeilnejad[2], Salar Zarrabi Ahrabi[3]
[1]Dr. Rezaei Veterinary Clinic, Farmahin, Iran
[2]Urmia University Faculty of Veterinary Medicine, Department of Pathobiology, Urmia, Iran
[3]Marmara University Faculty of Health Sciences, Department of Basic Health Sciences, İstanbul, Türkiye

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## ABSTRACT

**Objective:** Wild rodents act as important hosts and reservoirs for both zoonotic and non-zoonotic pathogens, playing a key role in maintaining and transmitting infectious agents in nature. Their presence can lead to contamination of food and water sources, affecting both humans and animals.

**Methods:** This study examined 138 dead rodents from six species (*Microtus socialis*, *Rattus norvegicus*, *Mus musculus*, *Meriones libycus*, *Apodemus witherbyi*, and *Ellobius lutescens*) collected from three regions in Iran. Fecal samples were analyzed for *Eimeria* spp. and *Cryptosporidium* spp. using potassium dichromate cultivation and sugar flotation for *Eimeria*, and modified Ziehl-Neelsen staining for *Cryptosporidium*.

**Results:** The infection rate for *Eimeria* spp. was 5.79%, and the identified species included *E. falciformis*, *E. papillata*, *E. miyairii*, *E. musculoidei*, and *E. hungaryensis*. For *Cryptosporidium* spp., a 4.34% infection rate was observed. While *Eimeria* infections were limited to three rodent species, *Cryptosporidium* was detected in all six.

**Conclusion:** This study presents the first morphological identification of *Eimeria* species in rodents in Iran, with findings consistent with host-parasite relationships reported globally. Additionally, the widespread presence of *Cryptosporidium* spp. in multiple rodent species emphasizes the epidemiological importance of these animals as potential reservoirs of zoonotic pathogens. These results contribute to a better understanding of protozoan diversity and distribution in rodent populations of Iran.

**Keywords:** Rodent, *Emeria, Cryptosporidium*, Iran

## ÖZ

**Amaç:** Yabani kemirgenler, hem zoonotik hem de zoonotik olmayan patojenler için önemli konakçı ve rezervuarlar olup, doğada enfeksiyöz etkenlerin devamlılığının sağlanması ve yayılmasında kilit rol oynamaktadır. Bu canlıların varlığı, hem insan hem de hayvanlar için gıda ve su kaynaklarının kontaminasyonuna neden olabilmektedir.

**Yöntemler:** Bu çalışmada, İran'ın üç farklı bölgesinden toplanan altı türe ait (*Microtus socialis, Rattus norvegicus, Mus musculus, Meriones libycus, Apodemus witherbyi* ve *Ellobius lutescens*) toplam 138 ölü kemirgen incelenmiştir. Dışkı örnekleri, *Eimeria* spp. için potasyum dikromat inkübasyonu ve şeker flotasyon yöntemi, *Cryptosporidium* spp. için ise modifiye Ziehl-Neelsen boyama yöntemi kullanılarak analiz edilmiştir.

**Bulgular:** *Eimeria* spp. enfeksiyon oranı %5,79 olarak belirlenmiş; tanımlanan türler arasında *E. falciformis, E. papillata, E. miyairii, E. musculoidei* ve *E. hungaryensis* yer almıştır. *Cryptosporidium* spp. için saptanan enfeksiyon oranı ise %4,34'tür. *Eimeria* enfeksiyonları yalnızca üç kemirgen türünde görülürken, *Cryptosporidium* altı türün tamamında tespit edilmiştir.

**Sonuç:** Bu çalışma, İran'daki kemirgenlerde *Eimeria* türlerinin ilk morfolojik tanımlamasını sunmakta olup, elde edilen bulgular dünya genelinde bildirilen konak-parazit ilişkileri ile uyumludur. Ayrıca, *Cryptosporidium* spp.'nin birçok kemirgen türünde yaygın olarak bulunması, bu hayvanların zoonotik patojenler için potansiyel rezervuarlar olarak epidemiyolojik önemini vurgulamaktadır. Elde edilen veriler, İran'daki kemirgen popülasyonlarında protozoon çeşitliliği ve dağılımının daha iyi anlaşılmasına katkı sağlamaktadır.

**Anahtar Kelimeler:** Kemirgen, *Eimeria, Cryptosporidium*, İran

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**Address for Correspondence/Yazar Adresi:** Asst. Prof. Salar Zarrabi Ahrabi, Ph.D, Marmara University Faculty of Health Sciences, Department of Basic Health Sciences, İstanbul, Türkiye
**E-mail/E-Posta:** salar.zarrabi@marmara.edu.tr **ORCID ID:** orcid.org/0000-0003-3543-061X



Türkiye PARAZİTOLOJİ Dergisi

## INTRODUCTION

Rodents are among the most common reservoirs of zoonotic infections, and they play an important role in the spread of infectious diseases to people and domestic animals. They comprise 42% of the world's mammalian biodiversity and are the biggest order of extant mammals. They are remarkably diverse. Rodents are thought to be the most successful and adaptable group of mammals (1,2). *Eimeria* spp. and *Cryptosporidium* spp. are protozoan genera of significant concern due to their impact on human and animal health. *Cryptosporidium* species cause cryptosporidiosis, a gastrointestinal disease that can be particularly severe in immunocompromised individuals. In contrast, the *Eimeria* species are primarily associated with coccidiosis, which affects a wide range of animal hosts and leads to substantial economic losses in the livestock industry (3,4).

There are about 2,000 species of *Eimeria* known to exist in invertebrate hosts as well as mammals, birds, reptiles, and other vertebrates. *Eimeria* species are found worldwide because their hosts are widely dispersed; over 200 species of coccidia, especially *Eimeria* spp., have been identified in rodents. Most species of *Eimeria* are homoxenous, which means they only infect one type of host. Nonetheless, certain avian *Eimeria* species have been found to have a restricted oligoxenous host range, a trait that could aid in the spread of the parasite by allowing livestock and wildlife to become infected (5,6). On the other hand, the protozoan genus *Cryptosporidium* spp. can infect a wide range of hosts, including humans, domestic animals, and wildlife. The oocysts of *Cryptosporidium* spp. are highly resistant to environmental conditions and can persist for extended periods, enabling transmission through contaminated food and water. Numerous rodent species have been identified as hosts of *Cryptosporidium* spp., making them significant reservoirs of the parasite. They may actively contribute to the spread of zoonotic *Cryptosporidium* species and play a role in cryptosporidiosis in both humans and animals (7,8).

The primary objective of this research is to identify and characterize *Eimeria* spp. and *Cryptosporidium* spp. species present in rodents from selected regions. This study aims to expand existing knowledge on the epidemiology of these parasites within rodent populations. The research is conducted in the central and northwestern regions of Iran, specifically in the cities of Arak, Saqqez, and Urmia, where rodent populations are abundant and frequently interact with human habitats.

## METHODS

A total of 138 rodents were collected from central and Northwestern Iran, specifically from Arak (n=45), Saqqez (n=41), and Urmia (n=52). The rodents were trapped in grain warehouses, cereal pastures, or accidentally captured in homes by rodenticide poisoning (Table 1). All rodents were found dead in the traps before being transported to the laboratory. The samples were securely transported in sealed bags following biosecurity protocols. Following the collection, the taxonomic classification of the rodents was performed and recorded. After the dissection of the gastrointestinal tract, fecal samples were collected from the distal part of the intestine, where the feces had a distinct shape and consistency. These samples were transferred to a 2.5% potassium dichromate solution. Additionally, a portion of the fecal sample was prepared for smear examination and identification of *Cryptosporidium*.

### Examination of *Eimeria* Oocysts

For this purpose, after fecal sample collection, a portion was placed in a 2.5% potassium dichromate preservative solution and stored at 4 °C in a refrigerator for 3 days to allow the oocysts to complete the sporulation process. After that, one slide was prepared from each sample using the direct smear method, and another was prepared using the sugar flotation method (9).

The *Eimeria* oocysts were identified using a light microscope at magnifications of ×10 and ×40, with each oocyst containing four sporocysts. After observing the target oocysts, further imaging and morphometric measurements of all oocyst samples on each slide were performed using a Dino-Eye digital camera. This camera, which allows simultaneous connection to a light microscope and a computer, facilitated detailed imaging at ×40 and ×100 magnifications using immersion oil.

To identify *Eimeria* species, images measured with Dino-Eye software were analysed based on key morphological features, including oocyst and sporocyst size, oocyst layers, and the presence of structures like micropyle, polar granules, and Stieda body and large or small refractile globules (6).

### Examination and Identification of *Cryptosporidium*

To identify *Cryptosporidium* spp. in fecal samples, the modified Ziehl-Neelsen acid-fast staining method was employed. Two fecal smears were prepared for each sample following standard protocols. Under ×100 magnification, *Cryptosporidium* oocysts were observed as minute red granules within a green-blue background, indicative of the characteristic acid-fast staining properties of the parasite (10).

**Table 1.** Distribution of rodent species by city and capture location

| Rodent species | Arak (n=45) | Saqqez (n=41) | Urmia (n=52) | Total (n=138) | Capture location |
|---|---|---|---|---|---|
| *Mus musculus* | 18 | 15 | 16 | 49 | Homes, Grain Warehouses |
| *Rattus norvegicus* | 12 | 10 | 10 | 32 | Homes, Grain Warehouses |
| *Microtus socialis* | 7 | 8 | 15 | 30 | Cereal Pastures |
| *Apodemus witherbyi* | 3 | 4 | 3 | 10 | Cereal Pastures, Grain Warehouses |
| *Ellobius lutescens* | 3 | 2 | 5 | 10 | Cereal Pastures |
| *Meriones libycus* | 2 | 2 | 3 | 7 | Cereal Pastures, Occasionally Grain Warehouses |
| Total | 45 | 41 | 52 | 138 | |

### Statistical Analysis

Descriptive statistical analyses were performed in the study. All data were entered into Microsoft Excel, and descriptive measures, including frequencies and percentages, were calculated using the software's built-in functions.

## RESULTS

A total of 138 rodents were examined, and six species were diagnosed: *Microtus socialis, Rattus norvegicus, Mus musculus, Meriones libycus, Apodemus witherbyi,* and *Ellobius lutescens* (11). These species are presented in Table 2.

Out of 138 rodents, 8 (5.79%) were infected with *Eimeria* spp. Among these, 5 were male and 3 were female. The infected rodents included *M. musculus* (n=4), *R. norvegicus* (n=2), and *A. witherbyi* (n=2). One case of simultaneous infection with *Eimeria* spp. and *Cryptosporidium* spp. was observed in *A. witherbyi*. In the 8 positive samples for *Eimeria*, 5 species were diagnosed: *E. musculoidei* (Figure 1) from *M. musculus* (n=1), *E. falciformis* (Figure 2) from *M. musculus* (n=2), *E. papillata* (Figure 3) from *M. musculus* (n=1), *E. miyairii* (Figure 4) from *R. norvegicus* (n=2), and *E. hungaryensis* (Figure 5) from *A. witherbyi* (n=2).

Additionally, 6 (4.34%) out of 138 rodents were infected with *Cryptosporidium* spp. (Figure 6). Among these, 3 were male and 3 were female. The infected rodent species included *M. musculus, R. norvegicus, M. socialis, E. lutescens,* and *A. witherbyi* (Table 3).

## DISCUSSION

This study examined the occurrence of *Emeria* spp. and *Cryptosporidium* spp. in wild rodents from Western, Central, and Northern Iran, identifying a diverse range of coccidian parasites, including *Eimeria* species *E. musculoidei, E. falciformis, E. papillata, E. miyairii,* and *E. hungaryensis*.

Rodents serve as hosts for a wide range of *Eimeria* species, most of which exhibit strict host specificity, even among different rodent species. Most *in vivo* studies on *Eimeria* spp. have been conducted in rodents, such as the house mouse, due to economic considerations (3).

*E. falciformis* is reported as the most prevalent *Eimeria* species in *M. musculus*, infecting the epithelial cells of the cecum and upper colon. This species can cause acute coccidiosis in rodents. *E. papillata* is commonly used in laboratory studies on anticoccidial agents, as it specifically infects *M. musculus*. *Eimeria hungaryensis* is a host-specific coccidian that primarily infects the genus *Apodemus*, consistent with our study findings. This species cannot be transmitted to *M. musculus, Microtus arvalis, Clethrionomys glareolus,* or *Cricetus cricetus*. *Eimeria miyairii* is an uncommon species that infects the epithelial cells of the villi and, occasionally, the glands of Lieberkühn in the small intestine of the Norway rat (*R. norvegicus*), its primary host. This species is host-specific and cannot infect *M. musculus, Sylvilagus floridanus, Cavia porcellus,* or *Spermophilus tridecemlineatus*. *E. musculoidei* has been identified in the upper ileum of *Mus (Leggada) musculoides* (its primary host) in Africa and is also suspected to infect the house mouse (*M. musculus*) in Asia (6,12,13).

There are few reports on *Eimeria* spp. infection in rodents in Iran, and most studies have been limited to genus-level identification.

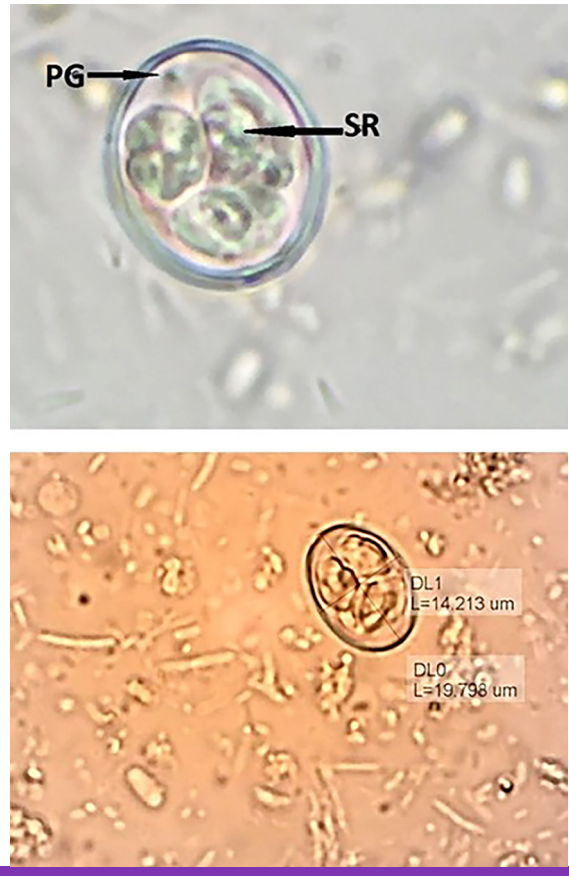



**Figure 1.** Oocysts of *E. musculoidiei* are spherical to ellipsoidal (16.9×19.7 µm; length-to-width ratio 1.16, average of 30 oocysts), with smooth, single-layered, pale yellow to brown walls (~1 µm thick), no micropyle, and 1-several polar granules (PG). Sporocysts are lemon-shaped (10.3×6.9 µm; length-to-width ratio 1.49), lack Stieda bodies, and contain coarse residual granules (SR). Host: *Mus musculus*

**Table 2.** Distribution of rodent species, including the number and percentage of males and females in the sample population (n=138)

| No | Rodent species | n/% | | Male | | Female | |
|---|---|---|---|---|---|---|---|
| 1 | *Mus musculus* | 49 | 35.50% | 37 | 26.81% | 12 | 8.70% |
| 2 | *Rattus norvegicus* | 32 | 23.20% | 20 | 14.49% | 12 | 8.71% |
| 3 | *Microtus socialis* | 30 | 21.73% | 19 | 13.77% | 11 | 7.97% |
| 4 | *Apodermus witherbyi* | 10 | 7.25% | 9 | 6.53% | 1 | 0.73% |
| 5 | *Ellobius lutescens* | 10 | 7.25% | 5 | 3.63% | 5 | 3.63% |
| 6 | *Meriones libycus* | 7 | 5.07% | 4 | 2.91% | 3 | 2.17% |
| Total | | 138 | 100% | 94 | 68% | 44 | 32% |

A study conducted in 2014 reported an *Eimeria* spp. infection rate of 22.5% in rodents from Meshkin Shahr, located in Northwestern Iran (14). Additionally, a study conducted in Iraq in 2022 reported a 28% infection rate in *M. musculus* (15). Another study in Egypt reports 19.9% *Emeria* spp. infection in *Psammomys obesus* (16). However, Reports on *Eimeria* infections in rodents from Iran and neighboring countries remain limited. This study provides the first detailed morphological identification of *Eimeria* species in rodents from Iran.

The first detection of *Cryptosporidium* spp. in mice dates back to 1907. Since then, more than 40 species and over 120 genotypes of *Cryptosporidium* spp. have been identified (17).

Many of these species pose a significant threat to human health and economically important animals, with rodents serving as crucial reservoirs for their transmission. Rodents can act as reservoirs for highly prevalent *Cryptosporidium* species such as *C. parvum*, which infects both humans and animals, and *C. meleagridis*, which primarily infects birds (18,19). Additionally, rodents can facilitate the adaptation of certain rare *Cryptosporidium* species to humans, including *C. ditrichi, C. mortiferum, C. tyzzeri,* and *C. viatorum* (20).

Most studies on rodents focus on their role as reservoirs of zoonotic agents, with *Cryptosporidium* being one of the most significant. Rodents can serve as reservoirs for *Cryptosporidium*, easily spreading thick-walled oocysts into food and water sources (8). Reports indicate that approximately 17-20% of rodents worldwide are infected with *Cryptosporidium* spp., with *C. parvum* being the most commonly detected species. Among rodent hosts, muskrats are considered the most relevant reservoir (4,21). Based on the results, all rodent species involved in the study can act as reservoirs for *Cryptosporidium* spp.

Several studies have reported *Cryptosporidium* spp. infections in rodents in Iran. A study conducted in 2016 in the Torkaman Sahra Region identified *Cryptosporidium* spp. in 6.6% of *R. norvegicus* samples (22). In the same year, another study reported that only 3% of rodent samples tested positive for *C. parvum* in Ahvaz City (22), while *Cryptosporidium* spp. was detected in just 1% of *R. norvegicus* specimens in Tehran (23,24). However, meta-analyses suggest that the overall prevalence of *Cryptosporidium* in rodents across Iran ranges from 18% to 20%. These findings indicate that *Cryptosporidium* spp. infection rates in Iranian rodent populations are consistent with those reported in other regions worldwide, highlighting the potential role of rodents as reservoirs for this parasite (25,26).


**Figure 2.** Oocysts of *E. falciformis* are spherical to slightly ellipsoidal (18.3×28.2 µm; length-to-width ratio ~1.7, average of 30 oocytes), with a single-layered wall, no micropyle, and a Stieda body (SB). Sporocyst residuum (SR) are present, with sporocysts measuring 12.2×7.2 µm. Oocysts are capable of enlarging to a size range of 13-24×15-26 µm. Host: *Mus musculus*

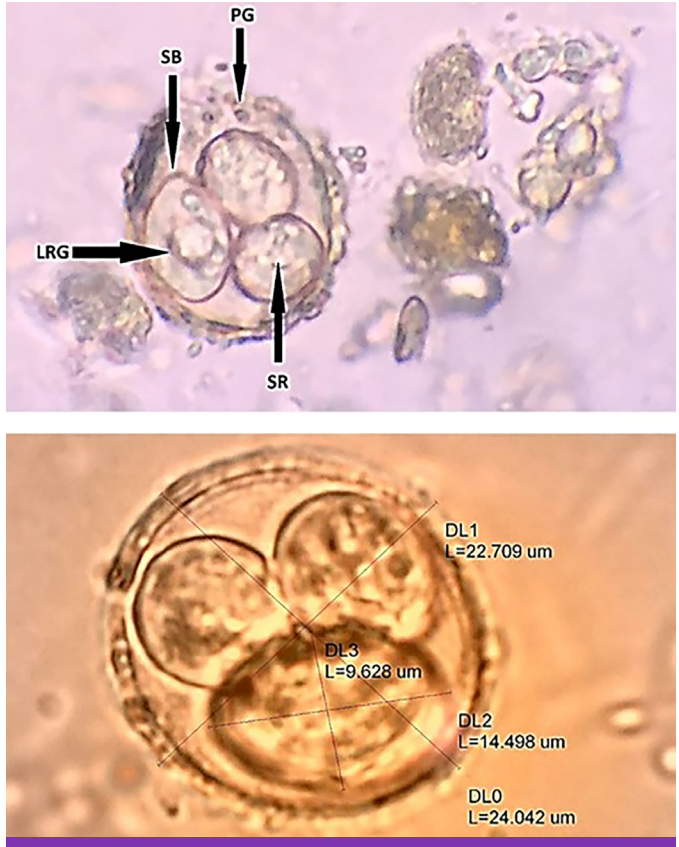


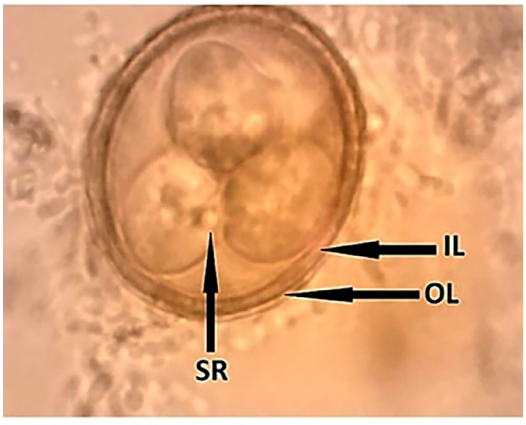

**Figure 3.** Oocysts of *E. papilata* are spherical to ellipsoidal, particularly at the poles, with a single-layered wall (~1.2 µm thick) and a yellowish-brown color. The wall may appear rough or coarse, sometimes exhibiting striations. Oocysts measure 19.2×22.4 µm (average of 30 oocysts), with a length-to-width ratio of ~1.16. They lack a micropyle, contain 1-3 oval-shaped polar granules (PG) and a large refractile globule (LRG), and possess Stieda bodies (SB) and sporocyst residuum (SR). Host: *Mus musculus*

### Study Limitations

The results of this study were based on morphometric measurements and taxonomic identification keys. However, molecular methods could not be applied, which represents a limitation since such approaches could have provided more accurate confirmation and deeper insights. Furthermore, although the presence of *Eimeria* and *Cryptosporidium* species in rodents was identified, their geographical and regional distribution patterns were not evaluated. Future studies should incorporate molecular techniques and spatial analyses to provide a more comprehensive understanding of the epidemiology of these parasites.

## CONCLUSION

In conclusion, the findings of this study provide the first detailed morphological descriptions of *Eimeria* species infecting rodents in Iran. The rodent host species identified in this study align with the results of similar studies conducted worldwide, further supporting the consistency of rodent species as hosts for various *Eimeria* species across different geographic locations. The results of *Cryptosporidium* spp. infection in terms of species were consistent with previous studies conducted in Iran and



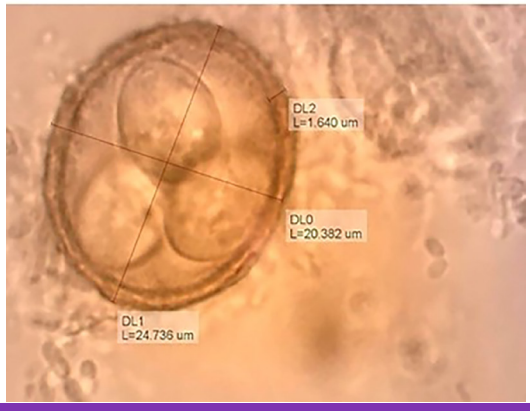
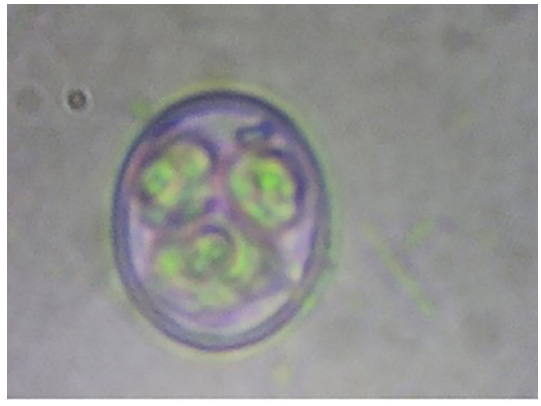




**Figure 4.** Oocysts of *E. miyairii* are spherical with a thick, rough, yellowish-brown wall, measuring 24.73 × 20.38 µm, an average of 30 oocysts. Sporocysts measure 11.23×8.6 µm. The wall is ~1.6 µm thick, radial, and striated, with two layers (the inner layer, IL, thicker than the outer layer, OL, and sometimes striated). Oocysts lack micropyle, polar granules, and oocystic remnants but contain sporocystic remnants (SR). Host: Rattus norvegicus



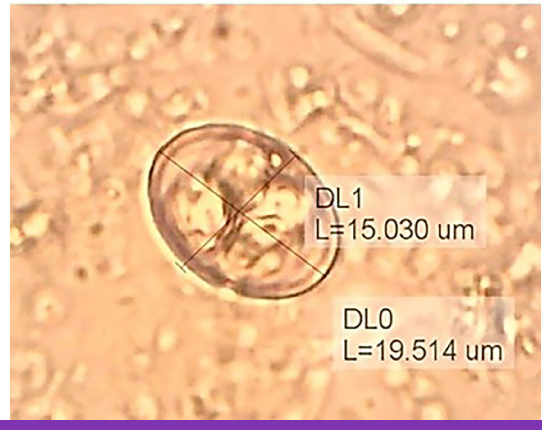



**Figure 5.** Oocysts of *E. hungaryensis* are spherical, with an average size of 18×20 µm. The wall is single-layered, thick, and yellowish-brown, occasionally appearing rough or coarse. They lack micropyle, oocystic remnants, and polar granules. In unsporulated oocysts, the internal space is filled with sporont granules. Host: *Apodemus witherbyi*

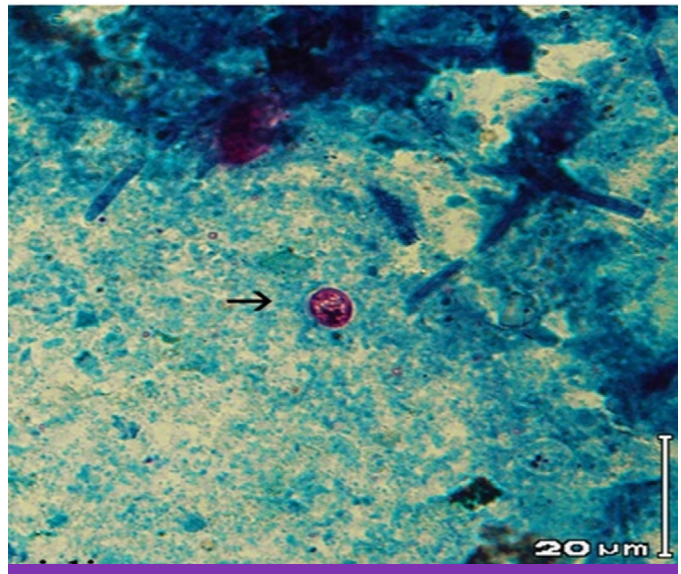



**Figure 6.** Oocyst of *Cryptosporidium* spp. in modified Ziehl-Neelsen staining: Oocyst is egg-shaped or spherical (4.35 µm), with a smooth wall, lacking a micropyle and sporocysts. They possess oocystic remnants and contain long, crescent-shaped sporozoites that are somewhat banana-shaped. Host: *Mus musculus*

**Table 3.** Infection rates of *Eimeria* spp. and *Cryptosporidium* spp. in different rodent species

| Rodent species | *Eimeria* spp. (n=) | *Eimeria* spp. (%) | *Cryptosporidium* spp. (n=) | *Cryptosporidium* spp. (%) |
|---|---|---|---|---|
| *Mus musculus* | 4 | 2.90% | 1 | 0.73% |
| *Rattus norvegicus* | 2 | 1.45% | 1 | 0.73% |
| *Apodemus witherbyi* | 2 | 1.45% | 1 | 0.73% |
| *Microtus socialis* | 0 | 0% | 1 | 0.73% |
| *Ellobius lutescens* | 0 | 0% | 1 | 0.73% |
| Total | 8 | 5.79% | 6 | 4.34% |

worldwide. However, the infection rate observed in this study was lower than the average rates reported in Iran, which could be attributed to factors such as the number of cases examined or the lack of molecular diagnostic techniques in this study.

## *Ethics

**Ethics Committee Approval:** This study did not require ethical approval, as no live animals were harmed or directly handled during the research process.

**Informed Consent:** Not necessary.

## Footnotes

## *Authorship Contributions

Surgical and Medical Practices: M.T., B.R., Concept: M.T., B.R., Design: M.T., B.R., B.E., S.Z.A., Data Collection or Processing: B.R. Analysis or Interpretation: M.T., B.R., B.E., S.Z.A., Literature Search: M.T., B.R., B.E., S.Z.A., Writing: S.Z.A., M.T.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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# Parasites and Parasite Eggs Detected in Laboratory Mice in Bursa

## *Bursa'da Laboratuvar Farelerinde Tespit Edilen Parazitler ve Parazit Yumurtaları*

Oya Girişgin[1], Dilara Karaman[2], Ahmet Onur Girişgin[3]
[1]Bursa Uludağ University Karacabey Vocational School, Department of Veterinary, Bursa, Türkiye
[2]Yıldız Technical University Faculty of Chemistry-Metallurgy, Department of Bioengineering, İstanbul, Türkiye
[3]Bursa Uludağ University Faculty of Veterinary, Department of Parasitology, Bursa, Türkiye

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## ABSTRACT

**Objective:** A mouse infected with parasites is not a suitable model for use in experiments, and therefore, it is necessary to know whether it is infected. In this study, the aim is to investigate the endo and exoparasites in BALB/c laboratory mice.
**Methods:** In this study, the presence of parasites in 250 mice obtained from Bursa Uludağ University Experimental Animal Centre was investigated by faecal flotation and cellophane tape methods. In addition, helminths recovered during necropsy of selected mice were examined to confirm species identification.
**Results:** According to the results of the research, ectoparasites found in mice were mites such as *Otodectes cynotis*, *Myobia musculi* and *Myocoptes musculinus*, and endoparasites were nematodes of the species *Syphacia obvelata*, *Syphacia muris* and *Aspiculuris tetraptera*. In addition to adults of these species, many unidentified parasite eggs were also found. An *Aspicularis tetraptera* nematode exhibiting an unusual cervical alae structure not previously described in the literature was detected.
**Conclusion:** Although helminths from the *Strongylidae* and *Heligmosomidae* families were not found in the necropsy, helminth eggs belonging to this family were found in the fecal flotation. This study has presented different parasites detected in laboratory mice and original images were presented for some samples with unusual morphological structures.
**Keywords:** *Aspicularis tetraptera*, Bursa, ectoparasites, laboratory mouse, *Syphacia*

## ÖZ

**Amaç:** Parazitler ile enfekte bir fare, deneylerde kullanmak için uygun bir model değildir ve bu nedenle enfekte olup olmadığının bilinmesi gerekir. Bu çalışmada BALB/c laboratuvar farelerinde bulunan endo ve ekzoparazitlerin araştırılması amaçlanmıştır.
**Yöntemler:** Bursa Uludağ Üniversitesi Deney Hayvanları Merkezi'nden temin edilen 250 faredeki parazitlerin varlığı fekal flotasyon ve selofan bant yöntemleriyle araştırılmıştır. Ayrıca, seçilen farelerin nekropsilerinden elde edilen helmintler tür teşhisi yapılması için incelenmiştir.
**Bulgular:** Yapılan araştırmanın sonuçlarına göre, farelerde bulunan ektoparazitler; *Otodectes cynotis*, *Myobia musculi* ve *Myocoptes musculinus* gibi akarlar, endoparazitler ise *Syphacia obvelata*, *Syphacia muris* ve *Aspiculuris tetraptera* türündeki nematodlardı. Bu türlerin erginleri yanı sıra çok sayıda teşhis edilemeyen parazit yumurtasına da rastlanmıştır. Ayrıca literatürde tanımlanmamış farklı bir servikal alae yapısı sergileyen *Aspicularis* cinsi bir nematoda rastlanmıştır.
**Sonuç:** Nekropside, *Strongylidae* ve *Heligmosomidae* ailesinden helmintler bulunamamış olsa da, fekal flotasyonda bu aileye ait helmint yumurtalarına rastlanmıştır. Bu çalışma ile Bursa'da laboratuvar farelerinde saptanan farklı parazitler ortaya konmuş ve ilginç morfolojik yapılara sahip bazı örnekler konusunda orijinal görseller sunulmuştur.
**Anahtar Kelimeler:** *Aspicularis tetraptera*, Bursa, ektoparazitler, laboratuvar faresi, *Syphacia*

## INTRODUCTION

The laboratory mouse is one of the most frequently used model organisms in experimental research due to its physiological similarity to humans. Some parasites carried by mice are known to affect the immune system (1). In addition, some parasitic infections cause observable changes in the behavior of mice (2). Coprophagic mice serve as ideal hosts for sustaining parasite life cycles; however, infected mice are unsuitable for experimental use, making infection screening essential. For these reasons, it is helpful to

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**Address for Correspondence/Yazar Adresi:** Dilara Karaman PhD, Yıldız Technical University Faculty of Chemistry-Metallurgy, Department of Bioengineering, İstanbul, Türkiye
**E-mail/E-Posta:** dilara.karaman@yahoo.com **ORCID ID:** orcid.org/0000-0003-4386-8531



Türkiye PARAZİTOLOJİ Dergisi

have information about the parasite load of mice before *in vivo* studies with mice.

Laboratory mice can be infested with different types of external parasites. Due to the environment they are in, infestation with mites is generally observed. *Myobia musculi*, *Myocoptes musculinus* and *Radfordia affinis* are more common among these mites, while *Demodex musculi* and *Ornithonyssus bacoti* are less common species. None of these ectoparasites are zoonotic. These mites feed on intercellular fluid and skin fragments (3).

Internal parasites that can be seen in laboratory mice are of two groups: protozoa and helminths. Cestode parasites are very rare in laboratory mice with modern conditions, while pinworms are much more common. *Hymenolepis diminuta*, *H. nana* and *Rodentolepis microstoma* are the three cestode species that can be seen in laboratory mice (3).

*Syphacia obvelata* is the oxyurid nematode that most commonly infects laboratory mice. Eggs are approximately 134x36 µm in size and are banana-shaped. *Syphacia muris* and *Trichuris muris* are other important species that less commonly infect laboratory mice. Simultaneous infection with both *S. obvelata* and *S. muris* is very rare. *Aspiculuris tetraptera* is another pinworm species most commonly found in laboratory mice. *Heligmosomoides polygyrus* is routinely used for parasitic immunology and the evaluation of the effects of anthelmintic compounds (3,4).

Protozoan parasites can be found in the intestines or tissues. Those found in tissues include *Klossiellla*, *Hepatozoon*, *Babesia*, *Toxoplasma* and *Plasmodium*. These are almost never found in modern laboratory mice. Only *Spironucleus muris* is occasionally found in modern laboratory mice, but *Giardia muris* and *Crytosporidium muris* are extremely rare. Non-pathogenic enteric protozoan species include *Entamoeba muris*, *Chilomastrix bettencourti* and various species of trichomonads (3).

The aim of this study is to determine the parasite species carried by some laboratory mice used in Bursa and to present original visual data on their morphology.

## METHODS

### Preparation of Experimental Animals

The experimental animals used in this study are the BALB/c type of mice known as laboratory mice, *Mus musculus albinus*. These albino mice are 1-2 years old, weigh approximately 28-40 g, are sensitive to light and are active at night. Fifty-four BALB/c mice of both sexes were obtained from the Bursa Uludağ University Experimental Animal Breeding and Application Center. Mice infected with oxyurid nematodes were selected from 250 mice by cellophane tape and fecal flotation methods. The reason for the special selection of mice infected with oxyurid nematodes was that these mice would be treated with extracts that were thought to have anthelmintic effects. For studies on extracts (5-7), images of various endo- and ectoparasite eggs observed in the selected mice were also recorded while the mice were being cared for. The animals were kept in standard polypropylene cages at 20-24 °C, 55% relative humidity, and fed ad libitum with standard pellets and tap water (Figure 1).

All experiments were approved by the Bursa Uludağ University Experimental Animals Local Ethics Committee (decision no: 2017-10/07, date: 11.07.2017). A total of 54 mice (approximately 30 cages) were kept by washing their cages every two days and renewing their food, water and bedding. The cages were washed with water only and dried with paper towels. The waterers were washed with boiling water, salt and alcohol and rinsed thoroughly.

### Detection of Parasite Eggs

### Perianal Tape Method

The cellophane tape method is recommended especially for the determination of *S. obvelata* infection (8). In order to apply this method, approximately 9 cm long cellophane tape was pressed on the perianal region of the mouse 10-15 times and stuck on the microscope slide, and then parasite eggs and ectoparasites were examined (Figure 2). After the tape was cut with a pair of scissors and stuck on the slide properly, all samples were brought to the parasitology laboratory. Eggs and parasites were examined along the slide with a 5x objective. Pictures of some parasite eggs were taken with a light microscope. The preparations are kept in the parasitology laboratory where the study was conducted.

### Flotation Method with Fulleborn Technique

To apply the fecal flotation method, mice were placed in separate cages and a single mouse was kept in each cage during the experiment. Fecal materials were collected from each mouse cage with forceps and fecal samples were placed in sterile plastic sample containers previously numbered according to the number of each mouse and wrapped in labeled nylon and transported from the Experimental Animal Center to the parasitology laboratory.

Fecal samples were collected in plastic cups numbered with each mouse number. Saturated salt water solution prepared and cooled beforehand was used for fecal flotation. A saturated salt water was added little by little to the stool and it was suspended with a plastic baguette. The suspension was filtered into another container with a wire strainer and salt water was added so there was a 1-2 cm gap in the container. Two coverslips were placed on it so that they would float and waited for 15-20 minutes. The coverslip was lifted parallel to the water surface with forceps and placed on the slide, taking care not to drop, and examined under a microscope at 5× and 10× magnifications.

The studies of Pritchett (4) were used for the identification of eggs, and Hedrich (3) was used for the identification of external parasites.

**Figure 1.** A group of experimental mice

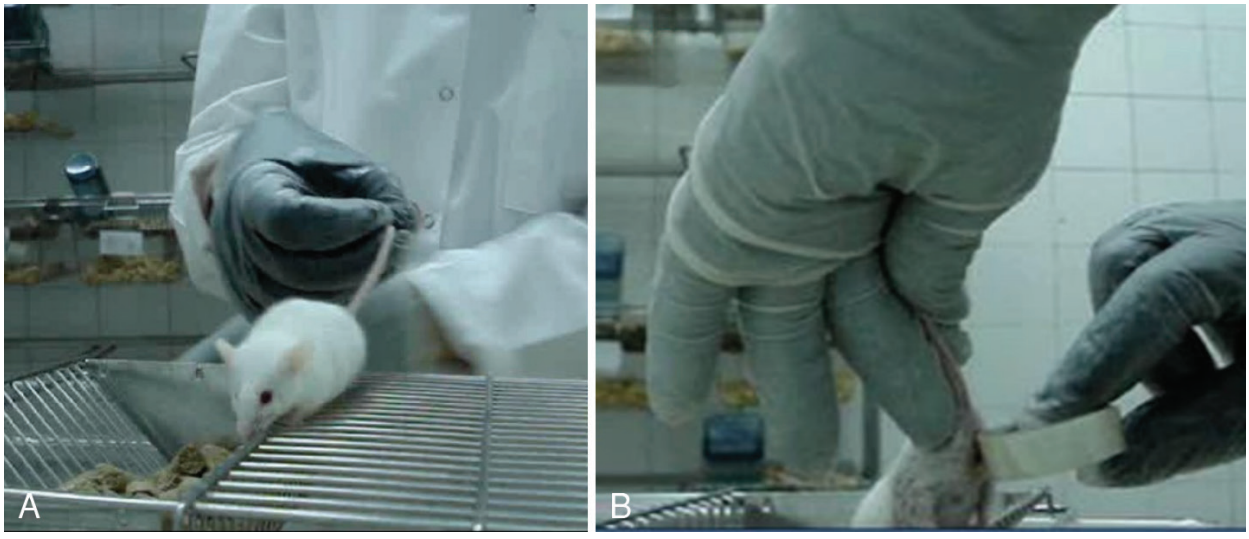


**Figure 2.** Application of the perianal tape method alone to laboratory mice. **A)** Each mouse is placed in its cage, **B)** The reverse-wound cellophane tape is pressed 15 times on the mouse rectum along the 9 cm tape

### Detection of Helminths at Necropsy

In order to show the location of the abdominal organs removed during the necropsy phase of this study, the abdominal organs of *Mus musculus* are schematically outlined and given in Figure 3.

Mice were euthanized by taking blood from the heart under sevofluron anesthesia. Each mouse was laid down with its abdomen facing up and opened with scissors from the anus to the anterior. All internal organs of the mice (esophagus, lungs, stomach, liver, kidneys, heart and intestines) except the brain, pancreas and reproductive organs were opened one by one and placed in petri dishes containing saline or 70% alcohol and examined under a stereomicroscope. Helminths in these organs were collected using a brush, dropper and needle tip and placed in labeled vials containing 70% alcohol. The date of collection, helminth species, mouse number from which they were collected and the name of the organ from which they were collected were written on each vial.

Helminths suitable for examination under the light microscope were used in glycerin water to identify species and take their pictures under a Nikon brand light microscope. The information given by Pritchett (4) was used as a key for identification. All helminths were fixed on slides with the numbers of mice they were taken from and turned into permanent preparations. In order for the glycerin to enter between the slide and the coverslip, alcoholic water was drawn from one end of the coverslip with blotting paper, while glycerin was slowly dropped from the other end to fill the coverslip and replace it with the alcoholic water. After this, the excess material on and around the coverslip was cleaned with blotting paper again, transparent nail polish was applied to the edges of the coverslip and left to dry. All preparations are kept in the Parasitology Laboratory of the Biology Department of the Bursa Uludağ University Faculty of Arts and Sciences.

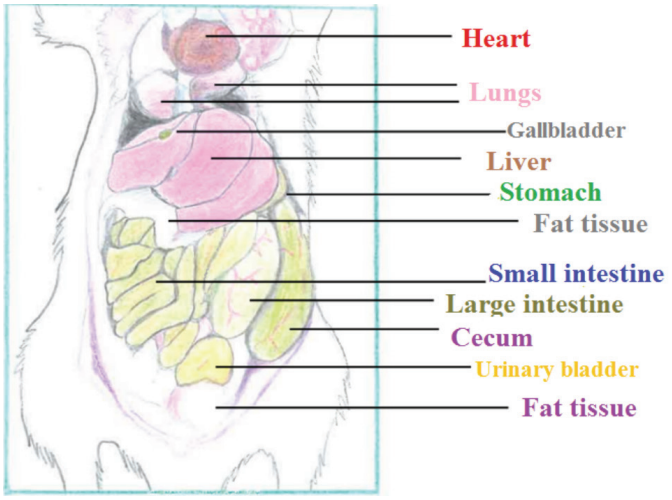


**Figure 3.** Abdominal organs of the mouse (original)

### Statistical Analysis

The infection rate with the oxyurid nematodes among laboratory mice was calculated, and a chi-square test was conducted on the data of infected mice.

## RESULTS

A total of 808 oxyurid nematodes were found in the small intestine, cecum and colon of laboratory mice. Of the 250 mice screened for infection, 54 (21.6%) were naturally infected with at least one nematode species. The infection rate in female mice was 16% (24/150), while the infection rate in male mice was higher than in females, at 30% (30/100). The significance of this data between genders was also confirmed by the chi-square test ($p<0.05$).

In fecal flotation tests, approximately 2000 parasite eggs were counted for some mice. These mice were numbered 36E, 43E, 21E, 42E and 40E. Since the letter E represents a male mouse, it was observed that all the most severely infected mice were male mice.

### Helminth Eggs Observed in Fecal Flotation

In this study, the main criterion for the investigation of parasite eggs was the morphology of the eggs. As can be seen in the images below, different morphologies were encountered in the

microscope images of the eggs. Although it is easy to distinguish the elliptical morphology of *Aspicularis tetraptera* eggs and the banana-shaped *Syphacia obvelata* eggs, it is quite difficult to distinguish ectoparasite eggs and some helminth eggs by looking only at their morphology. Nevertheless, when the egg sizes and shapes are considered, an egg belonging to the *Heligmosomoides polygyrus* species and another egg belonging to the *Strongylidae* family were found. Although these species are very rare in laboratory mice, these infections can still be encountered in mice. Images of these nematode eggs are shown in Figure 4.

Some parasite eggs could not be identified due to their different morphologies. Although the egg in Figure 5A resembles an *A. tetraptera* egg at first glance, genetic analyses are required for the identification of the two eggs in Figure 5.

When various parasite eggs stuck to the cellophane tape were examined, it was seen that the majority of them were *S. obvelata* eggs. Ectoparasite eggs were seen in some samples. Although they resembled *S. obvelata* eggs in shape, they were slightly larger in size and had dark granular structures inside (Figure 6).

## Ectoparasite Eggs Observed in Fecal Flotation

A large number of insect eggs were encountered in fecal flotation and some of them could be identified. Various egg types in the developmental stages of insects and the condition of the embryo inside the transparent egg shell are shown in Figure 7.

## Ectoparasites Observed in Fecal Flotation and Cellophane Tape

Some of the ectoparasites observed in fecal flotation and cellophane tape during the screening of mice for pinworm infection were photographed. Two different species of mite, *Myocoptes musculinus* and *Myobia musculi* were identified according to literature (3). Females can be seen carrying eggs, and are larger than males (Figure 8).

In insects, molting is inevitable at some stages of development. Figure 8G shows a *Myobia musculi* photographed while performing this process. The ectoparasite shown in Figure 8F, identified as a male *Myocoptes musculinus*, appears shrunken, likely due to osmotic effects of the saline solution. In addition to these insects, various mites were also encountered both in the fecal flotation and in the cellophane tape. A picture of the *Otodectes cynotis* mite is given in Figure 9.

## Helminths Observed at Necropsy

A large number of nematodes were found in the necropsies of mice. Some of them show morphological features different from those normally expected. Figure 10 shows the interesting anterior structure of a nematode shrinking in salt water. It is difficult to determine the sex of this specimen because its posterior part has been severed by the scalpel. Still, this type of annular cervical alae structure can be found in the genus *Aspicularis*. No nematode similar to this specimen was found in the literature review. Since nematodes other than *Syphacia obvelata*, *S. muris* and *A. tetraptera* are rarely detected in laboratory mice, the specimen in Figure 10 was not expected to be an alien species belonging to a genus other than *Aspicularis* or *Syphacia*.

Normal morphological characters are observed in the *A. tetraptera* specimens shown in Figure 11. There is a developed bulbous cervical alae around the lips. The body of the male individual is thinner and smaller than the female. The appearance of the tail in male *A. tetraptera* is very characteristic, the cloacal apparatus on the tail is clearly visible, allowing for reliable sex determination.

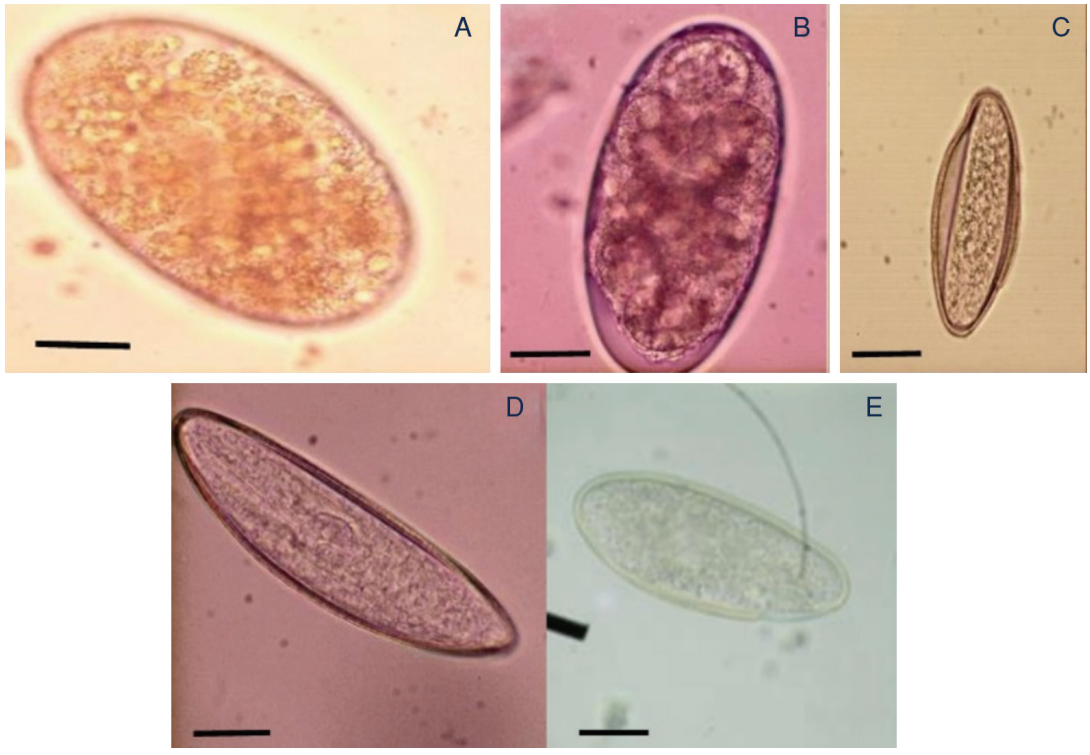


**Figure 4.** Helminth eggs detected in the faeces of laboratory mice (scale bar: 20 µm): **A)** *Heligmosomoides polygyrus*, **B)** *Strongylidae*, **C)** *Aspicularis tetraptera*, **D)** *Syphacia obvelata*, **E)** *Syphacia muris*

Females were carrying eggs in many of the examined specimens. The numerous eggs carried by this type of nematode under its transparent body cover could be distinguished by its characteristic elliptical appearance (Figure 11B). A veil-like cervical alae structure is characteristic in female *A. tetraptera* specimens (Figure 12B), but this structure was not observed in *S. obvelata* adults. Interestingly, an asymmetrically developed cervical alae structure was observed in the female *Syphacia* specimen (Figure 13A). In another female *Syphacia* nematode, a vulva structure that protrudes outwards was observed, unlike other females (Figure 13B). Many specimens of the genus *Syphacia* were egg-free, while one specimen was recorded during spawning (Figure 14).

In Figure 15, the body parts of female and male adults of *S. obvelata* and *A. tetraptera* are shown schematically.

## DISCUSSION

Oxyurids (pinworms) are cosmopolitan nematode parasites of public health importance (9). Mild infection in animals is usually subclinical, but intense parasite infection can cause decreased activity, weight loss and sometimes intestinal lesions (10,11). In addition to the negative impact on animal health, some members of the Oxyuridae family have zoonotic potential, but do not cause significant harm (4). Among the oxyurids, only *S. obvelata* is known to be transmitted to humans (12). Male mice carry more parasites in pinworm infections than females (13), and this finding was confirmed in our study, where 30% of males and 16% of females were affected.

*A. tetraptera* and *Syphacia* sp. are located in the large intestine (14). In this study, the fact that in some examples *Syphacia* eggs were not found on cellophane tape and that such eggs were found in the fecal flotation of mice indicates that *S. obvelata* does not always migrate to the anus to lay eggs. In addition, a picture showing that it lays eggs in the large intestine (Figure 14) supports this.

In a previous study, laboratory rats and mice in Ankara were investigated for *Syphacia* infection and only *S. obvelata* was found in the mice, and the infection rate was found to be between 21 and 100% (15). In the current study, both *S. obvelata* and *S. muris* eggs were observed in laboratory mice in Bursa. However, *S. muris* infection was detected in only one out of the 250 mice (0.4%) examined. Among the 250 mice scanned, at least one instance of either *S. obvelata* or *A. tetraptera* infection was found in 54 of them (21.6%).

Another study conducted in Ankara between 2011 and 2012 revealed that all 283 BALB/c mice cared for in the laboratory were infected with at least one of the helminths *A. tetraptera*, *S. obvelata* and *H. nana*. *A. tetraptera* was found in all mouse cages (16). As for the current study, although the majority of infected mice were infected with *A. tetraptera*, this rate is not 100%.

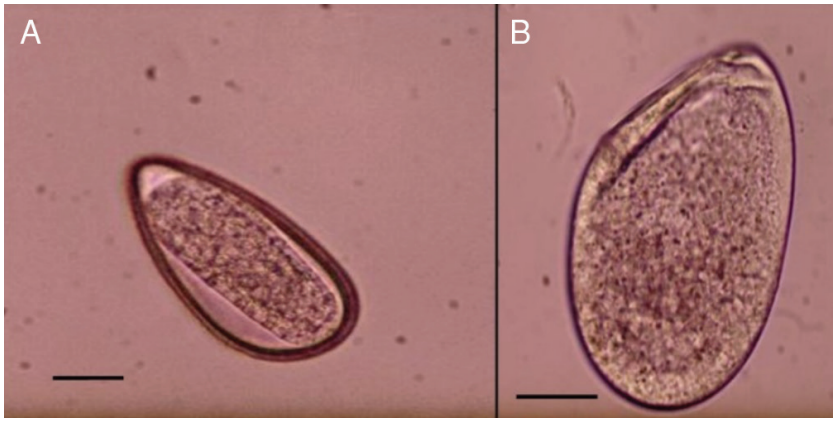


**Figure 5. A,B)** Two unidentified eggs detected in faecal flotation (scale bar: 20 µm)

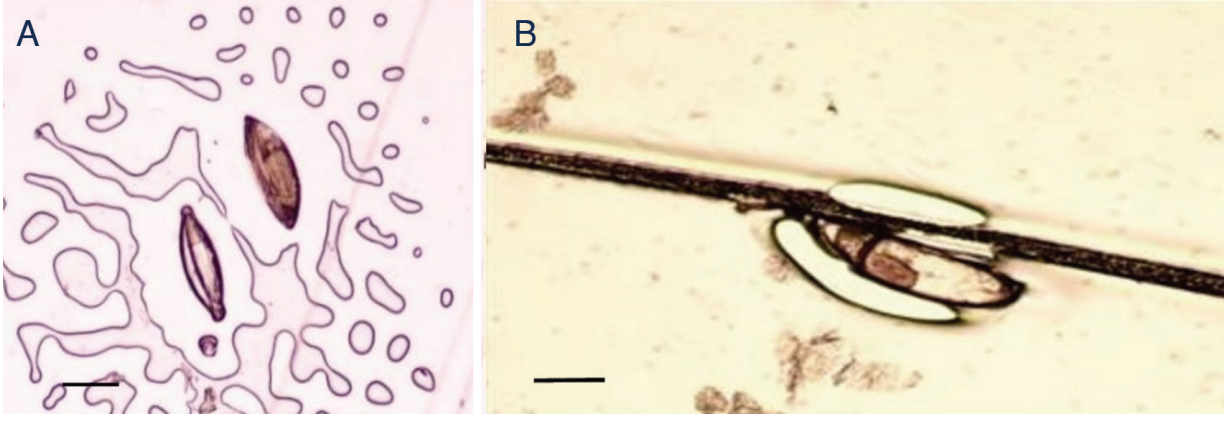


**Figure 6.** Parasite eggs detected on cellophane tape (scale bar: 50 µm). **A)** *Syphacia obvelata*, **B)** A mite egg stuck to a mouse hair

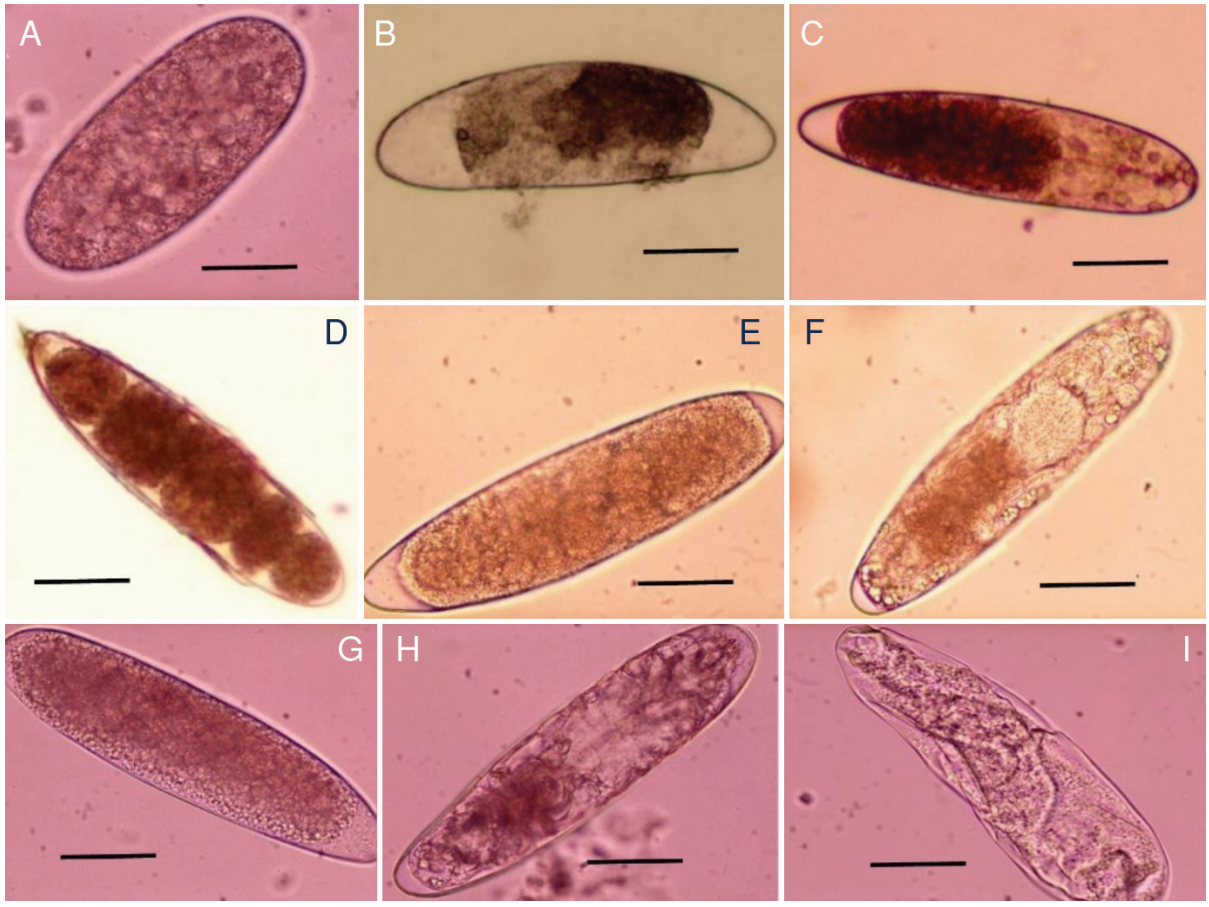


**Figure 7.** Various ectoparasite eggs seen in fecal flotation (scale bar: 25 µm). **A)** *Otodectes* (mite) egg, **B-H)** Various stages of mite eggs, probably of the genus *Chirodiscoides*, **I)** An abandoned eggshell of a mite

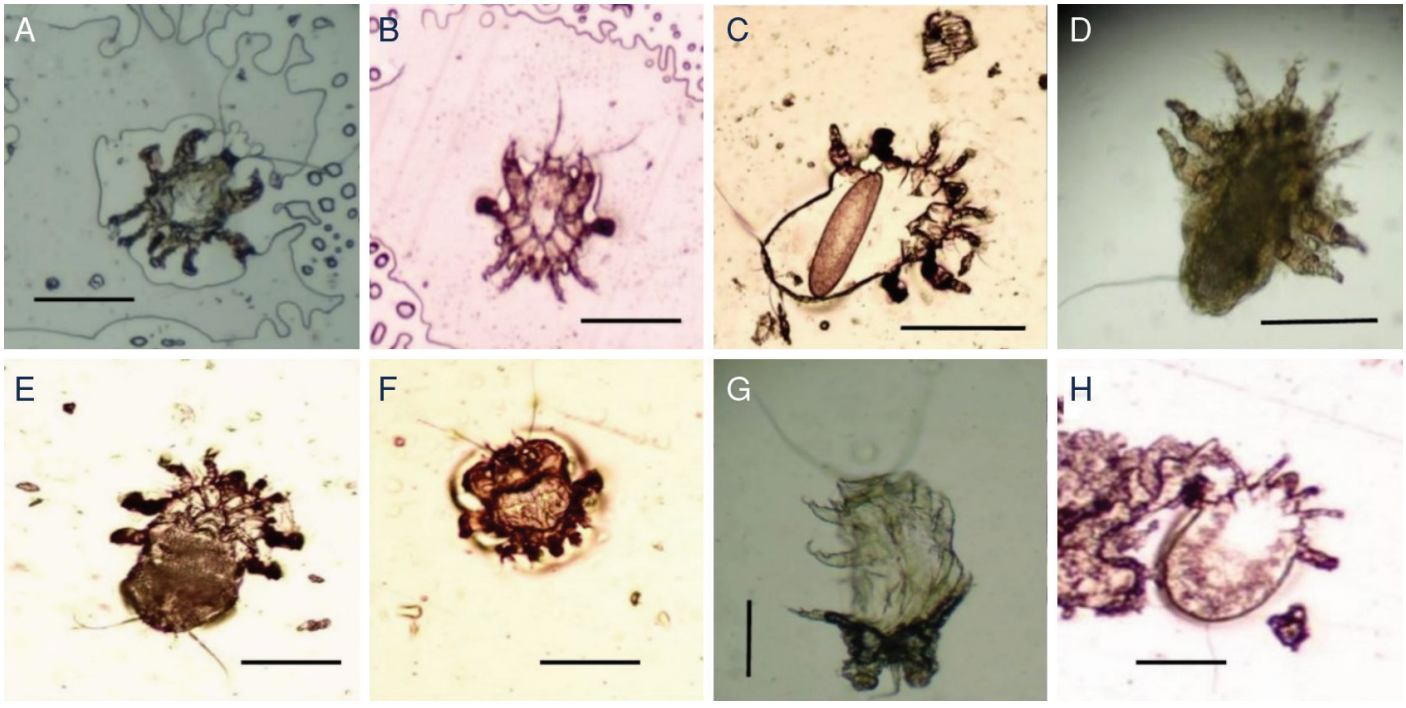


**Figure 8.** Some ectoparasites observed in mice (scale bar: 100 µm). **A)** and **B)** A *Myocoptes musculinus (M. musculinus)* male seen on cellophane tape, **C)** The remains of a *M. musculinus* with eggs developed inside, **D)** and **E)** *M. musculinus* female seen in fecal flotation, **F)** Shrunken *M. musculinus* male seen in fecal flotation, **G)** *Myobia musculi* shedding its coat, and **H)** An unidentified ectoparasite on cellophane tape

*Myocoptes musculinus* infestation in laboratory mice was observed in mice brought from abroad to Akdeniz University's laboratory (17). The agents were detected in all mice from the same herd (100%) that showed clinical signs, and were treated with the addition of ivermectin to their drinking water. In our study, no clinical signs were observed in the mice; this mite was detected during the cellophane tape or flotation technique.

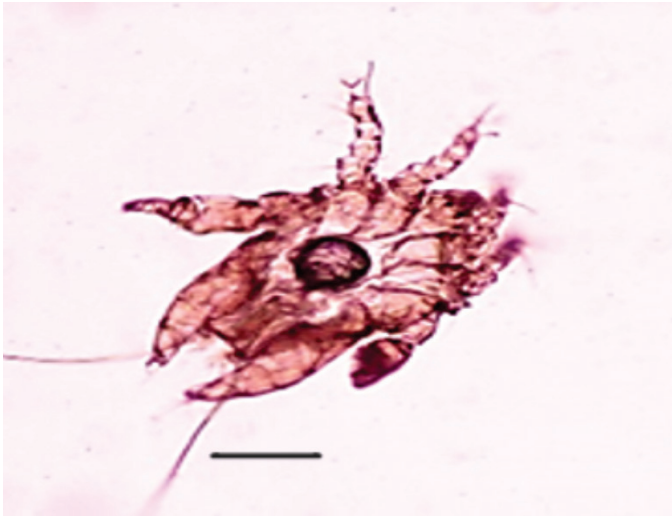

**Figure 9.** *Otodectes cynotis* (ear mite), female (scale bar: 100 µm)

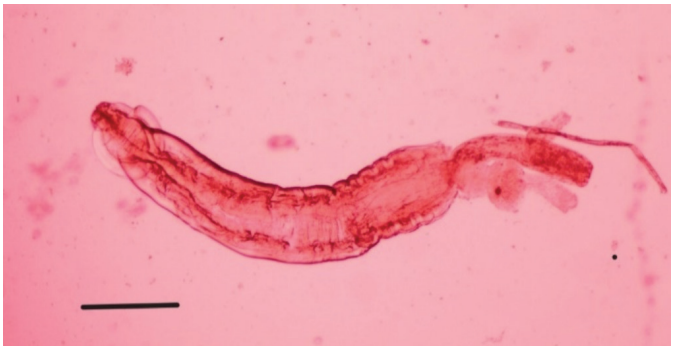

**Figure 10.** An *Aspicularis* with a different cervical alae structure. The posterior part was severed by a scalpel blow while opening the intestine of a mouse (scale bar: 0.5 mm)

According to the literature review, it was concluded that *Mus musculus* can be the host of at least 109 different species of parasites. Among the ectoparasites carried by the house mouse, *Xenopsylla cheopis*, *Nosopsyllus* spp. and *Rhipicephalus* spp. are vectors for important zoonotic diseases. Among the endoparasites, twelve species of helminths and two species of protozoa are species that can also cause disease in humans (18). However, trematodes are not found in laboratory mice because trematodes require intermediate hosts for their development, which cannot survive in laboratory animal breeding areas.

Laboratory mice can only be infected with a limited number of parasites. Among these parasites, *A. tetraptera*, *S. obvelata*, *S. muris*, *Strobilocercus fasciolaris*, *H. nana* and *H. diminuta* species of helminths have been identified by various researchers (14). In the present study, two parasite eggs were considered to belong to *Heligmosomoides polygyrus* and *Strongylidae* because they were morphologically similar in appearance. *H. polygyrus* is a nematode species widely used for research purposes and is not found in mice raised in modern laboratories (3). Therefore, in order to reach a definitive conclusion, it is necessary to develop the eggs or compare the sequence similarity by genetic analysis. In addition to these eggs, a large number of *A. tetraptera* and *S. obvelata* eggs were found, and the eggs of these oxyurid species can be easily distinguished by their specific appearance, and adults of these species were also seen at necropsy.

*S. obvelata* is zoonotic, and the first report of human infection occurred in a child from the Philippines, when eggs and two mature females of the parasite were identified (19). In the same year, oxyurid eggs were reported in 429 cases among 140,000 soldiers examined in Texas, Oklahoma, New Mexico, and Arizona in the United States (20). In 2009, 25 of 200 patients (12.5%) screened for infection had unexplained abdominal pain and eosinophilia, which were reported to be associated with *Syphacia* spp. infection (21). There are also records of *S. obvelata* eggs in mummified human bodies from Nubia dating from 700-300 BC (22).

According to the study on the morphology of *S. obvelata*, the body of females was measured as 2.9-4.6 (3.5±0.1) mm in length and 0.12-0.23 (0.15±0.001) mm in width based on 10 mature specimens (23). In the present study, the length of the

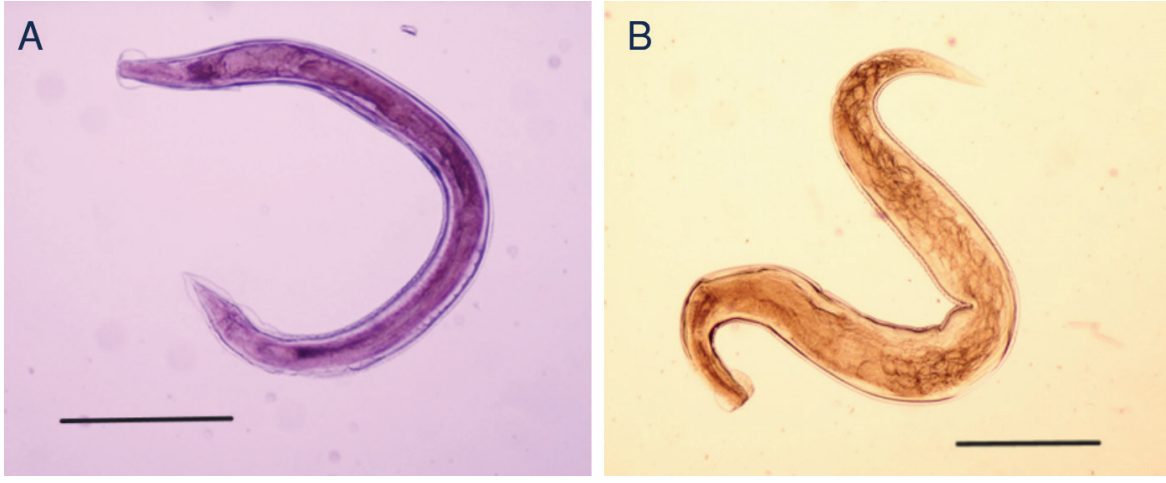


**Figure 11.** Two examples of the *Aspicularis* genus. **A)** Male *Aspicularis tetraptera*, **B)** Female *Aspicularis tetraptera* (scale bar: 0.7 mm)

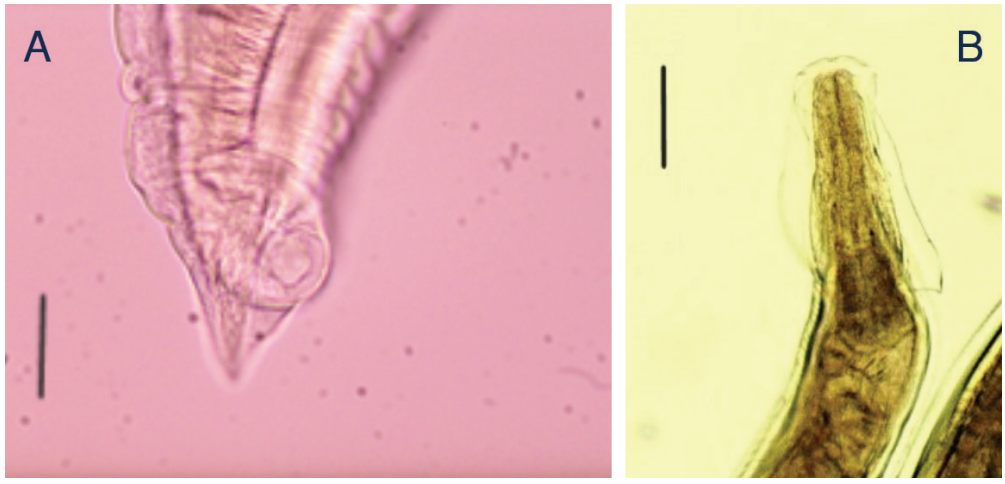


**Figure 12.** Some morphological observations in *Aspiculuris tetraptera (A. tetraptera)*. **A)** Male *A. tetraptera* tail (scale bar: 0.1 mm), **B)** Veil-like cervical alae of a female *A. tetraptera* (scale bar: 0.2 mm)


**Figure 13.** Two female individuals of the genus *Syphacia*. **A)** Asymmetrically developed cervical alae (scale bar: 0.5 mm), **B)** Vulva clearly protruded (scale bar: 0.4 mm)

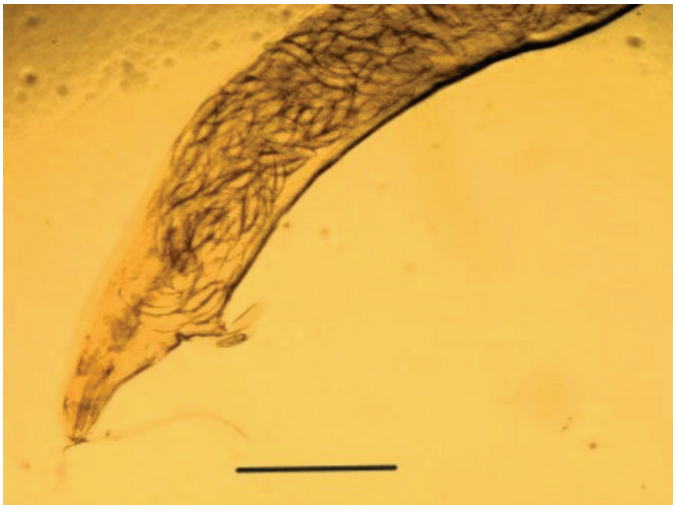

**Figure 14.** An egg-laying *Syphacia obvelata* (scale bar: 0.5 mm)

helminths was not measured; hundreds of pinworms of various sizes, including newly hatched larvae, were collected. All collected specimens were first identified under a stereomicroscope and then under a light microscope, and these specimens are preserved in the parasitology laboratory.

*A. tetraptera* is the only species of the genus *Aspicularis* and has been found to infect mice extensively in this study, as in many other studies (14-16). *A. tetraptera* females are characterized by having a veil-like cervical alae, which was seen under the microscope in almost all females in this study, but in one specimen the cervical alae were quite different, the picture of which is shown in the results (Figure 10). For the specimen with the severed posterior shown in Figure 10, since a severed posterior image filled with *Aspicularis* eggs was also taken, this species is possibly a different species of *Aspicularis* with its very different circular cervical alae. A similar difference was also seen in an adult of *Syphacia* (Figure 13A). Although a definitive conclusion cannot be reached regarding species identification since polymerase chain reaction analysis of these two samples could not be performed,

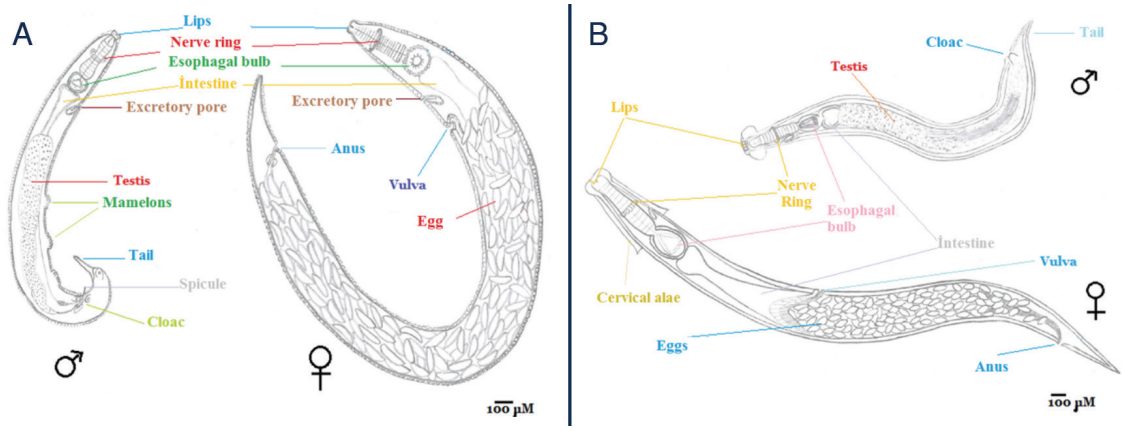


**Figure 15.** Schematic representation of oxyure nematodes. **A)** *Syphacia obvelata*, **B)** *Aspicularis tetraptera* female and male individuals (original)

if oxyurids similar to these two samples are encountered, their genetic analysis may be useful for the discovery of new species.

## CONCLUSION

With this study, some examples of the most common parasites found in laboratory mice, showing different morphologies, are presented with pictures and new visual data provided to researchers working in this field. Even if clinical signs aren't present, different types of parasites can be observed in laboratory animals. Scientists conducting studies in mice are advised to first treat potential parasites in the herd before beginning experimental studies.

### *Ethics

**Ethics Committee Approval:** All experiments were approved by the Bursa Uludağ Experimental Animals Local Ethics Committee (decision no: 2017-10/07, date: 11.07.2017).

**Informed Consent:** This study is an animal experiment, so patient consent is not required.

### Footnotes

### *Authorship Contributions

Surgical and Medical Practices: D.K., Concept: D.K., A.O.G., Design: O.G., D.K., A.O.G., Data Collection or Processing: D.K., Analysis or Interpretation: O.G., D.K., A.O.G., Literature Search: O.G., D.K., Writing: O.G., D.K., A.O.G.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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# Effectiveness of Oral Ivermectin Treatment in Cases Unresponsive to Classical *Demodex* Blepharitis Treatment

## *Klasik Demodeks Blefarit Tedavisine Yanıtsız Olgularda İvermektin Etkinliği*

Oktay Alver[1], Derya Doğanay[2], Selim Doğanay[3]

[1]Bursa Uludağ University Faculty of Medicine, Department of Medical Microbiology, Bursa, Türkiye
[2]Çekirge State Hospital, Clinic of Ophthalmology, Bursa, Türkiye
[3]Bursa Uludağ University Faculty of Medicine, Department of Ophthalmology, Bursa, Türkiye

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## ABSTRACT

**Objective:** *Demodex* blepharitis is a common inflammatory eye condition caused by an overgrowth of *Demodex* mites on the eyelids, and lash follicles, Zeiss' glands and Meibomian glands that is often overlooked. The aim of this study is to investigate the effectiveness of ivermectin tablet oral treatment in cases unresponsive to classical *Demodex* blepharitis treatment.
**Methods:** A total of 35 patients (17 men and 18 women) with chronic symptomatic blepharitis were included in the study. Patients were admitted to Çekirge State Hospital and Bursa Dünyagöz Hospital Ophthalmology Clinics between December 12, 2017, and April 15, 2021, and were found to have Cylindrical dandruff resistant to classical *Demodex* blepharitis treatment. Following the classical *Demodex* treatment in these cases, oral ivermectin was started in two doses of 0.2 mg/kg, 30 days apart. The presence of *Demodex* in the eyelashes, ocular surface disease index (OSDI) score was evaluated before the classical *Demodex* blepharitis treatment and after the ivermectin treatment. A detailed biomicroscopic eye examination was performed in all cases.
**Results:** Of the total 35 patients admitted to the hospital, 17 (48.6%) were men [mean ± standard deviation (SD): 48.41±15.62, min-max: 18-75] and 18 (51.4%) were women (mean ± SD: 51.17±14.98, min-max: 19-73). There was no statistically significant difference between men and women in terms of average age (p=0.598). OSDI scoring mean ± SD values before classical *Demodex* blepharitis treatment and after oral ivermectin treatment were 77.47±5.74 and 6.69±4.71, respectively.
**Conclusion:** In the treatment of *Demodex* related blepharitis, the use of oral ivermectin is an effective treatment option in cases that do not respond to classical treatment.
**Keywords:** *Demodex* sp., blepharits, oral ivermectin, Bursa, Türkiye

## ÖZ

**Amaç:** *Demodeks* blefariti, göz kapaklarında, kirpik diplerinde, Zeiss bezlerinde ve Meibomian bezlerinde *Demodeks* akarlarının aşırı çoğalmasıyla oluşan ve genellikle gözden kaçan yaygın bir göz iltihabı rahatsızlığıdır. Bu çalışmanın amacı klasik *Demodeks* blefarit tedavisine yanıtsız olgularda ivermektin tablet oral tedavisinin etkinliğini araştırmaktır.
**Yöntemler:** Çalışmaya kronik semptomatik blefarit tanısı almış toplam 35 hasta (17 erkek ve 18 kadın) dahil edildi. Çalışmaya Çekirge Devlet Hastanesi ve Bursa Dünyagöz Hastanesi Göz Hastalıkları Klinikleri'ne 12 Aralık 2017 ile 15 Nisan 2021 tarihleri arasında başvuran ve klasik *Demodeks* blefariti tedavisine dirençli Cylindrical dandruff 'lu hastalar dahil edildi. Bu olgularda *Demodeks* klasik tedavisinin ardından 30 gün arayla 0,2 mg/kg'lık iki dozda oral ivermektin başlandı. Klasik *Demodeks* blefariti tedavisi öncesi ve ivermektin tedavisi sonrası kirpiklerde *Demodeks* varlığı, oküler yüzey hastalığı indeksi (OSDI) skoru değerlendirildi. Tüm olgulara detaylı biyomikroskopik göz muayenesi yapıldı.
**Bulgular:** Hastaneye kabul edilen toplam 35 hastanın 17'si (%48,6) erkek [ortalama ± standart sapma (SS): 48,41±15,62, min-maks: 18-75] ve 18'i (%51,4) kadındı (ortalama ± SS: 51,17±14,98, min-maks: 19-73). Erkekler ve kadınlar arasında yaş ortalamaları açısından istatistiksel olarak anlamlı bir fark saptanmadı (p=0,598). Klasik *Demodeks* blefariti tedavisi öncesi ve oral ivermektin tedavisi sonrası OSDI skorlama ortalama ± SS değerleri sırasıyla 77,47±5,74 ve 6,69±4,71 idi.
**Sonuç:** *Demodeks* ilişkili blefarit tedavisinde klasik tedaviye yanıt vermeyen olgularda oral ivermektin kullanımının etkili bir tedavi seçeneği olabileceği kanaatine varıldı.
**Anahtar Kelimeler:** *Demodeks* sp., blefarit, oral ivermektin, Bursa, Türkiye

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**Address for Correspondence/Yazar Adresi:** Assoc. Prof. Oktay Alver, Bursa Uludağ University Faculty of Medicine, Department of Medical Microbiology, Bursa, Türkiye
**E-mail/E-Posta:** oktayalver@uludag.edu.tr **ORCID ID:** orcid.org/0000-0002-5559-3590



Türkiye PARAZİTOLOJİ Dergisi

## INTRODUCTION

*Demodex* mites are arthropods that belong to the class Arachnida, the order Prostigmata and family Demodicidae (1). It is the most common host-dependent microscopic ectoparasite in humans (2). The family Demodicidae is represented by eight genera and 116 species that live as parasites on mammals (3), while only two of them (*Demodex folliculorum* and *Demodex brevis*) live in the pilosebaceous units of human skin (4). While *D. folliculorum* is 0.35-0.4 mm long and lives singly or in groups in hair follicles, *D. brevis* is 0.15-0.2 mm long and usually lives alone in the depths of the sebaceous glands. In the eyelids (Zeiss' glands) and the lobules of the eyelid glands (Meibomian glands) (5,6) and cause anterior and posterior blepharitis, respectively; *D. folliculorum* and *D. brevis* (7). *Demodex* blepharitis is a chronic inflammatory disease that can cause severe eye problems affecting the lid margin and ocular surface (7). The primary cause of demodectic blepharitis is no daubt the increase in the number of *Demodex* mites in the area. The plug and tension that occurs in the follicles due to increased number of *Demodex* mites can not only cause deformities in the eyelashes and dysfunction in the Meibomian glands, but also can cause a foreign body sensation and the development of an immune response against the chitin skeleton, as it deepens into the Meibomian glands (8). Accordingly, if left untreated, it may lead to severe lesions such as eyelid and eyelash abnormalities, blurred vision and corneal damage (9). It has been reported that 41.6-81.25% of blepharitis patients have concurrently developed symptoms related to *Demodex* mites, and this rate reaches 100% among people over 70 years (10).

The aim of this study is to probe further the response of systemic ivermectin treatment in blepharitis cases with *Demodex* infestation that do not respond to the classical treatment.

## METHODS

The study was carried out in Bursa, the fourth most industrialized city in Türkiye. A total of 35 patients (17 men and 18 women) aged between 18 and 75 years, with chronic blepharitis, diagnosed with Cylindrical dandruff (CD) resistant to classical *Demodex* blepharitis treatment, were admitted to the Ophthalmology Clinics in Çekirge State Hospital and Bursa Uludağ University Faculty of Medicine, Bursa between December 12, 2017 and April 15, 2021, were included in the study. Data on demographic (age and gender), clinical characteristics (diabetes, hypertension, etc.), ocular symptoms (dry eye symptoms, eyelash loss, ingrown eyelashes, etc.), *Demodex* mite density, and ocular medications were obtained from patient records.

Ethical approval was obtained from the Clinical Research Ethics Committee of Bursa Uludağ University (permission no: 2021-14/18, date: 13/10/2021).

### Collection and Examination of the Samples

A specialist medical parasitologist examined the eyelash samples. A total of 12 eyelash samples, three from the upper and lower lids of each eye, were taken by epilation method. Before taking samples, care was taken to avoid cosmetic products such as mascaras, eye shadows, eye liners. Eyelashes were placed on the slide, and then 2-3 drops of Hoyer's medium were dropped on them and covered with a coverslip. The area was scanned using a light microscope with x40 magnification, and then inspected with a slightly closed diaphragm at x10 and x40 magnification, and the findings were noted. Participants whose sample materials are found to contain at least one egg, larva, nymph or mature forms of *D. folliculorum* or *D. brevis* were considered positive for *Demodex* (Figure 1).

### Evaluation of Ocular Surface Parameters

The ocular surface examinations were performed in the following order: Slit lamp examination was conducted on palpebral margin, and checked for any congestion and shape irregularity of palpebral margin, blockage on the openning of the Meibomian gland, and backward displacement of the Meibomian gland orifice opening were evaluated using the scoring criteria reported in (Figure 2) (11).

### Statistical Analysis

Statistical analysis of data was carried out using IBM SPSS Version 28.0 (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp). The descriptive statistics are given as mean and standard deviation for quantitative data, and frequency and percentage for the qualitative data. Shapiro-Wilk test was used to examine the normality of variables. It is found that the collected data samples are normally distributed. T-test was used to compare the comparison of the two independent groups, and the paired t-test was used to compare the dependent variables. Pearson correlation coefficient was used to evaluate the relationship between variables. The statistical significance level was determined to be 0.05.

## RESULTS

Of the total of 35 cases, 17 (48.6%) were men [mean ± standard deviation (SD): 48.41±15.62, min-max: 18-75] and 18 (51.4%) were women (mean ± SD: 51.17±14.98, min-max: 19-73). There was no statistically significant difference between men and


**Figure 1. (a)** *Demodex folliculorum* egg form (arrow) and **(b)** *Demodex folliculorum* adult located on an eyelash follicle under light microscope

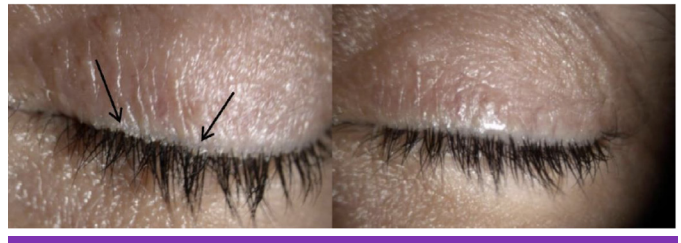

**Figure 2.** A *Demodex*-positive patient with Cylindrical dandruff (arrow) before and 2 months after ivermectin treatment

women in terms of average age (p=0.598). All patients were over 18 years of age, while their mean age was 49.8±15.1 years. At their first visit to the clinic, 27 (77.1%) of patients were found to have dry eye complaints, 4 (11.4%) have eyelash loss, 3 (5.7%) have ingrown eyelashes (trichiasis), and 2 (5.7%) have itching complaints. In the study, each patient stated that they had had chalazion at least once. One patient stated that she was operated on once for chalazion, and another patient stated that she was operated on 4 times. Biomicroscopic examination revealed anterior blepharitis in 5 (14.3%) patients, posterior blepharitis in 6 (17.1%), anterior and posterior blepharitis in 23 (65.7%), and allergic conjunctival papillae in 5 (14.3%). *D. folliculorum* was detected in 32 of 35 cases (91.4%). Coexistence of *D. folliculorum* and *D. brevis* was detected in three patients (8.6%). *Demodex* sp. was detected in all CD patients in the biomicroscopic study, while keratitis was present in three patients. Three patients were taking prescribed medication for diabetes and hypertension, while two patients were taking medication for hypertension only. In 18 of the cases, *Demodex* sp. was found on the upper and lower eyelids of both eyes. There was no significant relationship between age and *Demodex*/eyelash in women (r=0.117; p=0.644), men (r=-0.175; p=0.503) and in all patients in general (r=-0.025; p=0.887). Before the application of ivermectin, the patients were treated doxycycline (tetradox® capsule 100 mg) 2x1 oral for 20 days, levofloxacin (levolon® 0.5% drops) 4x1 for two months, oxytetracycline hydrochloride+polymyxin b sulfate eye ointment (terramycin® 5 mg/10.000 IU eye ointment) 2x1 for two months, pilocarpine (pilosed® 2% drops) (massage to the eyelash roots with earwax 5 times a day) for two months, eye wash shampoo containing tea tree oil (TTO thermal®) (every evening eyelid cleaning) for six months. *Demodex* sp. was detected in the eyelash examinations of patients whose blepharitis symptoms did not completely disappear at the end of the second month despite the previous treatment given above. Two doses of oral ivermectin tablet (0.2 mg/kg) were administered, one month apart. As shown in Figure 1, after two months of oral ivermectin treatment, the blepharitis symptoms completely resolved and the parasite load was reduced in all patients. A statistically significant difference was found between ocular surface disease index (OSDI) values before and after treatment (p<0.001).

## DISCUSSION

In a multicenter retrospective study involving six optometry and ophthalmology clinics in the USA, the prevalence of *Demodex* blepharitis was reported as 57.7% (12). Many studies have reported an increase in the number of mites with age (13) which was explained by the decrease in the activity of the Rusiecka-Ziółkowska et al. (14) glands with aging. In the studies conducted on patients with blepharitis, as discussed by Sędzikowska et al. (15), Mongi et al. (16), and Wesolowska et al. (17) that they detected the highest *Demodex* sp. infestation rate on patients aged 50 years and above (p<0.005), 60 years and over (p<0.001), and 70 years and over (p<0.001), respectively. This may be associated with the lack of good eyelid hygiene in elderly patients and certain factors such as immunosuppression, vasodilatation factors, diabetes mellitus, and/or sebaceous gland hyperplasia (18) or malignancy, or malnutrition (19). As age increases, *Demodex* infestation rates tend to rise not only in the eyelashes but also on the face and other areas of the body (18). In the present study, the highest *Demodex* sp. infestation rate was determined in people aged 45 and over (68.5%), while no significant differences were found between age and *Demodex* sp. This finding was consistent with the results of numerous previous studies (p=0.598) (20,21). Although there are many studies in the literature relating the number of *Demodex* to the gender of the patients, it still remains controversial. However, in this study, no relationship was found between the number of *Demodex* and the gender of the patients, supporting the results discussed by Lee et al. (6) and Kemal et al. (21). In other words, Okyay et al. (22) reported that *Demodex* sp. infestation was higher in men, while Forton et al. (23) and Zeytun (24) reported higher infestation rates in women, and Zhao et al. (25) reported equal rates in men and women. These differences between studies may be associated with the differences in skin care, type of skin, increase in the number of mites and hygiene practices of patients included in the study. Itching, burning sensation, excessive lacrimation, feeling of heaviness in the eyelids and mucous discharge are frequently encountered symptoms in blepharitis cases with *Demodex* infestation (26). Kheirkhah et al. (27) reported decreased vision in 50% of blepharitis cases caused by *Demodex* sp., and Meibomian gland dysfunction in 83.3%. Gao et al. (28) reported that they detected eyelash loss in 45.5% of the eyelid demodicosis cases, and Meibomian gland dysfunction in 63.6%. It is stated that the microabrasions caused by *D. folliculorum* at the base of the eyelashes cause reactive hyperkeratinization and epithelial hyperplasia, resulting in the formation of CD, which is accepted as pathognomonic for *D. folliculorum* infestation (28,29). In Thailand, Kasetsuwan et al. (30) reported that they detected CD by microscopy in 69% and 15.5% of cases with and without ocular *Demodex* infestation, respectively. It has been stated that CD formation may be clinically valuable in monitoring the response to treatment in patients with chronic blepharitis (31). In the present study, all patients diagnosed with CD with chronic blepharitis, resistant to classical treatments, were found to be *Demodex* sp. positive. The number of *Demodex* sp. per eyelash before treatment was 1.63 (652/398). Today, the ambiguity in etiopathogenesis makes the treatment of blepharitis complicated. One of the primary aims of ocular demodicosis treatment is to reduce parasitic overpopulation in the lids and eyelashes and reduce inflammation by providing a better environment for the eye surface. There are many treatment options in the literature for this purpose. Although XDEMVY™ (lotilaner ophthalmic solution) is an FDA-approved preparation for the treatment of *Demodex* blepharitis, it is not being used in our country. In the treatment of blepharitis, the American Academy of Ophthalmology Blepharitis Preferred Practice Pattern recommends a combination of antibiotics, topical anti-inflammatories, and daily eyelid hygiene (32). The most frequently used approach to the eyelid hygiene is the use of gel, cream and shampoo forms of TTO, which is obtained by steam distillation from the leaves of Melaleuca Alternifolia (Indian laurel), or its most important acaricidal component, terpinen-4-ol (26). In previous studies Junk et al. (33) reported a remission rate of 71.6% in the fourth week of combined treatment of oral ivermectin and metronidazole (3x250 mg/day for three weeks) in patients with anterior blepharitis and valve lesions caused by *Demodex* sp. and also showed that combined treatment was more effective than ivermectin alone. In the treatment of inflammation on the palpebral edges, it was reported that local application of yellow mercury ointment, sulfuric ointment, anticholinesterase,

antifungal drugs or antibiotics provided clinical improvement, but the best results were obtained after the application of metronidazole, mercury and erythromycin ointments (33). Holzchuh et al. (34), treated their patients with one dose of oral ivermectin (200 μg/kg) and repeated it after 7 days, and reported that the treatment had improved the symptoms of chronic blepharitis, with a 35.3% mite eradication rate. Symptoms caused by *Demodex* mites are difficult to treat and can take several months. This can be partly explained by the fact that the life cycle of *Demodex* sp. is approximately 14 days and the total life span is several weeks (4). Therefore, in this study, 8 weeks of treatment (approximately 4 life cycles of *Demodex*) were administered to evaluate recurrence of infestation. In another study, the mite eradication rate was expressed as 36% after four months of TTO shampoo treatment at low concentration (7.5%) to minimize the risks of possible side effects of TTO (35). In our study, there was little improvement in symptoms after the classical treatments of *Demodex* chronic blepharitis, when the presence of *Demodex* sp. was detected and a single dose of ivermectin was prescribed. No drug-related side effects were observed after treatment. At the end of the second month, improvement in the symptoms of blepharitis and a significant decrease in parasite load were observed. OSDI score is usually (generally) used in ophthalmology practice as it can directly indicate the degree of ocular surface inflammation. Zhang et al. (20) and Zeytun (24) reported that they found a significant increase in OSDI score in the ocular *Demodex* positive patient group. Pan and Chen (36) reported a significant correlation between *Demodex* count and OSDI score. Liu et al. (8) stated that there was a significant relationship between the number of *Demodex*, the OSDI score, and the severity of eye disease. Mergen et al. (37) reported that they found a negative relationship between the *Demodex* count and OSDI scores in patients with seborrheic blepharitis. In this study, a significant relationship was also found between the *Demodex* count and the OSDI score by performing correlation analysis.

## CONCLUSION

Our study shows that oral ivermectin treatment may be beneficial especially in cases that do not respond to classical *Demodex* blepharitis treatments. However, we think that our results should be supported by more comprehensive randomized and prospective studies.

### *Ethics

**Ethics Committee Approval:** Ethical approval was obtained from the Clinical Research Ethics Committee of Bursa Uludağ University (permission no: 2021-14/18, date: 13/10/2021).

**Informed Consent:** Retrospective study.

### Footnotes

### *Authorship Contributions

Surgical and Medical Practices: A.O., D.D., S.D., Concept: A.O., D.D., S.D., Design: A.O., D.D., Data Collection or Processing: A.O., D.D., Analysis or Interpretation: A.O., S.D., Literature Search: A.O., D.D., Writing: A.O., S.D.

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# Assessment of Knowledge and Personal Hygiene Practices Regarding Scabies Among High-risk Students in a Non-formal Education Boarding School

## *Uyuzun Önlenmesinde Bilgi ve Kişisel Hijyen Uygulamaları: Gayriresmi Yatılı Okuldaki Yüksek Riskli Öğrenciler Üzerine Kesitsel Bir Çalışma*

Sri Wahdini[1], Ika Puspa Sari[2], Fajaria Nurcandra[3], Saleha Sungkar[2]

[1]Doctoral Program of Biomedical Science, Universitas Indonesia Faculty of Medicine, Jakarta, Indonesia
[2]Department of Parasitology, Universitas Indonesia Faculty of Medicine, Jakarta, Indonesia
[3]Public Health, Faculty of Health Science, Universitas Pembangunan Nasional Veteran Jakarta, Depok Jawa Barat, Indonesia

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## ABSTRACT

**Objective:** Scabies is a common skin disease, especially prevalent in densely populated environments such as a boarding school. This study assesses knowledge and practices related to scabies among students in a non-formal education boarding school.
**Methods:** This cross-sectional study was conducted in a non-formal boarding school Daarul Ishlah in South Jakarta, Indonesia, in December 2023. The population was all male students (127 respondents) who were recruited using consecutive sampling. Data were collected through a semi-structured and self-administered questionnaire to assess students' knowledge and personal hygiene practices. The collected data were analyzed descriptively using SPSS version 20.
**Results:** The respondents' levels of knowledge were categorized as follows: 59.1% had good knowledge, 34.6% had moderate knowledge, and 6.3% showed poor knowledge. However, their hygiene practices indicated a different trend, with only 22% exhibiting good practices, 72.4% showing moderate practices, and 5.5% demonstrating poor practices. Most respondents (88.2%) were unaware of the etiology of scabies. Regarding personal hygiene practices for preventing scabies, 78% used personal towels, and 74.8% slept on their own mattresses. Yet, only 18.9% maintained the cleanliness of their bed linens.
**Conclusion:** While most respondents demonstrated good knowledge about scabies, their hygiene practices were generally at a moderate level. To address this gap, knowledge and behavior can be enhanced through regular health education, the provision of adequate facilities, and the enforcement of internal regulations to foster better student discipline.
**Keywords:** Scabies, boarding school, knowledge, hygiene practic

## ÖZ

**Amaç:** Uyuz, özellikle yatılı okul gibi yoğun nüfuslu ortamlarda yaygın olarak görülen bir deri hastalığıdır. Bu çalışma, yatılı bir gayriresmi eğitim kurumundaki öğrencilerin uyuz hakkındaki bilgi ve uygulamalarını değerlendirmektedir.
**Yöntemler:** Bu kesitsel çalışma, Aralık 2023'te Endonezya'nın Güney Jakarta'daki yatılı bir gayriresmi okul olan Daarul Ishlah'ta gerçekleştirilmiştir. Çalışma popülasyonu, ardışık örnekleme yöntemiyle seçilen 127 erkek öğrenciden oluşmaktadır. Öğrencilerin bilgilerini ve kişisel hijyen uygulamalarını değerlendirmek için yarı yapılandırılmış ve kendileri tarafından doldurulan bir anket kullanılarak veri toplanmıştır. Toplanan veriler, SPSS sürüm 20 kullanılarak tanımlayıcı olarak analiz edilmiştir.
**Bulgular:** Katılımcıların bilgi düzeyleri şu şekilde kategorize edilmiştir: %59,1'i iyi bilgi düzeyine sahipken, %34,6'sı orta düzeyde bilgiye sahip ve %6,3'ü düşük bilgi düzeyinde bulunmuştur. Ancak, kişisel hijyen uygulamaları farklı bir eğilim göstermiştir; yalnızca %22 iyi uygulamalar sergilerken, %72,4 orta düzeyde uygulamalar göstermiş ve %5,5 düşük düzeyde uygulamalara sahiptir. Katılımcıların çoğu (%88,2) uyuzun etiyolojisini bilmiyordu. Uyuz önleme için kişisel hijyen uygulamalarına bakıldığında, %78'i kişisel havlu kullanmış, %74,8'i kendi yataklarında uyumuş, ancak yalnızca %18,9'u çarşaf temizliğini sağlamıştır.
**Sonuç:** Katılımcıların çoğu uyuz hakkında iyi bilgiye sahip olmasına rağmen, kişisel hijyen uygulamaları genellikle orta düzeydedir. Bu boşluğu gidermek için, düzenli sağlık eğitimi, uygun tesislerin sağlanması ve öğrencilerin disiplinini artırmak adına iç düzenlemelerin uygulanması yoluyla bilgi ve davranış geliştirilebilir.
**Anahtar Kelimeler:** Uyuz, yatılı okul, bilgi, hijyen uygulamaları

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**Address for Correspondence/Yazar Adresi:** Saleha Sungkar, Department of Parasitology, Universitas Indonesia Faculty of Medicine, Jakarta, Indonesia
**E-mail/E-Posta:** saleha.sungkar@ui.ac.id **ORCID ID:** orcid.org/0000-0002-6570-8793



Türkiye PARAZİTOLOJİ Dergisi

## INTRODUCTION

*Sarcoptes scabiei* is an ectoparasite triggering the occurrence of scabies, a skin disease designated by the World Health Organization as a neglected tropical disease (NTD) targeted for elimination by 2030 (1). In Indonesia, scabies is classified as one of the 13 NTDs and is still endemic (2), posing a significant public health challenge in densely populated environments such as boarding schools, orphanages, nursing homes, prisons, refugee camps, and daycare centres. High population density increases physical and non-physical contact between people, facilitating the transmission and infestation of scabies mites. Other factors contributing to scabies incidence include socioeconomics, knowledge about scabies, personal hygiene practices, and infrastructure availability (3). A study among university graduates in Saudi Arabia found a generally good level of knowledge about scabies, yet only 22.1% could correctly identify its signs and symptoms (4). In contrast, a study of Syrian refugees revealed inadequate knowledge about the disease (5).

The prevalence of scabies in boarding schools has been reported to range from 36.8% to 76.9% (6-8), primarily in Islamic boarding schools (pesantren), which are integral to Indonesia's national education system. These non-formal institutions often design curricula and learning methods tailored to specific needs, contributing significantly to the development of Islamic religious education. However, many pesantren face challenges related to limited facilities, infrastructure, and hygiene standards among administrators and students, increasing vulnerability to infectious diseases like scabies (9). Wahdini et al. (10) identified parasitic diseases, including blastocystosis, giardiasis, enterobiosis, scabies, and head lice, among certain groups of students. Risk factors for high scabies incidence include shared clothing, joint sleeping arrangements, repeated use of dirty clothes, insufficient washing, and inadequate bedding materials and room space (6-8).

Scabies is characterized by a high cure rate. Sungkar et al. (11) reported a 95.7% cure rate using the standard treatment method (whole-body topical application of permethrin) and a 91.3% cure rate with modified treatment (lesion-only permethrin application combined with soap usage) by the third week. However, reinfection is common, emphasizing the need for a comprehensive understanding of vulnerable populations' knowledge and behavior to inform effective intervention strategies. This study aims to assess the knowledge and hygiene practices related to scabies transmission among students in non-formal Islamic boarding schools.

## METHODS

The research employed a descriptive survey method with a cross-sectional approach. This study was conducted among male students in a non-formal education boarding school (Islamic boarding school or Pesantren Daarul Ishlah) in South Jakarta, Indonesia, in December 2023. The research employed a descriptive survey method with a cross-sectional approach. Sampling was carried out using a consecutive (non-random) sampling method.

The inclusion criteria included all students actively attending classes, residing in the school dormitory, and voluntarily agreeing to complete the provided questionnaires. Incomplete questionnaires were excluded from the analysis. The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the study under protocol number 21-11-1921, date: 09.11.2023.

Before the study began, all respondents were informed about its purpose. Written consent was obtained from participants aged 18 and above, who retained the right to decline participation. For respondents under 18 years old, consent was provided by their parents after a comprehensive explanation of the study procedures. Only those who consented to participate were included in the study.

The questionnaire, adapted from a study by Binti Mohd Yusof et al. (12), was divided into two sections. The first section consisted of ten multiple-choice questions assessing knowledge about scabies, each with one correct answer. A score of one point was awarded for each correct response, while incorrect answers or "don't know" responses received zero points, making the maximum achievable score 10.

The second section of the questionnaire comprised 20 statements assessing personal hygiene practices related to scabies risk factors, with yes or no answer options. One point was assigned for each good personal hygiene practice, while poor practices received zero points. Statements 11, 13, 15, and 18 were reverse questions, requiring responses to be interpreted inversely. The total score for each part was calculated, with scores above 74% categorized as good, 40-74% as moderate, and below 40% as poor.

### Statistical Analysis

The data were analyzed using the SPSS statistical package for Windows version 20.0 (IBM Corp., Armonk, NY). Descriptive statistics were employed to summarize the key features of the data, presenting results as frequencies and percentages in tables or figures.

## RESULTS

A total of 127 male students from a non-formal education boarding school participated in this study (Table 1). Most respondents (55.1%) were young adults aged 18-24, and 59.1% had previously received information about scabies.

**Table 1.** Characteristics of respondents (n=127)

| Characteristics | n | % |
|---|---|---|
| **Age** | | |
| Early adolescence (10-13 years old) | 7 | 5.5 |
| Middle adolescence (14-17 years old) | 43 | 33.9 |
| Late adolescent or young adult (18-24 years old) | 70 | 55.1 |
| Adult (>24 years old) | 7 | 5.5 |
| **Last education** | | |
| Elementary | 28 | 22.0 |
| Junior high school | 24 | 18.9 |
| Senior high school | 29 | 22.8 |
| Bachelor degree | 5 | 3.9 |
| Non-formal/not in school | 41 | 32.3 |
| **Information about scabies before** | | |
| Yes | 75 | 59.1 |
| No | 52 | 40.9 |

Among the participants, 59.1% demonstrated good knowledge, 34.6% had moderate knowledge, and 6.3% showed poor knowledge. However, personal hygiene practices displayed a different pattern, with only 22% of respondents exhibiting good practices, 72.4% showing moderate practices, and 5.5% demonstrating poor practices (Figure 1).

Regarding knowledge, most respondents gave correct answers related to clinical symptoms, treatment, and prevention of scabies. However, only 30 respondents (11.8%) correctly answered questions about the etiology of scabies. Additionally, 38.6% were unaware of how the disease is transmitted, and 31.5% did not know the specific areas of the skin that may present abnormalities (Table 2). Nevertheless, most participants correctly identified the body parts affected by scabies, its signs, major symptoms, susceptible populations, the risk of transmission, the treatability of scabies, preventive measures, and the need for quarantine for scabies patients.

The questionnaires on personal hygiene practices revealed several behaviors requiring improvement. These included bathing after exercise or profuse sweating (35.4%), washing towels weekly (31.5%), drying mattresses or bedding at least once a month (44.9%), changing or washing bed linens weekly (18.9%), and washing clothes after a single use (33.1%). On a positive note, the majority of respondents refrained from exchanging clothes with friends (66.9%) and frequently washed their hands with soap and running water before eating (59.8%) and after using the toilet (67.7%). Furthermore, all respondents are encouraged to maintain good practices, such as avoiding the use of shared or alternating towels (Table 3).

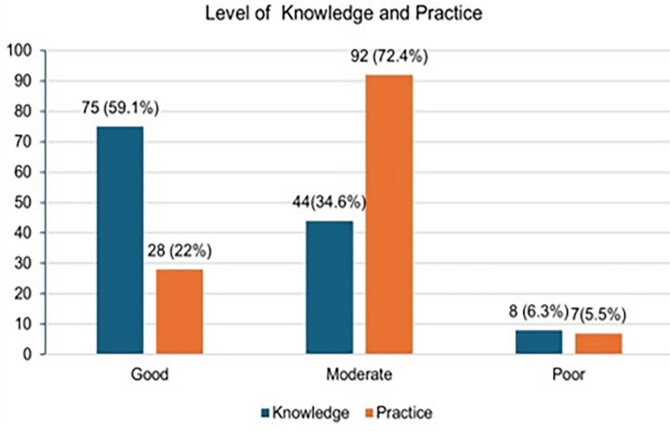


**Figure 1.** Level of knowledge on scabies and personal hygiene practice

## DISCUSSION

Scabies transmission occurs through direct and indirect contact, highlighting personal hygiene as the key preventive measure. Direct contact involves the skin of a healthy individual coming into contact with someone infested with mites. In contrast, indirect contact occurs when the skin touches items belonging to a patient, such as mattresses, towels, clothing, or other personal items shared interchangeably. Mites can survive outside the human skin for several days, making contaminated objects or belongings intermediaries for parasite transfer (13). Conducting a study on knowledge and personal hygiene practices is crucial to support scabies eradication and prevention programs in specific communities, with potential applications to other similar populations.

A non-formal education boarding school is an environment where students study and live in the dormitory area. Male respondents who lived in dormitories were selected in this study to minimize bias, as knowledge and personal hygiene practices can be influenced by factors such as gender, residential environment, family educational and occupational status, and economic level (4). By focusing on students from an area with limited access to information, specifically Islamic boarding schools (pesantren) with restricted device use and social media access, this study gathered uniform and specific data to aid the development of targeted intervention programs. Ararsa et al. (14) reported that male students often engage in more physically demanding activities, resulting in excessive sweating and increased physical contact, such as playing sports or field activities. Consequently, hygiene practices tailored to male students' needs differ from those of female students.

The respondents demonstrated a good level of knowledge about scabies, as reflected in the analysis of their responses to each question. Most participants provided correct answers; however, only 11.8% correctly identified the etiology of scabies when given

**Table 2.** Knowledge of respondents regarding scabies

| Questions about knowledge | Right answer | | Wrong answer | |
|---|---|---|---|---|
| | n | % | n | % |
| What is the cause (etiology) of scabies? | 15 | 11.8 | 112 | 88.2 |
| Which body parts are commonly affected by scabies? | 87 | 68.5 | 40 | 31.5 |
| What are the signs of scabies? | 116 | 91.3 | 11 | 8.7 |
| What are the primary symptoms of scabies? | 109 | 85.8 | 18 | 14.2 |
| Who is at risk of being infected with scabies? | 117 | 92.1 | 10 | 7.9 |
| How is scabies transmitted? | 78 | 61.4 | 49 | 38.6 |
| Which behavior can increase the risk of scabies transmission? | 118 | 92.9 | 9 | 7.1 |
| What is the recommended treatment for scabies? | 100 | 78.7 | 27 | 21.3 |
| What measures can be taken to prevent scabies? | 100 | 78.7 | 27 | 21.3 |
| What steps should be taken to care for a person infected with scabies? | 92 | 72.4 | 35 | 27.6 |

**Table 3.** Personal hygiene practices

| Personal hygiene practices | Yes | | No | |
|---|---|---|---|---|
| | n | % | n | % |
| Bathe regularly, at least once a day | 91 | 71.7 | 36 | 28.3 |
| Use soap routinely while bathing | 98 | 77.2 | 29 | 22.8 |
| Bathe after exercising or sweating | 45 | 35.4 | 82 | 64.6 |
| Cut fingernails weekly | 111 | 87.4 | 16 | 12.6 |
| Regularly wash hands with soap before eating | 76 | 59.8 | 51 | 40.2 |
| Always wash hands with soap after using the toilet | 86 | 67.7 | 41 | 32.3 |
| Dry hands thoroughly after washing, using a tissue or a clean towel | 44 | 34.6 | 83 | 65.4 |
| Use your personal towel | 99 | 78.0 | 28 | 22.0 |
| Ensure towels are properly dried after use | 99 | 78.0 | 28 | 22.0 |
| Wash towels at least once a week | 40 | 31.5 | 87 | 68.5 |
| Sharing towels with others | 9 | 7.1 | 118 | 92.9 |
| Ironing towels after washing or washing with warm water | 12 | 9.4 | 115 | 90.6 |
| Sleep in separate beds/mattresses | 32 | 25.2 | 95 | 74.8 |
| Sun-dry mattresses at least once a month | 57 | 44.9 | 70 | 55.1 |
| Multiple students sleeping on the same mattress | 67 | 52.8 | 60 | 47.2 |
| Wash bed linens at least once a week | 24 | 18.9 | 103 | 81.1 |
| Change clothes at least once daily | 87 | 68.5 | 40 | 31.5 |
| Share unwashed clothes with other students | 85 | 66.9 | 42 | 33.1 |
| Wash clothes after a single use | 42 | 33.1 | 85 | 66.9 |
| Ironing clothes after washing | 33 | 26.0 | 94 | 74.0 |

the options of mites, viruses, or bacteria. This finding aligns with the report by Binti Mohd Yusof et al. (12), which also noted high levels of knowledge among respondents, coupled with a tendency to give incorrect answers regarding the cause of scabies. Similarly, a study by Amoako et al. (15) conducted through interviews with respondents—including those actively suffering from scabies, those with prior experience, and individuals with no history of the condition—revealed varied responses. Most respondents answered that scabies was caused by parasites, microorganisms, or transmitted by other people.

Additionally, both healthy individuals and respondents who consulted or received treatment from health workers believed that scabies was caused by supernatural forces such as witchcraft or curses. In a prior study by Lopes et al. (16), which utilized open-ended questions, only a few respondents identified mites as the organisms responsible for scabies. Instead, the majority attributed the disease to dirtiness, poor personal hygiene, or contaminated water. In contrast, this study employed closed questions with certain unfamiliar answer options, leading many respondents to provide incorrect answers (17).

Knowledge requiring improvement includes understanding the body regions commonly affected by itching and skin disorders due to scabies, as well as the transmission patterns of *S. scabiei* mites from infected individuals to others. These two aspects serve as key indicators of students' level of scabies awareness. A lack of awareness may result in delays in seeking medical attention for diagnosis and treatment, increasing the risk of severe clinical complications, secondary infections, or misdiagnosis as other skin conditions. Moreover, the limited infrastructure in the school environment contributed to the small percentage of respondents demonstrating good personal hygiene practices. An analysis of statements related to personal hygiene revealed that respondents did not exhibit certain behaviors due to dormitory constraints. These limitations included inadequate provision of clothes irons, insufficient mattresses to accommodate all students, lack of hot water for washing, unavailability of towels or tissue paper, and restricted space for drying mattresses and personal belongings. Such factors significantly influenced personal hygiene practices. The findings of this study contrast with those of Sungkar et al. (18), which reported that instances of students sharing clothes or beds with friends stemmed from the belief prevalent among students in Islamic schools that lending personal items is a way to assist others.

Hygiene practices that could be improved through healthy living education include bathing after exercising or sweating, washing towels and bed linens at least once a week, and regularly washing used clothes. Enhancing knowledge about healthy living is expected to encourage positive behavioral changes among students (19). Education on disease prevention and clean-living practices can be delivered directly through lectures or indirectly via flyers and posters. The understanding of scabies among students was notably improved by displaying pictorial posters in various schoolrooms and dormitories, allowing for frequent viewing and easier retention of the information. A study by Hasanica et al. (20) on elementary school students who received clean and healthy living education through posters demonstrated an increase in correct answers from 58.07% to 62.76% one month after implementation. Furthermore, Kulkarni et al. (21) emphasized that posters designed with simple and localized language are a cost-effective health education tool, proven to capture and sustain public attention effectively.

### Study Limitations

The study identified several limitations, including inadequate infrastructure in the boarding school environment, such as a lack of clothes irons, sufficient mattresses, hot water for washing, towels, tissues, and space to dry mattresses and personal belongings, which negatively impacted personal hygiene practices. Conducted in a non-formal Islamic boarding school with restrictions on device use and social media access, the closed environment further limited students' exposure to information about scabies. Additionally, using closed-ended questions with unknown response options in the questionnaire may have led to erroneous responses. The study also demonstrated gender bias by focusing only on male students, limiting the generalizability of its findings to female or mixed-gender populations. Cultural beliefs, such as lending personal belongings to help others, likely influenced hygiene practices and complicated improvement efforts. These findings underscore the need for improved infrastructure, culturally sensitive intervention strategies, and tailored health education to address these challenges effectively.

## CONCLUSION

This study concluded that boarding school students possessed a good level of knowledge, though many struggled to identify scabies' etiology correctly. Inadequate facilities within the dormitory environment primarily influenced the moderate personal hygiene practices observed. To address these gaps, structured health education programs are recommended to enhance further students' knowledge and awareness of scabies and healthy living practices. Such initiatives could significantly reduce the risk of disease transmission in enclosed settings like boarding schools.

### *Ethics

**Ethics Committee Approval:** The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the study under protocol number 21-11-1921, date: 09.11.2023.

**Informed Consent:** Written consent was obtained from participants aged 18 and above, who retained the right to decline participation.

### Footnotes

### *Authorship Contributions

Concept: S.W., S.S., Design: S.W., S.S., Literature Search: S.W., F.N., Writing: S.W., I.P.S.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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# The First Report of *Dirofilaria immitis* from a Dog in Ilgaz, Çankırı

## *Çankırı Ilgaz'da Bir Köpekten İlk Dirofilaria immitis Raporu*

Sümeyra Yırtıcı[1], Kader Yıldız[2]

[1]Kırıkkale University Faculty of Veterinary Medicine, Kırıkkale, Türkiye
[2]Kırıkkale University Faculty of Veterinary Medicine, Department of Parasitology, Kırıkkale, Türkiye

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### ABSTRACT

In this study, *Dirofilaria immitis (D. immitis)* detected in the necropsy of an Izci Kopegi Zagar breed dog in Ilgaz, Çankırı was reported. After being attacked by stray dogs, the owner of an 8-year-old male Izci Kopegi Zagar from Ilgaz, Çankırı presented to the veterinary clinic with serious injuries. The dog did not get well even after receiving treatment. Two nematodes were found in the dog's right heart chamber after postmortem investigation. Light microscopic examination revealed that these parasites were adult female *D. immitis*. As far as the authors' knowledge, this is the first observation of dogs residing in Ilgaz, Çankırı. Furthermore, it was noteworthy that the affected dog only had two female parasites. Due to occult infection, veterinarians are recommended to perform serological tests as well as blood examinations on dogs suspected of having heartworm.
**Keywords:** *Dirofilaria immitis*, dog, Zagar, Ilgaz, Çankırı, Türkiye

### ÖZ

Bu çalışmada, Ilgaz, Çankırı'da İzci Köpeği Zagar ırkı bir köpeğin nekropsisinde tespit edilen *Dirofilaria immitis (D. immitis)* rapor edilmiştir. Ilgaz, Çankırı'da sokak köpeklerinin saldırısına uğrayan 8 yaşındaki erkek İzci Köpeği Zagar'ın sahibi yaralı olarak veteriner kliniğine başvurmuştur. Tedaviye rağmen köpek iyileşmemiştir. Postmortem incelemede köpeğin sağ kalbinde iki nematod bulunmuştur. Işık mikroskobu ile incelenmesi sonucunda bu parazitlerin erişkin dişi *D. immitis* olduğu görülmüştür. Yazarların bilgisine göre bu çalışma Ilgaz, Çankırı'da yaşayan köpeklerden ilk kayıttır. Üstelik enfekte köpekte sadece iki dişi parazitin bulunması kayda değer bulunmuştur. Occult enfeksiyon sebebiyle veteriner hekimlerin kalp kurdu yönünden şüpheli köpeklere kan muayenesinin yanı sıra serolojik test de uygulaması önerilir.
**Anahtar Kelimeler:** *Dirofilaria immitis*, köpek, Zagar, Ilgaz, Çankırı, Türkiye

## INTRODUCTION

*Dirofilaria immitis* (*D. immitis*) (Spirurida: Onchocercidae), infects some animals, including dogs, cats, and other wild carnivores (1). These animal species act as the final hosts in life cycle of parasites (1,2). Adult parasites primarily settle in the right heart (atrium and ventricle), pulmonary artery, and vena cava of the final hosts. They are also found in the peritoneal cavity, central nervous system, eyes, and lungs of the final hosts (1,2). In endemic places, *D. immitis* can accidentally infect humans, leading to a condition known as human pulmonary dirofilariosis which is an emerging zoonotic disease (3).

Some mosquito species are intermediate hosts in the parasite biology (1). They are infected with microfilariae by sucking blood from the infected final host. Microfilariae develop into infective stage larvae (L3) in the female mosquitoes within 13 days. The L3 is transferred to the final host during sucking blood by infective mosquitoes (1,2). The L3 reach the fourth stage under the skin of the final host within 3-12 days and then they arrive at the right heart through venous circulation. They develop into adult parasites in the right side of the heart within 70-120 days postinfection. After copulation, the female *D. immitis* begins producing microfilariae in the 6$^{th}$ month after infection and can continue producing microfilariae for a long time. Microfilariae pass to the uterus via placenta and they may cause prenatal infection (1,2). Sometimes microfilariae may not be present in the bloodstream of dogs infected with *D. immitis*, which is a situation called occult heartworm infection (4).

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**Address for Correspondence/Yazar Adresi:** Kader Yıldız, Kırıkkale University Faculty of Veterinary Medicine, Department of Parasitology, Kırıkkale, Türkiye
**E-mail/E-Posta:** kaderyildiz@hotmail.com **ORCID ID:** orcid.org/0000-0001-5802-6156



Türkiye PARAZİTOLOJİ Dergisi

Heartworm infection is a systemic disease affecting heart, lungs, liver, and kidneys in dogs. It may be subclinical in general, but 25 adult parasites are sufficient to cause clinical signs (1,2). During necropsy, adult parasites in the heart are considered typical in terms of morphology (1,2,5). The female has a blunt posterior end and is 25-31 cm in length and 1-1.3 mm in width. The male has a spirally curved posterior end and is 12-20 cm in length and 0.7-0.9 mm in width. Microfilariae are approximately 205-280 µm long and can be observed in the light microscopic examination of venous blood samples (1,5). However, the species of microfilariae should be distinguished from those of other filariform parasites of dog such as *Dirofilaria repens* and *Acanthocelionema* spp. using some morphological criteria, including the staining characteristics of the excretory and anal orifice. Serological tests can be performed in addition to microscopic examination of blood samples (1).

Çankırı is located between 32° 30' and 34° east longitudes and 40° 30' and 41° north latitudes in the north of Central Anatolia. It has an altitude of 723 meters above sea level. It is geographically surrounded by Bolu, Karabük, Kastamonu, Çorum, Ankara, and Kırıkkale provinces. The province has a continental climate. Winters are cool and summers are warm in the central, Ilgaz and Yapraklı districts of Çankırı. Precipitation occurs almost every season in this region, and the average annual rainfall varies between 392-538 (kg/m$^2$) (6).

Izci Kopegi Zagar is one of the native dog breeds in Türkiye (Official Gazette of the Republic of Türkiye, registration date: 25.08.2021, number: 28036) (2). These dogs are also called by different regional terms such as kopay, kopoy, tavşanci, izsüren, and çakir in different regions of Türkiye, including Thrace, West and central Anatolia. Izci Kopegi Zagar is a smart, loyal and energetic dog breed and is especially used for hare hunting. Its coat is typically black or brown. The height of this breed is approximately 49-52 cm, and the weight is approximately 18-20 kg (2,7). In this study, *D. immitis* was observed in the necropsy of an Izci Kopegi Zagar in Çankırı/Ilgaz. To the knowledge of the authors, this is the first report of *D. immitis* in a dog in this region.

## CASE REPORT

In December 2024, an 8-year-old male Izci Kopegi Zagar living in Ilgaz, Çankırı was brought to the veterinary clinic by its owner. The dog was seriously injured after being attacked by roaming dogs. Despite the treatment, the dog could not recover. During necropsy, two large, white parasites were detected in the right heart chamber. The parasites were brought to Parasitology Department of Kırıkkale University Faculty of Veterinary Medicine. The parasites were rinsed with sterile saline solution and subsequently were preserved in 70% alcohol. Morphological identification was performed under a light microscope (Leica ICC50) after clearing them in lactophenol with related reference (5). Light microscopic examination indicated that these parasites were adult female *D. immitis*. The parasites were measured as 25.9-26.5 cm long. There was a small round mouth at the anterior end (Figure 1A and B), without lips. The vulva is 2.4-2.6 mm longer than the anterior end (Figure 1C and D), just behind the posterior end of oesophagus. The blunt posterior end of female parasites was observed in Figure 1E and F.

Clinical cases of heartworm infection have increased in dogs. This increase mainly depends on certain epidemiological factors, including the presence of potential vectors and infected animals,

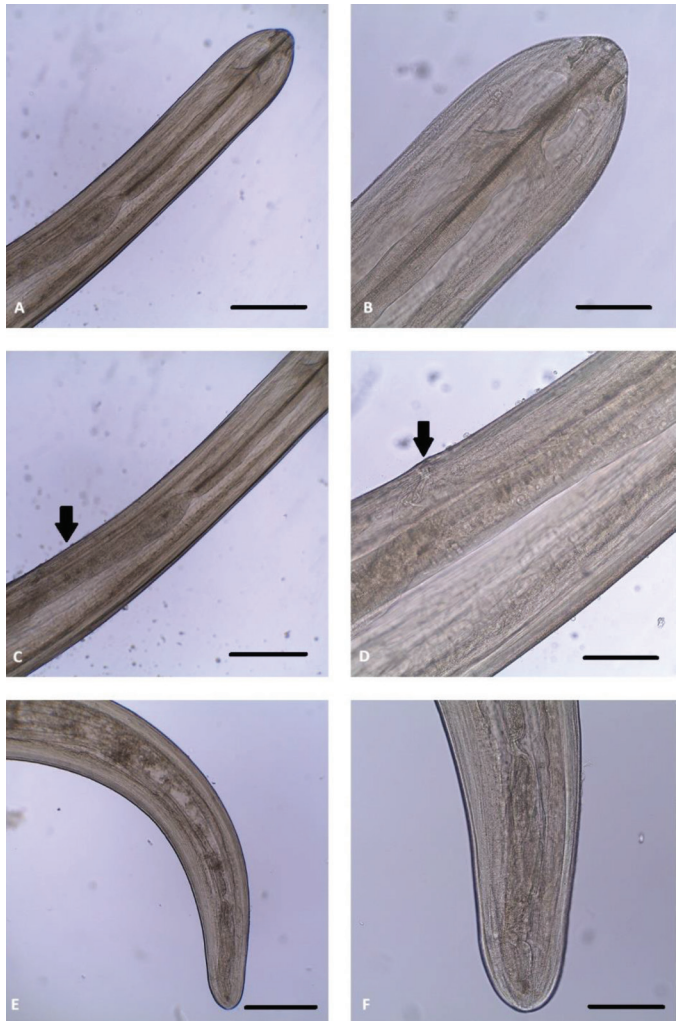


**Figure 1.** Adult female *Dirofilaria immitis*. A: Anterior part of the parasite, B: Small round mouth in the anterior end, C: The vulva is 2.4-2.6 mm longer than the anterior end (arrowhead), D: The genital pore (arrowhead), E: Posterior part of the parasite, F: Blunt posterior end of the parasite. Bar =500 µm (A, C, E), 200 µm (B, D, F)

and the absence of regular chemoprophylaxis in dogs (8). Depending on the diagnostic technique performed (serologic tests, blood examination or polymerase chain reaction), *Dirofilaria* spp. have been detected at varying rates in dogs (8-13). *D. immitis* has been reported between 0.08-40% from dogs in Türkiye (9,12,13), including in some neighbouring provinces of Çankırı (Ankara and Kırıkkale) (10,11). According to authors' knowledge it is the first report of *D. immitis* in dogs from Ilgaz, Çankırı.

Occult heartworm infection characterised amicrofilaremia can be seen in infected dogs due to several reasons, including the presence of only male or only female (single sex) parasites, the parasites not yet having become adults, the adult parasite becoming sterile depending on treatment, and the microfilariae being controlled by immunological mechanisms in the final host (4). Serological tests should be preferred to detect occult heartworm infection. Occult heartworm infection has been detected between 26.5-29.6% of positive dogs (8,9,14). In this study, only two female parasites were found in the right heart.

Some mosquito species play the role of intermediate hosts in *D. immitis* life cycle. *D. immitis* DNA has been mostly detected in *Aedes vexans* and *Culex pipiens* in Kayseri, 51.7% and 42.1%, respectively (15). Similarly, *Dirofilaria* spp. DNA has been reported in *Ae. vexans* (6.66%) in Aras Valley, located in north-eastern Türkiye (16). Çankırı is one of the areas in the Kızılırmak river basin where rice farming is intensively carried out (17). There is no information on which mosquito species can transmit *D. immitis* in this region. In a previous study, *Anopheles maculipennis* s.s was determined as the most common mosquito species in this region (17). *Anopheles maculipennis* is determined as vector of *D. immitis* in different countries in Europe (18). Similarly, *An. maculipennis* sl is claimed as a potential vector of *D. immitis* and *D. repens* due to their DNA being found in head-thorax pools of this species sampled in Aras Valley (17). However, vector competence differs in vectorborne parasites, including *Dirofilaria* species and is influenced by various factors. Thus, further studies are required to determine which mosquito species serve as vectors for *D. immitis* in this region.

## CONCLUSION

In conclusion, vector-borne infections are expected to increase with global warming. In this study, *D. immitis* was seen for the first time in an Izci Kopegi Zagar necropsy in Ilgaz, Çankırı. Moreover, the presence of two female parasites in this dog was considered noteworthy. Due to the possibility of occult heartworm infection, it is recommended that veterinarians perform serological tests as well as blood examinations on suspected dogs in endemic areas.

### *Ethics

**Informed Consent:** The dog owner was informed of the study and a signed consent form was obtained.

### Footnotes

#### *Authorship Contributions

Design: S.Y., K.Y., Data Collection or Processing: S.Y., K.Y., Analysis or Interpretation: S.Y., K.Y., Literature Search: S.Y., K.Y., Writing: K.Y.

**Conflict of Interest:** No conflict of interest was declared by the authors.

**Financial Disclosure:** The authors declared that this study received no financial support.

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# 2025 Hakem Dizini - 2025 Referee Index

Abdurrahman Ekici
Ahmet Özbilgin
Ahmet Özkeklikçi
Alican Bilden
Alireza Chavshin
Alparslan Yıldırım
Ayşe Caner
Ayşe Gül Ünver
Ayşegül Taylan Özkan
Aysu Değirmenci Döşkaya
Banuçiçek Yücesan
Cem Vuruşaner
Ceylan Polat
Cihangir Akdemir
Derya Dirim Erdoğan
Emre Öz
Erdoğan Malatyalı
Evren Tileklioğlu
Eylem Akdur Öztürk
Gülay Aral Akarsu
Gülnaz Çulha
Hamza Avcıoğlu
Hatice Ertabaklar
Hüseyin Ayhan
Kader Yıldız
Kosta Y. Mumcuoğlu
Levent Aydın
Mehmet Ziya Alkan
Mehmet Aykur
Muhammed Nalçacı
Mükremin Özkan Arslan
Mustafa Karatepe
Mustafa Köse
Mustafa Necati Muz
Muttalip Çiçek
Nafiye Koç İnak
Nuran Aysul
Oktay Alver
Onur Köse
Orçun Zorbozan
Özgür Kurt
Özlem Çakıcı
Özlem Makbule Kaya
Özlem Miman
Özlem Ulusan Bağcı
Salar Zarrabi Ahrabi
Sami Şimşek
Samira Firooziyan
Sefer Özer Babat
Selma Usluca
Sema Ertuğ
Semra Özçelik
Serdar Düşen
Şinasi Umur
Tonay İnceboz
Yener Özel
Zeph Nelson Omondi