# Introduction

## Epidemiology

The various forms of Leishmaniasis exhibit a global incidence of 12 million cases, while the prevalence of cutaneous leishmaniasis about 2 million cases per year. In recent years, the prevalence of leishmaniasis has been increasing, due to human infiltration of the habitats of the animal vectors of the parasites for use as living space or part of political clashes in these areas.

Cutaneous leishmaniasis is generally found in tropical and sub-tropical regions, with particular species endemic to specific geographic areas. “Old World” leishmaniasis is typically encountered in Afghanistan, Algeria, Iraq, Iran, Saudi Arabia, Ethiopia, and the Middle East, while “New World” Leishmania species are found in Brazil, Mexico, Bolivia, and Peru.

The species responsible for Old World leishmaniasis include *L. major, L. tropica,* and *L. aethiopica*, while New World leishmaniasis is categorized into muco-cutaneous and visceral types. The muco-cutaneous type is caused by *L. mexicana*, *L. venezuelensis*, and *L. amazonensis*, while visceral leishmaniasis is predominantly caused by *L. infantum*, *L. donovani (Old World), and L. chagasi (New World),* which can rarely also cause muco-cutaneous leishmaniasis.

The purpose of this work is to evaluate the sensitivity of *L. major* (most common in Israel) to paromomycin (Leshcutan) treatment in a laboratory setting and determine if there is a possible significance to the location the disease was contracted.

**Methods:** 20 frozen samples of *L. major* parasites were thawed and cultured in their promastigote forms. Sensitivity testing to paromomycin was performed by analyzing the metabolic activity of the parasite, a marker of its viability as well as the effectiveness of the treatment. This activity was measured using chromatography methods.

**Results:** Only 10 of the 20 *L. major* samples thawed successfully reproduced, reaching adequate concentrations. Additionally, 2 samples of *L. tropica* were successfully cultured; this small number of species cultured did not allow for statistically significant comparison of the different locations from which the parasites originated. Data showed that in general, *L. tropica* was more sensitive to paromomycin than *L. major.*

**Discussion:** The purpose of this study was to evaluate sensitivity of *L. major* to treatment with paromomycin in a laboratory setting and determine whether there was a link between the location where the infection was contracted and the parasites sensitivity. We were unable to achieve this goal because of a lack of viable results and the inconsistencies of our findings with previous knowledge on the subject. Clinical experience has shown that *L. tropica* has a higher resistance to paromomycin treatment than *L. major*, yet our findings were the opposite. This work emphasizes the need for sensitivity testing of *L. major* in its amastigote form to paromomycin.

## Pathogenesis

### Vector:

Leishmania is transmitted by a group of arthropod vectors, commonly known as sandflies. Old World leishmania is transmitted by flies of the *Phlebotomus* family while New World leishmania is transmitted by various *Lutzomyia* species. Their habitats of these flies are diverse and include desert regions, rainforests, plains, and hilly regions. Sandflies are short-range flying insects, meaning they can only fly a few hundred meters from their breeding grounds. Their small size (2-3 mm) allows them to pass through insect nets, however they are very sensitive to pesticides.

### Reservoir

Most species of Leishmania are zoophiles - wild animals such as rodents, rock hyraxes, and marsupials, as well as house-pets such as dogs, act as the main reservoirs for leishmania species.

Cutaneous leishmaniasis in humans generally occurs when humans enter natural habitats of infected sandflies. Testing of people living in endemic areas reveal positive skin testing in 10-32% of the population. Skin testing, similar to tuberculin testing, indicates exposure to the parasite.

Identification and treatment of infected animals may provide an effective measure at controlling the disease.

### Life cycle:

The complete life-cycle of the leishmania parasite involves a mammal reservoir and the sandfly vector. The parasite generally exists in two main forms – in the promastigote stage the parasite lives in the sandfly as an extracellular parasite with a characteristic motile flagellum, while the intracellular amastigote form occurs in mammals, and does not have a flagellum.

The female sandfly is infected by the amastigote form while feeding on an infected mammal’s blood. In the posterior stomach of the fly, the parasite differentiates into its promastigote form and begins reproducing rapidly. During this stage the parasite propels itself towards the anterior stomach, and is then injected into the new host during the sandfly’s next feeding.

### Clinical Manifestations of Cutaneous Leishmaniasis:

The area of the sandfly bite (usually on exposed skin), presents with an erythematous papular lesion of 3-5 mm, and is otherwise asymptomatic. This lesion slowly enlarges and develops into a 3-5 cm nodule within a few months to years, and leaves behind a characteristic scar.

The incubation period, clinical presentation, number of lesions, and the speed at which they develop is dependent on the particular species of parasite. For example, *L. major* has an incubation period of 2-8 weeks after the bite, while *L. tropica* exhibits a longer incubation of about 8 months.

### Treatment:

Many treatment options for cutaneous leishmaniasis are suggested in the literature, however these recommendations are based on only small number of case studies. There are no available double-blind randomized control trials. Often, clinicians must treat patients using treatment, dosage, and durations appropriate for completely different geographic locations, sometimes even for different species than those previously studied, and response rates are accordingly inconsistent.

Treatments available include: pentavalent antimony, sodium stibogluconate, meglumine antimonite, hexadecylphosphocholine, amphotericin B, pentamidine, dapsone, azoles, paromomycin, imiquimod, cryotherapy, and photodynamic therapy. There have been reports of resistance to most of the se treatments.

This study focuses on treatment with the antibiotic paromomycin, developed by an Israeli group with the cooperation of Hadassah Medical Center and the Parasitology Department at Hebrew University. This topical preparation is thought to be particularly effective against *L. major*. However, 3 years after publication, new studies found lack of efficacy of paromomycin. We hypothesize this may be due to variants of the parasite from different geographic regions.

## Objectives:

1. Test the sensitivity of *L. major* to treatment with paromomycin (Leshcutan) in a laboratory setting

2. Determine whether geographical source of the infection is a significant factor in the parasite’s sensitivity to paromomycin treatment

## Methods:

### Parasites:

Parasites were taken from frozen laboratory samples of lesions from patients diagnosed with cutaneous leishmaniasis cause by *L. major*. The samples were thawed, and the parasites were cultured in a temperature of 26 degrees Celsius, in an appropriate medium, until the parasite concentration reached 2 × 107 promastigotes per ml.

### Growth medium:

Medium-199 (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM L-gIutamine, 100 PM adenosine, 23 PM folic acid, antibiotics (100 IU penicillin G and 100 pg/ml streptomycin), 1 × BME vitamin mix, 25 mM2-(N-morphoIino) ethanesulfonic acid (MES), 4.2mMNaHC03 and heat-inactivated fetal calf serum (fcs, 10% v/v) adjusted to pH 6.8.

### Sensitivity Testing:

Testing for sensitivity of the promastigotes was performed by analyzing the metabolic activity of the parasite. This is a marker of the parasite’s viability and thus of the effectiveness of the treatment. Metabolic activity was measured using chromatography with the following mediums:

1. (Plain) growth medium – control
2. Growth medium with promastigotes – set to represent 100% metabolic activity of the organism
3. Growth medium with promastigotes and amphotericin B – representative of 100% suppression of parasite activity
4. Growth medium, promastigotes, and 5 different concentrations of paromomycin, in order to calculate the percent suppression of parasite metabolic activity.

Each growth plate contains 3 separate cells, allowing for more accurate measurement and acting as an additional control in the experiment.

### Calculation of Percent Suppression:

Percent suppression of the parasite was calculated using the following formula for each concentration of paromomycin:

Y = [(k-y)/k] \* 100

From this, the EC50 can be derived for a particular sample. A parasite with a significantly higher EC50 is considered resistant to paromomycin

## Results:

20 samples of *L. major* from different patients across the country were thawed and cultured with the appropriate medium. Only 10 samples successfully grew to sufficient concentrations of 2x10^7 promastigotes per ml. despite repeated attempts to culture the parasites from different batches of samples or new growth plates, which were unsuccessful.

2 samples of *L. tropica* were also successfully cultured to sufficient concentrations.

The following table details the results of sensitivity testing of *L. major* and *L. tropica* to paromomycin, expressed as values of EC50. The lower the EC50, the higher its sensitivity to paromomycin. Sensitivities of *L. major* ranged from 0.019mM to 0.259mM, regardless of geographical location – from northern regions, the Galilee and Beit She’an regions, to the southern regions of Shizafon and Arabah, in the west from Tze’elim to the eastern Jordan valley. The small number of samples we were able to culture and test, did not allow for statistically significant comparison of these different regions.

Sensitivity of *L. tropica* to paromomycin ranged from 0.021 to 0.025. These samples originated from the Shomron and Jerusalem Mountain areas. It can be seen that there was no significant differences between the sensitivity ranges of *L. major* and *L. tropica*, although *L. tropica* sensitivities were on the higher range compared to *L. major.*

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| Sample | Origin | Species | EC50 (mM) |
| L137 | Jericho | *L. major* | 0.259 |
| Beit She'an 1 | Beit She'an | *L. major* | 0.202 |
| Beit She'an 2 | Beit She'an | *L. major* | 0.118 |
| P2317 | Sea of Galilee | *L. major* | 0.0497 |
| P2777 | Yeruham | *L. major* | 0.0426 |
| P2855 | Israel Trail\* | *L. major* | 0.0413 |
| P2783 | Tze’elim | *L. major* | 0.0408 |
| P2399 | Gitit | *L. major* | 0.0388 |
| P2374 | Arabah | *L. major* | 0.037 |
| P2805 | Shizafon | *L. major* | 0.019 |
| P2858 | Ma'ale Adumim | *L. tropica* | 0.025 |
| P2400 | Ma’ale Efraim | *L. tropica* | 0.021 |

Table 1: Sensitivity to paromomycin

\*Precise location unknown

## Discussion:

The purpose of this study was to evaluate the sensitivity of *L. major* to treatment with paromomycin in a laboratory setting, and determine if the location the infection was contracted had a significant impact on its apparent sensitivity to paromomycin. We were unable to achieve this goal, because of the small data set we were able to produce, and because our results were contradictory to previous clinical experience, which has shown higher rates of resistance to paromomycin in *L. tropica* compared to *L. major*, while our results showed the opposite. This suggests that laboratory sensitivity testing does not correlate well with the parasite’s sensitivity to paromomycin, similar to the conclusions of other researchers in an investigation of *L Danovi* sensitivity to sodium stibogluconate (Pentostam) treatment in a laboratory.

Possible explanations for the inconsistency between the laboratory findings and the actual sensitivity of the parasite to paromomycin may be due to the different environment of the parasite within a host, which contains an immune system not found in the laboratory environment. Additionally, the *in vitro* laboratory testing is performed on the promastigote form, while *in vivo* the parasite exists as an intracellular amastigote. Growth requirements and measurement of treatment sensitivity for the amastigote forms are problematic. The method for testing sensitivity of amastigotes to treatment is by infecting macrophages using promastigotes. However, a number of factors can influence these results, such as the wide variance in the parasites ability to infect and reproduce within macrophages. Therefore, this method is quite inadequate; attempts in recent years to improve on these methods have been unsuccessful. These considerations led us opt for an alternative to testing the amastigote forms, by testing *L. major* sensitivity to paromomycin while in its promastigote form for the first time. The significance of this work is in highlighting the importance of testing the amastigote forms, and the development of those methods for better sensitivity testing of Leishmania.