

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 Özel¹, İbrahim Çavuş², Ahmet Özbilgin²

¹Balıkesir University Faculty of Medicine, Department of Medical Microbiology, Balıkesir, Türkiye

²Manisa 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 parasitocidal 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 parasitocidal activity at concentrations of $\geq 0.5 \mu\text{g/mL}$. 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 denemesi amaçlanmıştır.

Yöntemler: AmpB'nin *L. infantum* suşuna karşı etkinliğini ifade eden minimum parazitisit konsantrasyon (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, $0,5 \mu\text{g/mL}$ 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



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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 parasitocidal 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.

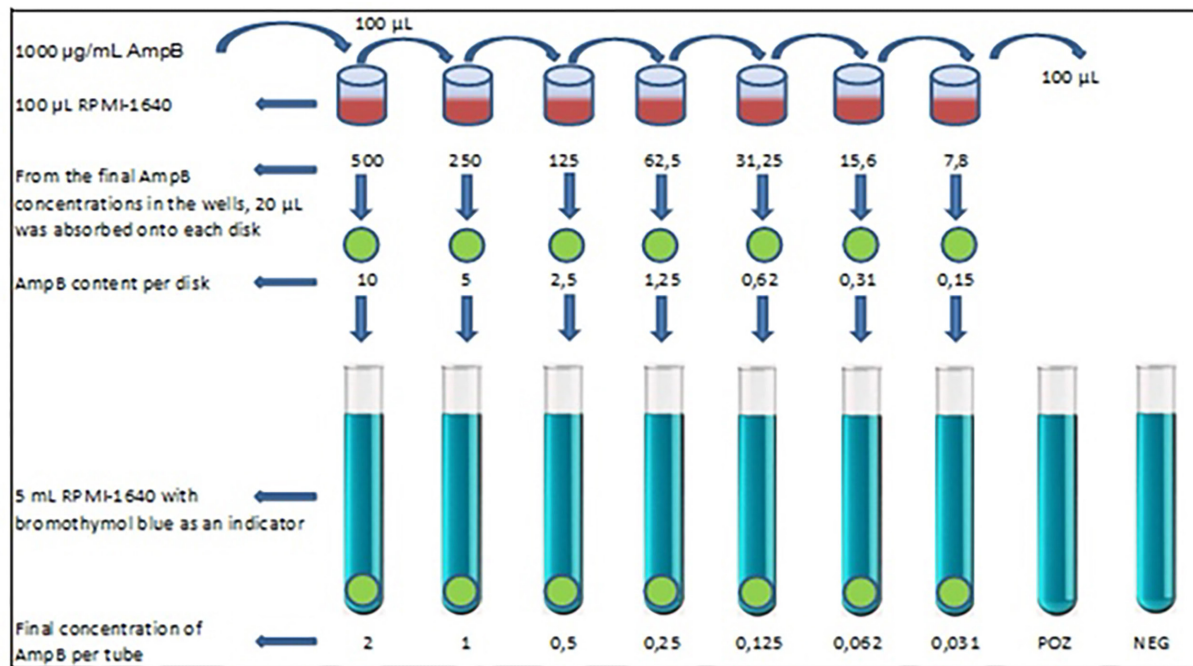


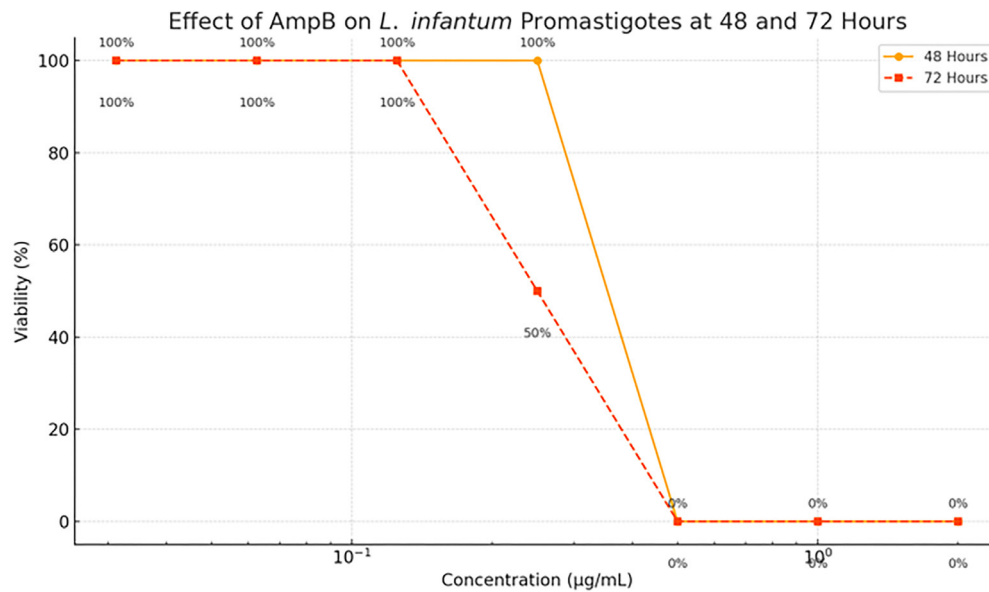
Figure 1. Schematic representation of the application process of AmpB into tubes using the modified disk elution method. Initially, serial dilutions were prepared from the 1000 µg/mL stock solution to obtain different AmpB concentrations. From each well, 20 µL of the AmpB solution was absorbed onto sterile 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



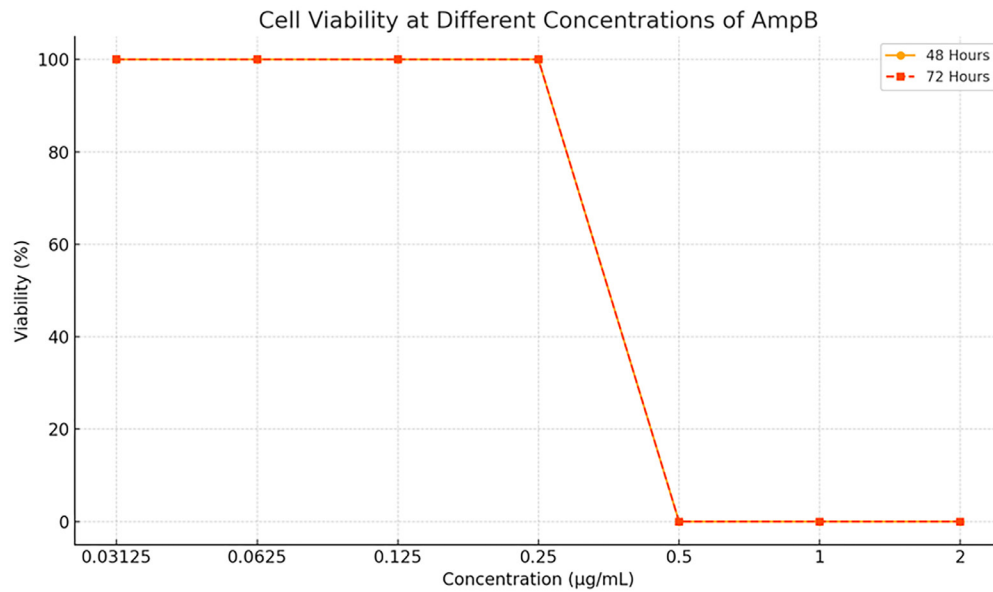


Figure 3. Line graph comparing the effects of different concentrations of AmpB on *L. infantum* promastigotes, as evaluated by the disk elution method, in terms of parasite viability (%) at 48 and 72 hours. The X-axis represents AmpB concentrations (µg/mL), and the Y-axis represents viability rates (%). 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*

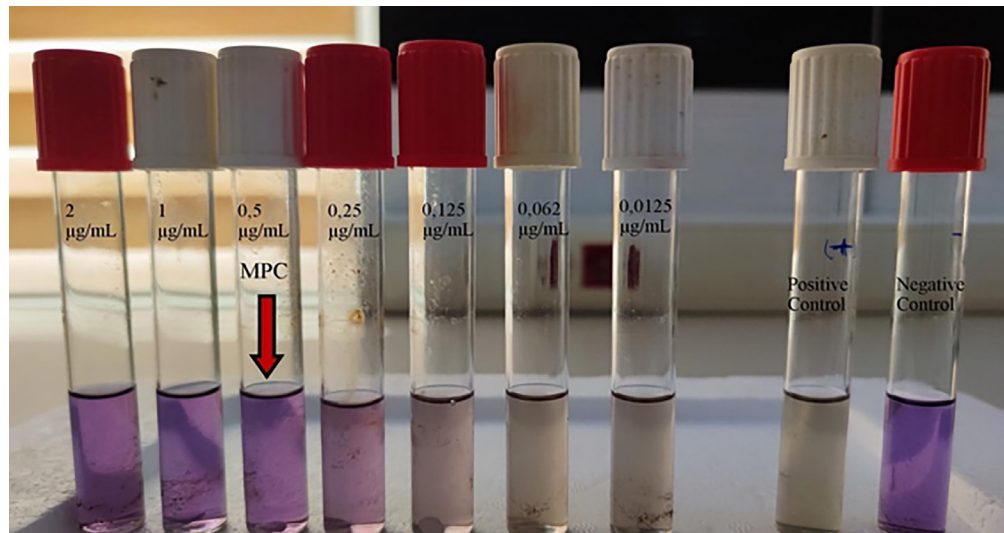


Figure 4. Appearance of the tubes prepared using the modified disk elution method at 48 hours. Each tube contains an AmpB disk at a different concentration. The indicator in the medium (normally purple) turns yellow in the presence of viable promastigotes. The third tube (indicated by the yellow arrow)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

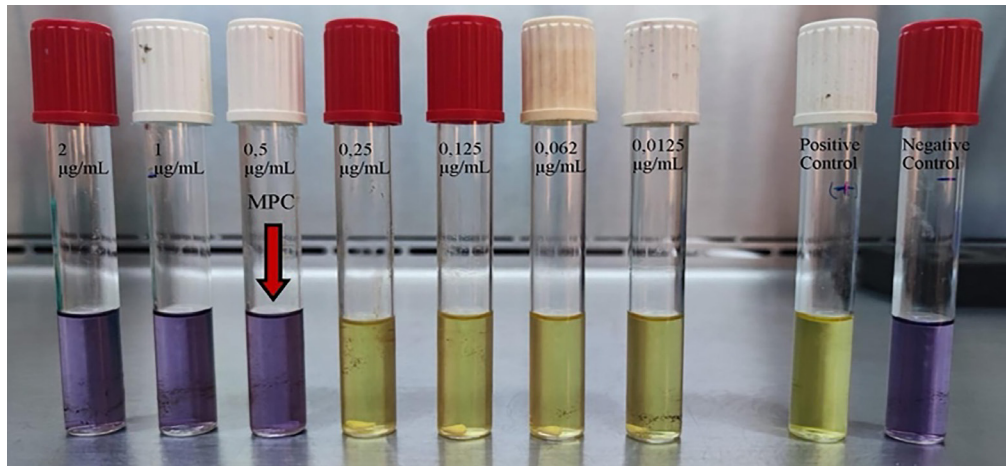


Figure 5. Appearance of the tubes prepared using the modified disk elution method at 72 hours. Each tube contains an AmpB disk at a different concentration, and the color of the indicator in the medium changes depending on parasite viability. A distinct yellow color is observed in tubes containing live 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 $\geq 0.5 \mu\text{g/mL}$, 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 $0.22 \mu\text{g/mL}$, 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

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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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