

Research Note

Antileishmanial activity of drug infused mini-agar plates on *Leishmania donovani* promastigotes

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Abstract. Drug infused mini agar plates were found to be a better alternative of broth dilution method in the determination of antileishmanial susceptibility of two commonly used drugs, Sodium antimony gluconate and Amphotericin B against *Leishmania donovani* promastigotes. These two drugs were used here as models for antileishmanial compounds. The stability of the drugs in the stored agar plates was also tested for six months and found that they were same as fresh plates. Determination of antileishmanial susceptibility of *Leishmania donovani* promastigotes to compounds of screening by this method is quite inexpensive, simple to perform even in under-sophisticated laboratories of developing countries where the disease is endemic.

Leishmaniasis, a group of diseases endemic in 88 countries, is a major public health problem worldwide, with approximately 2-3 million new cases occurring each year and that about 350 million people are at risk (Singh, 2006). The current scenario for the treatment of leishmaniasis is more promising than it has been for several decades, mainly due to the availability of new drugs and new formulations of old drugs. However, still, the emergence of drug resistant strains of leishmania parasite has imposed those who are in the field to search for novel antileishmanial agents from different sources. In order to handle a large number of drug candidates, primary screening requires tests that are simple to manipulate, reproducible and easily quantifiable (Serenio *et al.*, 2007). Primary screening programmes for antileishmanials

are based either on random screening, which relies on a high throughput (approximately one compound moves on to advanced development per 10,000 compounds screened), or on directed screening, which relies on informed selection criteria to limit the numbers of compounds screened. The philosophy of the screen (random versus directed) currently has a direct impact on the choice of the *Leishmania* model (promastigote vs intracellular amastigote) (Callahan, 1997). Although, there are significant differences between promastigotes and clinically more relevant amastigotes in biochemistry and susceptibility to standard and experimental drugs, the promastigotes assays are still useful as cytotoxicity indicators in testing antileishmanials (Croft *et al.*, 2006) and being used as routine in most antileishmanial works (Habtemariam,

2003; Mantyla *et al.*, 2004; Kedzierski *et al.*, 2007). For *in vitro* screening of new antileishmanial drugs, there is a need for reliable, reproducible and simple assays. Indeed, existing assays present disadvantages: direct counting is time-consuming and hard to perform; other techniques such as ³[H] thymidine, FACS, and transgenic parasites need particular tools and specific experience; moreover, spectrophotometric methods have not been sufficiently validated. Although animal models are well established for drug testing, they are unsuitable (Fumarola *et al.*, 2004). Although, the promastigote stage is easily cultivatable, the available techniques for drug screening are still complex and need to be simplified. The main objective of this study was not to determine the drug sensitivity of any particular drug, but to find out the susceptibility of promastigotes to any particular drug candidate by a simple method.

Although, the growth of *Leishmania* promastigotes on the surface of agar based semi solid media and form macroscopic colonies has already been well documented (Hill, 1983; Hill & Fahey, 1987; Muniaraj *et al.*, 2005), we report the use of drug infused agar plates in the determination of drug susceptibility of *Leishmania* promastigotes. In the present study, a simple agar plating technique was used in the evaluation of susceptibility of *Leishmania donovani* promastigotes to commonly used drugs, sodium antimony gluconate (SAG) and amphotericin B (AmB), both were used here as models for drug candidates. This method is economical, easy to perform and suitable for even under-sophisticated laboratories of the developing countries where the diseases are endemic. The drug infused agar plates can be prepared once in bulk and can be stored for a long time in a refrigerator and readily used whenever there is a need, especially in field conditions. Moreover, the size of the petri plates used in this work is only 35 mm in diameter (Tarsons, India) which can hold as little as 2 ml of medium – an added

advantage. The reliability of agar plating method in the determination of drug susceptibility was compared with standard but laborious broth dilution technique and found that agar plating was giving the similar susceptibility pattern.

Drug infused agar plating. The promastigotes used in this study were isolated from the splenic / bone marrow aspirates of patients with kala-azar / visceral leishmaniasis who had been admitted to the inpatient ward at the Rajendra Memorial Research Institute of Medical Sciences, Patna, India, in NNN medium overlain with Locke's solution (Muniaraj *et al.*, 2005). Only recent isolates (in the second passage) from 6 different patients were selected for this study. The speed of multiplication of the promastigotes may be a key to the success of agar plating as a method to determine the drug susceptibility; we used chocolate agar plates in this study, since the colonies of *L. donovani* were reported to grow more quickly on chocolate agar than blood agar medium (Muniaraj & Das, 2008). The plates were prepared by the addition of 10% (v/v) defibrinated & pooled rabbit blood to the sterile, molten blood agar base (HiMedia, India), at 80°C -90°C. 100 µl of the serially diluted drug was added to each plate (except control) and 4ml of the molten agar was poured at around 45-50°C and then the plates were rotated in clock & anticlockwise to mix both the medium and the drug. The final concentration of drug after the addition of molten agar was 10, 5, 2.5, 1.25, 0.62, 0.31, 0.15, 0.078, 0.039 mg/ml for SAG (Albert David Ltd, Kolkata, India) and µg/ml for AmB (Sarabhai Chemicals, Vadodara, India). The plates were then kept at 35°C for 24 hrs for drying and sterility checking. After drying, each plate was inoculated separately with two isolates of *L. donovani* promastigotes culture (from different patients, one was inoculated in one half and the other was inoculated in the other half of the plate) (adjusted to 1x10⁶ parasites/ml) by 0.01ml inoculation loop, and incubated at 24±1°C for 7 days. The experiment was repeated at

least three times with different promastigotes isolates. After preparing chocolate agar plates with different concentration of SAG and AmB, they were kept under refrigeration at 4-6°C for six months. After this storage period, the plates were again tested with the same but cryopreserved isolates (after revival) to check whether the plates were stable or not.

Broth dilution (Direct counting)

method. The SAG and AmB were serially diluted in Locke's solution containing 20% FBS in 96 well flat bottom plate (Tarsons products, India). Each well was then seeded with 200µl of promastigotes culture, at an initial concentration of cells equivalent to that of early log phase (2×10^5 cells/ml). After seeding, the final concentration of SAG (in mg/ml) was 10, 5, 2.5, 1.25, 0.62, 0.31, 0.156, 0.078, 0.039 and AmB (in µg/ml) was 10, 5, 2.5, 1.25, 0.625, 0.31, 0.15, 0.078, 0.039. The medium without any drug was kept as control. Plates were incubated at 25°C for 72 hr. After incubation, the viability of promastigotes was determined by counting the number of motile cells under a light microscope, using hemocytometer. The experiments were repeated at least three times with different isolates of promastigotes.

The growth of *Leishmania* promastigotes colonies on the surface of chocolate agar, in response to different concentration of the drugs SAG and AmB is given in the Fig. 1. In the agar plates, added with serially diluted SAG/ AmB, distinct, macroscopic colonies of *Leishmania* promastigotes started appearing on 3rd day and reached the maximum size on 6th day of incubation, after which, there was no further increase in size of the colonies. In the plates added with SAG, colonies of promastigotes have developed in concentration of the drug ranging from 0.395 to 5 mg SAG/ml. At the concentration of 10 mg SAG/ml, the plates were found virtually free from any promastigotes colonies. Similarly, in the plates added with AmB, the macroscopic colonies have developed only in the plate with 0.039µg AmB/ml. The remaining plates with concentration of drug ranging from 0.078 to 10µg/ml were found to be free from any promastigotes colonies. The appearance of promastigotes colonies, their texture and morphology were found as similar at both highest and lowest concentration of drugs.

The exact result of agar plating was reflected in broth dilution method (Direct counting). In the wells with serially diluted SAG, the inhibition was 95.63% at 5 mg/ml

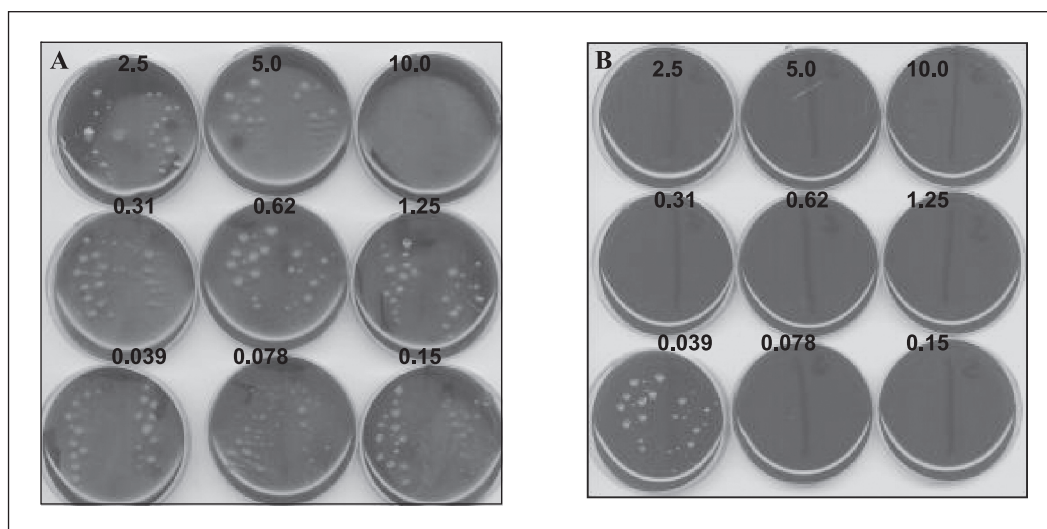


Figure 1. Drug susceptibility of promastigotes to the two common drugs, SAG and AmB. A: sodium antimony gluconate (SAG) mg/ml; B: amphotericin B (AM-B) µg/ml

Table 1. Growth of *Leishmania donovani* promastigotes in response to different concentration of sodium antimony gluconate (SAG) and amphotericin B (AM-B) by standard broth dilution and drug infused agar plates

Concentration SAG mg/ml or AM-B µg/ml	Growth of promastigotes			
	Standard broth dilution		Drug infused agar plates	
	SAG	AM-B	SAG	AM-B
0.039	+	+	+	+
0.078	+	-	+	-
0.156	+	-	+	-
0.13	+	-	+	-
0.62	+	-	+	-
1.25	+	-	+	-
2.5	+	-	+	-
5	+	-	+	-
10	-	-	-	-

concentration. Similarly, in the wells with AmB, the inhibition of promastigotes was 91.70% at 0.039 µg/ml and the AmB concentration higher than 0.039 µg/ml showed 100% inhibition (Table 1). This one step inoculation of parasites in drugs added agar plates would largely reduce the expenses, complexity of promastigotes testing by broth dilution. Although, the promastigotes is not clinically relevant stage, the microscopic counting of motile cells after treatment with particular compound is considered to be the Gold standard for the screening of compounds for antileishmanial activity (Habtemariam, 2003). When comparing with the technically complex conventional culture method in liquid broth, though which can provide results in 3 days (Callahan *et al.*, 1997), the present method is technically simpler and easy to perform, except, the results will be available only on 6th day. The broth dilution method is comparatively cumbersome as it requires good expertise in the technique, well equipped laboratory and lengthy and often painful microscopic observation. However, the agar plating technique with added chemotherapeutic agents (pre-prepared plates) require only one step inoculation of *Leishmania* promastigotes culture and the result will be

available on 6th day of incubation. This agar plating method could also be used for similar protozoans than *Leishmania* such as *Trypanosoma* spp. which can also form colonial growth on the agar based medium. This method could be a better alternative to the broth dilution method in the screening of antileishmanial compounds. The only disadvantage of this method seems to be that, it requires 6 days to find out the result as compared with 3 days of broth dilution method.

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