# Introduction

The purpose of this work is to evaluate the sensitivity of *L. Tropica* to paromomycin (Leshcutan) ) and to sodium stibogluconate (Pentostam) treatment in a laboratory setting.

**Methods:** 18 frozen samples of *L. Tropica* parasites were thawed and cultured in their promastigote forms. Sensitivity testing to paromomycin (Leshcutan) and to sodium stibogluconate (Pentostam) was performed by analyzing the metabolic activity of the parasite, a marker of its viability as well as the effectiveness of both treatments. This activity was measured using chromatography methods.

**Results:** Only 11 of the 18 *L. Tropica* samples were7 successfully reproduced, reaching adequate concentrations. This small number of species cultured did not allow for statistically significant comparison. Data showed that, in general, *L. tropica* was more sensitive to paromomycin than to sodium stibogluconate*.*

**Discussion:** The purpose of this study was to evaluate sensitivity of *L. Tropica* to treatment with both paromomycin and sodium stibogluconate in a laboratory setting and determine whether *L.Tropica* is more sensitive to paromomycin or to sodium stibogluconate in a laboratory setting. Clinical experience has shown that *L. tropica* has a higher resistance to paromomycin treatment than to sodium stibogluconate, yet our findings were the opposite. This work emphasizes the need for sensitivity testing of *L. Tropica* in its amastigote form to paromomycin.

## Introduction to Leshmaniasis:

## Epidemiology

The various forms of Leishmaniasis exhibit a global incidence of 12 million cases, while the prevalence of cutaneous leishmaniasis amounts up to 2 million cases per year. In recent years, the prevalence of leishmaniasis has been increasing, due to a number of factors, most importantly, human infiltration of the habitats of the animal vectors of the parasites for use as living space or part of political situation in some areas, and mass migration.

Cutaneous leishmaniasis is usually found in tropical and sub-tropical regions, with particular species endemic to specific geographic areas. Geographically, cutaneous leishmaniasis has been categorized into “Old World” leishmaniasis,which is typically encountered in countries like Afghanistan, Algeria, Ethiopia, Iraq, Iran, Saudi Arabia, and other countries in the Middle East, while “New World” Leishmania species, that are usually found in Brazil, Mexico, Bolivia, and Peru.

The species responsible for Old World cutaneous leishmaniasis include *L. major, L. tropica,* and *L. aethiopica*, while visceral leishmaniasis in the Old World is predominantly caused by *L. infantum*, *L. donovani,* which can rarely also cause muco-cutaneous leishmaniasis*.*  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*, *and L. chagasi .*

## Pathogenesis

### Vector:

Leishmania is transmitted by a group of arthropod vectors, known as sandflies. The female sandfly, belonging to the genera Phlebotomus in the Old World and Lutzomyia in the New World, is the sole vector responsible for transmitting leishmaniasis . Ninety three of the around 800 known sandfly species spread leishmaniasis. The haematophagous sandfly is a noiseless 2-3 mm long arthropod whose colour ranges from black to white. Their small size allows them to pass through insect nets, however they are very sensitive to pesticides. Sandflies are short-range flying insects, meaning they can only fly a few hundred meters from their breeding grounds

The habitats of these flies are diverse and include desert regions, rainforests, plains, and hilly regions.

### 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 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 reservoir (mammal) and the vector (sandfly). The parasite generally has two main forms : Promastigote and Amastigote.

 The promastigote form is the stage in which 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:**

In the Old World CL manifests initially as a painless erythematous papular lesion of 3-5 mm, which progresses over weeks to months into a 3-5 cm nodule with a central crust underneath which is an indurated dry ulcer. Healing occurs over several months/years depending on the species of the parasite and on the hosts immunity and may leave a scar and permanent alterations in the skin pigmentation.

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

### **Treatment:**

Many treatment options for cutaneous leishmaniasis are prescribed in the literature, however these are usually based on only small number of case studies, with very limited 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 topical, lesional and systemic treatments:

 Lesional/Topical :sodium stibogluconate(interlesional), paromomycin, , imiquimod, cryotherapy, electrotherapy, thermotherapy and photodynamic therapy.

Systemic: pentavalent antimony, sodium stibogluconate, meglumine antimonite, hexadecylphosphocholine, amphotericin B, pentamidine, dapsone, azoles.

There have been reports of resistance to most of these treatments.

This study focuses on treatment of *L.Tropica* with sodium stibogluconate and the antibiotic paromomycin, developed by an Israeli group with the cooperation of Hadassah Medical Center and the Department of Parasitology, The Hebrew University, Jerusalem. This topical preparation is considered to be particularly effective against *L.Major* and less effective against *L. Tropica* than sodium stibogluconate.

## Objectives:

 Test the sensitivity of *L. Tropica* to treatment with paromomycin (Leshcutan) and with sodium stibogluconate (Pentostam) in a laboratory setting

## Methods:

### Parasites:

Parasites were taken from frozen laboratory samples of lesions from patients diagnosed with cutaneous Leishmaniasis from the location of *Ma’aleh Adumim*, which is known geographically as an *L.Tropica* endemic area. 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.
5. Growth medium, promastigotes, and 5 different concentrations of sodium stibogluconate, 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:

18 samples of *L. Tropica (according to the geographical location of Ma’aleh Adomim and one sample from Karmel -and not by PCR)*  were thawed and cultured with the appropriate medium. Only 11 samples successfully grew to sufficient concentrations of 2x10^7 promastigotes per ml.

The following table details the results of sensitivity testing of *L. tropica* to paromomycin and to sodium stibogluconate , expressed as values of EC50. The lower the EC50, the higher its sensitivity to the treatment. Sensitivities of *L. Tropica* to Paromomycin ranged from 250 to 17 and to of sodium stibogluconate from 250 to 22, with an overall sensitivity to Paromomycin ,8 strains having a lower EC50 in the Paromomycin treated strains against 2 having a lower EC50 with the of sodium stibogluconate treated strains and one having an equal EC50 to both treatments.

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|  | Strain | Geographical Location | Sodium Stibogluconate (Pentostam) EC50 | Paromomycin (Leiscutan) EC50 |
| 1 | ***L1638*** | Karmel | 67 | 18 |
| 2 | ***L1504*** | Ma'aleh Adumim | 55 | 56 |
| 3 | ***L1534*** | Ma'aleh Adumim | 69 | 48 |
| 4 | ***L1566*** | Ma'aleh Adumim | 53 | 17 |
| 5 | ***L1567*** | Ma'aleh Adumim | 60 | 30 |
| 6 | **L1559** | Ma'aleh Adumim | 170.6 | 76.9 |
| 7 | **L1579** | Ma'aleh Adumim | >250 | 152.1 |
| 8 | **L1601** | Ma'aleh Adumim | 92.91 | 33.17 |
| 9 | **L1607** | Ma'aleh Adumim | >250 | >250 |
| 10 | **L1623** | Ma'aleh Adumim | 22 | >27 |
| 11 | **L1558** | Ma'aleh Adumim | >80 | 68.96 |

## Discussion:

The purpose of this study was to evaluate the sensitivity of *L. Tropica* to treatment with paromomycin and sodium stibogluconate in a laboratory setting.

Previous clinical experience has shown higher rates of resistance to paromomycin in *L. tropica* compared to treatment with sodium stibogluconate, 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 and of *L.Major* sensitivity to Paromomycin (Leshcutan).

Possible explanations for the inconsistency between the laboratory findings and the actual sensitivity of the parasite to paromomycin and of sodium stibogluconate 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. Tropica* sensitivity to paromomycin and to sodium stibogluconate while in its promastigote form. 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.