

CHAPTER III

*A Rapid And Sensitive Method To Quantify Leishmania
Infection In Macrophages By Episomal Expression Of
Luciferase Reporter Gene*

1.0 Introduction

Leishmaniasis is a parasitic disease caused by a haemoflagellate *Leishmania*. There are more than 21 species causing human infection and are transmitted to humans through the bite of female sandflies. The disease manifests mainly in 3 forms: the visceral, cutaneous and mucocutaneous leishmaniasis. The diagnosis of visceral form is conventionally made by the demonstration of amastigotes of the parasite in the aspirated fluid from bone marrow, spleen and rarely from lymph node or liver. The parasite demonstration and isolation rates are rather poor from cutaneous and mucocutaneous lesions due to low parasite load and high rate of culture contamination. Different species of *Leishmania* can be maintained easily as promastigotes in a wide variety of culture medium. In case of certain species it is even possible to grow amastigotes in axenic culture (Pan, Duboise and Eperon, 1993; Bates, 1994; Sereno and Lemesre, 1997a). Axenic amastigotes have since been reported for several strains of *Leishmania* using temperature elevation as a trigger, either alone or in combination with a reduction in pH (Bates, 1993; Zilberstein and Shapira, 1994).

Number of methods have been developed to measure *Leishmania* infections in animal tissues or in infected macrophages *in vitro*. *In vitro* infection of parasite is normally determined by staining the parasite nucleus with Giemsa staining (Berman, 1984). *L. donovani* amastigotes in tissues are most efficiently measured by microscopic examination, following Giemsa staining of spleen and liver biopsy smears (Olivier, Proulx and Tanner, 1989). Although, disadvantages of this technique are that loss of viability could precede the inability to react with Giemsa, and that the counting procedure is manual and potentially subjective.

Molecular genetic studies in parasitic protozoa have been hindered by the lack of methods for the introduction and expression of modified or foreign genes in these organisms. Generally, targeted recombination in mammalian cells is less frequent than non-homologous integration (Mansour, Thomas and Capecchi, 1988; Thomas and Capecchi, 1987). In the Kinetoplastidae *Trypanosoma* and *Leishmania*, as in yeast cells (Rothstein, 1991), nearly all integration events proceed by homologous recombination (Cruz et al., 1990; Eid and Sollner-Webb, 1991; Lee and van der Ploeg, 1990; Ten Asbroek, Ouellette and Borst, 1990; Tobin, Laban and Wirth, 1991). Recently, genetic manipulations in protozoan parasites have become possible by introducing exogenous DNA by electroporation, yielding stably transfected parasite lines. In *Leishmania* the transfected DNA can either remain episomal or can integrate into the genome by homologous recombination (Cruz et al., 1990; Eid and Sollner-Webb, 1991; Lee and van der Ploeg, 1990; Ten Asbroek, Ouellette and Borst, 1990; Tobin, Laban and Wirth, 1991; Curotto de Lafaille and Wirth, 1992; Dumans, et al., 1997; Papadopoulou, et al., 1994; Papadopoulou, et al., 1996). The use of reporter genes such as the firefly luciferase (Gay, Wilson and Donelson, 1996), chloramphenicol acetyl transferase (Laban and Wirth, 1989), the β -galactosidase and β -glucuronidase genes (LeBowitz, Coburn and Beverley, 1991), and the green fluorescent protein, has considerably facilitated the screening of antimicrobial agents against intracellular pathogens such as *Mycobacterium tuberculosis* (Collins, Torrero and Franzblau, 1998; Jacobs et al., 1993), *Trypanosoma cruzi* (Buckner, et al., 1996), *Toxoplasma gondii* (McFadden, Seeber and Boothroyd, 1997), and *Leishmania* (Serenio, et al., 2001). The aim of the present study was to stably express the firefly luciferase gene (LUC) in *Leishmania* in order to develop a method that allows easy and sensitive method of detection of infection in infected macrophages. Firefly luciferase represents one of the most

efficient available biological reporter molecules because it catalyzes the reaction of luciferin with adenosine triphosphate (ATP) to generate photons with a quantum yield of 0.85 photons per molecule of substrate reacted. Because of the availability of a variety of sensitive light-detection systems, luciferase has become the standard assay for measuring ATP. Since the molecular cloning of its cDNA (DeWet, et al., 1985), the firefly luciferase gene has been used directly as a molecular reporter in cells of a variety of animal, plant, and bacterial species.

2.0 Materials and Methods

2.1 Chemicals

The tissue culture chemicals like M-199, RPMI-1640, penicillin-G, streptomycin, HEPES, sodium bicarbonate etc. were procured from Sigma Chemical Co., USA. Fetal Calf Serum (FCS) was purchased from Gibco-BRL Ltd., USA. The LUC-expressing episomal vector pGL- α NEO α LUC was kindly gifted by Marc Ouellette, University of Laval, Canada. Luciferase assay buffer was procured from Promega, (Madison, USA). Tri reagent was purchased from Sigma Chemical Co., USA. All other chemicals were purchased locally and were of analytical grade.

2.2 Parasite culture

Leishmania donovani AG83 (MHOM/IN/1983/AG83) promastigotes were cultured at 22°C in modified M199 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% heat inactivated fetal calf serum (FCS). AG83-W strain used for antimony susceptibility studies has been growing in the laboratory for several months and was non-virulent in the animal model system. The wild type AG83-W strain used here has been passaged several times in the laboratory and was found to have low virulence as it had low infectivity in macrophages and in the animal model system. Strains 41-R, CK2-R and

NS2-R are the clinical isolates, which were supposedly resistant to antimony. The isolates were obtained from IICB, Calcutta.

2.3 Macrophage culture

A murine macrophage cell line J774A.1 (American Type Culture Collection, Rockville, Maryland) was used in this study. The macrophages were maintained at 37°C in RPMI-1640 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% FCS in an atmosphere of 5% CO₂ in air. The macrophages were seeded onto tissue culture plates (60 mm) at a density of 1×10^6 cells/plate and incubated for 24 h before being used for the requisite assay.

2.4 Luciferase expressing vector

The LUC-expressing episomal vector pGL- α NEO α LUC consists of neomycin phosphotransferase gene (NEO) and two α -tubulin intergenic regions, together with the relevant restriction sites for Bg/II, HindIII and pfm1I (Fig. 1). In the LUC-expressing vectors, the maturation of the firefly luciferase transcript is under the control of the intergenic region of the α -tubulin (α) gene (Papadopoulou, Roy, Ouellette, 1994). LUC-containing episomal vector were electroporated into the *L. donovani* and transfectants were selected for resistance to G418 (Sigma).

2.5 Electroporation of *Leishmania donovani* expressing luciferase vector

An appropriate number of cells were grown to stationary phase ($\sim 2 \times 10^7$). For each experiment, 1×10^8 cells were used. An appropriate number of cells were spun down in a sterile centrifuge tube at 3000 rpm for 10 minutes at 4°C. The supernatant was poured off and the pellet was resuspended in 1 ml of phosphate buffered saline (PBS). The contents were transferred to a new tube and the spin was repeated at 3000 rpm for 10 min and at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml of

electroporation buffer (EP buffer) (21 mM HEPES (pH 7.5), 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄, 6 mM Glucose). The cells were spun down as above. The supernatant was removed and the pellet was again resuspended in EP buffer to 2 x 10⁸ cells/ml. 0.8 x 10⁸ cells were transferred to the cuvette (BTX 2 mm gap electroporation cuvettes) and placed on ice. DNA (50 µg) was added to the cuvette with a sterile pipette. The cuvettes were kept on ice for 10 min. The cells were zapped using the following BTX settings: 450 V/cm and 500 µF capacitance. After shock, the cuvettes were again kept on ice for 10 min. The cells were transferred to tissue culture flask containing 10 ml of M-199 (20% FCS) without selection antibiotic. The cells were allowed to grow overnight at 22°C. The cell concentration was determined on the following day. After 24 h, drug was added to both the control and the transfected cells G418 (40 µg/ml) in case of neomycin resistance gene. All the flasks were kept at 22°C for a week or two till control cells die and transfectants started growing.

2.6 Luciferase activity

Leishmania cells expressing the luciferase gene were harvested by centrifugation, washed, and diluted from 10¹ to 10⁷ parasites in a luciferase assay buffer (Promega). The samples were lysed with a lysis buffer (5X) at a final concentration of 25 mM Tris pH 7.8, 2 mM DTT, 10% glycerol and 1% Triton-X-100, incubated at room temperature for 30 min, and then kept at -80°C until assayed. An aliquot of 5 µl of parasite lysate was thawed completely, mixed with 20 µl of luciferase assay buffer (20 mM tricine, 1.07 mM (MgCO₃)₄ . Mg (OH)₂. 5 H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 200 µM ATP, 33.3 mM DTT) and transferred to a luminometer plate (Barbeau, Bernier, Dumais et al., 1997). Luciferase activity was measured using tube scintillation counter. Light output was measured for 20s/well after a 2-5s delay. Values were expressed as RLU (relative light

units). To measure the luciferase activity in intracellular amastigotes isolated from infected macrophages, macrophage cells were washed to remove non-internalized promastigotes and incubated with the luciferase lysis buffer as described above.

2.7 Infectivity assay

Macrophages (1×10^6) were plated in 60 mm tissue culture plate and were infected with parasite/macrophage ratio of 10:1. Macrophages were kept at 37°C in an atmosphere of 5% CO₂ in air. Non-internalized parasites were washed off after different incubation periods (i.e. 3, 12, 24, 48, 72 h). Cells were then fixed with methanol and stained with Giemsa or lysed for measuring luciferase activity. The percentage of infected macrophages, the mean number of amastigotes per macrophage were determined by Giemsa staining. Results were then expressed as total number of amastigotes per macrophage. Alternatively, parasites were detected by measuring luciferase activity. A total of 10^6 murine macrophage derived from the cell line J774 A.1 were infected with *L. donovani* stationary promastigotes at a parasite/macrophage ratio of 10:1 (Dumas et al., 1997). Total number of amastigotes per plate was counted by the luciferase assay and values were expressed as RLU (relative light units).

2.8 Drug study

J774A.1 macrophages were plated as mentioned above. Antimony resistant isolates 41-R, CK2-R, NS2-R and the wild-type strain AG83-W were transfected with luciferase episomal vector (pGL- α NEO α LUC) and were used for infectivity assay as described above. The infected macrophages were treated with different concentrations of pentavalent antimonial compound (Sb^V-Cl₂) for a period of five days and the cells were lysed on day 5 after drug treatment and luciferase assay was carried out as described.

2.9 RNA extraction

Total RNA was isolated from *Leishmania donovani* transfected with luciferase episomal vector (pGL- α NEO α LUC), using Tri reagent (Sigma, USA) according to the manufacture's instructions as described in General Materials and Methods section.

3.0 Results

3.1 *Leishmania donovani* expressing the firefly luciferase gene

The firefly luciferase (LUC) (Gould and Subramani, 1988) has been a useful reporter gene to monitor gene expression (Welsh and Kay, 1997) and to probe host-microbe interactions (Valdivia and Falkow, 1997). We studied the possibility of stably expressing the firefly luciferase gene in the protozoan parasite *Leishmania*. The LUC-expressing vector (pGL- α NEO α LUC) (Fig. 1) was electroporated into *L. donovani*, which were then selected in the presence of G418. To test whether luciferase activity correlated well with the number of parasites, we made serial dilutions of promastigotes and measured luciferase activity. An excellent correlation was observed with the number of *L. donovani* promastigotes and luciferase activity (Fig. 2).

3.2 Growth curve of *L. donovani* transfected with luciferase expressing episomal vector

Figure 3 depicts the growth profile of *L. donovani* promastigotes *in vitro* transfected with LUC (Luciferase) gene containing, episomal vector pGL- α NEO α LUC, at different time intervals in M-199 medium. LUC transfected promastigotes were inoculated into the medium at a density of 1×10^6 cells/ml and incubated at 22°C for 144 h. The cell densities were determined every 24 h both by hemocytometer counting and measuring luciferase activity as described above. There was an initial lag phase of 24 h and then the cell number

relatively peaked at 120-144 h of growth. These results are mean of duplicate samples (Fig. 3).

3.3 Luciferase activity of intracellular parasites within macrophages *in vitro*

Intracellular *Leishmania* infection was visualized either microscopically by Giemsa staining or by measuring luciferase activity. Luciferase activity estimates the number of parasites per macrophages (Fig. 4). Indeed, infection of the murine J774A.1 macrophage cell line with *L. donovani* promastigotes was maintained stably for 3 days and estimation of the number of parasites by both luciferase activity and microscopic examination correlated well (Fig. 4). The initial decrease in parasite number in Fig. 4 depicts the probable intramacrophage killing of the late-stationary promastigotes that did not differentiate.

3.4 Measuring resistance of leishmanial isolates to pentavalent antimonial compound (Sb^V-Cl₂) *in vitro* by luciferase assay

J774A.1 macrophages were plated as mentioned above. Strains 41-R, CK2-R, NS2-R and AG83-W were transfected with luciferase episomal vector (pGL- α NEO α LUC) and were used for infectivity assay. It was observed that strain AG-83-W, 41-R and CK2-R were resistant to pentavalent antimonial compound (Sb^V-Cl₂). Interestingly, field isolate NS2-R that was supposedly resistant was found to be sensitive to this pentavalent antimonial compound (Sb^V-Cl₂) (Fig. 5). IC₅₀ values for clinical isolates (41-R, CK2-R and NS2-R) and wild type (AG83-W) were calculated. IC₅₀ values for CK2-R, 41-R, NS2-R and AG83-W were 68 μ g, 84 μ g, 14 μ g and 50 μ g respectively (μ g of Sb^V). The AG83 strain used here has been passaged several times in the laboratory and showed reduced infectivity in macrophages and in the BALB/c mice. The number of amastigotes per 100 macrophages at 24 h after infection with this strain was found to be 100 ± 10 . It is known that parasite when passaged several times in the laboratory tend to loose their virulence.

3.5 Northern blot analysis of *Leishmania* LUC-transfectants

Total RNA was isolated from the *Leishmania* LUC-transfectants and wild type non-transfected AG83 strain using TRI-reagent (Sigma) according to the manufacturer's instructions. The isolated RNA was dissolved in Diethylpyrocarbonate (DEPC) treated water and stored in small aliquot at -80°C. Approximately 15 µg of total RNA was separated on MOPS/formaldehyde agarose gel as described in the General Materials and Methods section and transferred to nylon membrane. Labeling of the probe was done using Prime-a-Gene Labeling System (Promega). Northern blot of LUC transfected *Leishmania donovani* was hybridized with the LUC probe (Fig. 6). Hybridization was carried out for 16-18 h at 42°C (General Material and Methods). Result indicates the stable expression of episomal vector pGL-αNEOαLUC (Fig. 6) compared with the control (AG83).

4.0 Discussion

In this study, we have evaluated the efficiency of luciferase-encoding *Leishmania* cells expressing the LUC gene as part of an episome. New bioluminescent assays using LUC-expressing recombinant *Leishmania* gained acceptance as routine analytical techniques for evaluating *Leishmania* infections in macrophages and was also used efficiently in *in vitro* drug screening. The firefly luciferase enzyme has had a long history of use in biology, especially for the detection of ATP. The cloning of the firefly luciferase gene (De Wet et al., 1985) and its expression in cells from different organisms (De Wet et al., 1985) has generated a great deal of interest in possible applications of the gene as a tool in biological studies. Its first use was as a reporter for monitoring promoter activity. Though available for only a short time, the gene has already been widely applied in this role, due to the great sensitivity, ease of use, and cost efficiency of the luciferase assay. Also, the observation that this protein is sorted to peroxisomes in several eukaryotes has

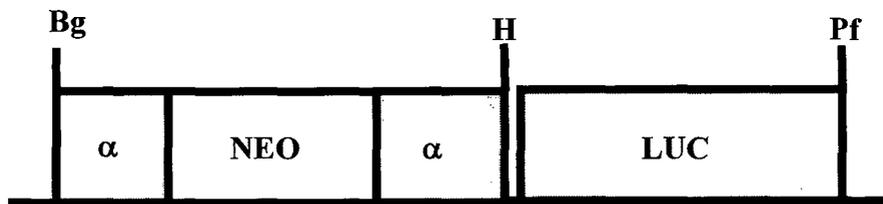
allowed the development of the luciferase gene as a model for elucidation of signals required for targeting proteins to peroxisomes. Moreover, measuring luciferase activity is straightforward and basically requires cell lysis, incubation with the luciferase substrate luciferin and counting luciferase activity with a luminometer.

The growth profile of *L. donovani* promastigote transfected with episomal vector pGL- α NEO α LUC, at different time intervals resulted in relative increase in parasite number with time. An excellent correlation between the number of *L. donovani* promastigotes and luciferase activity was observed. We have also evaluated the intracellular survival of the promastigotes using *in vitro* infection systems. With *Leishmania* promastigotes we have observed an initial decrease in the parasite burden, which probably corresponds to the non-transformed parasites that are eliminated once inside the macrophage cells. As the parasite differentiation progresses however, the level of infection is maintained.

The strain 41-R, CK2-R and AG83-W were found to be resistant to pentavalent antimonial compound ($\text{Sb}^{\text{V}}\text{-Cl}_2$) (Fig. 5). AG83-W strain used in the present study was passaged in the laboratory for several months and was non virulent in the animal model system. It was observed that AG83-W had become resistant to antimonial compound. Clinical isolate NS2-R strain, which was supposedly resistant, was found to be sensitive in our studies. IC₅₀ values for CK2-R, 41-R, NS2-R and AG83-W were 68 μg , 84 μg , 14 μg and 50 μg respectively (μg of Sb^{V}). The stable expression of luciferase reporter gene in *Leishmania* and infection in macrophage could be an excellent model to evaluate the response of drugs to the parasite and to find out if the field isolates are actually resistant to the drug or not.

Ideally, to use luciferase activity as a marker of intracellular infection for longer time points, in *in vitro* model systems, it will be preferable to quantify simultaneously the host cells. This could be done, by generating recombinant macrophage cells expressing another reporter gene that could be easily assessed in parallel to the luciferase activity derived from the parasites. A ratio of the two reporters would give the number of parasites per macrophages.

Our results confirm the stable expression of the reporter firefly luciferase gene in *L. donovani*. These recombinant parasites represent useful tools to investigate pathogen-host interactions and also in developing tools for new and effective therapeutic approaches against protozoan parasites. This model will be very successful for determining antimony-resistance of *L. donovani* clinical isolates.



pGL- α NEO α LUC

Fig. 1: Luciferase-expressing vectors for transfection in *Leishmania*. Constructs for episomal expression of the luciferase (LUC) gene. No specific promoters are required for gene expression in *Leishmania*. The only requirements are intergenic sequences that serve in RNA maturation by trans-splicing and polyadenylation. The maturation of the neomycin phosphotransferase (NEO) and LUC genes in these vectors is assessed by the intergenic region of the α tubulin gene (α). Relevant restriction sites are shown. Bg, Bg/II; H, HindIII; Pf, pfm1I

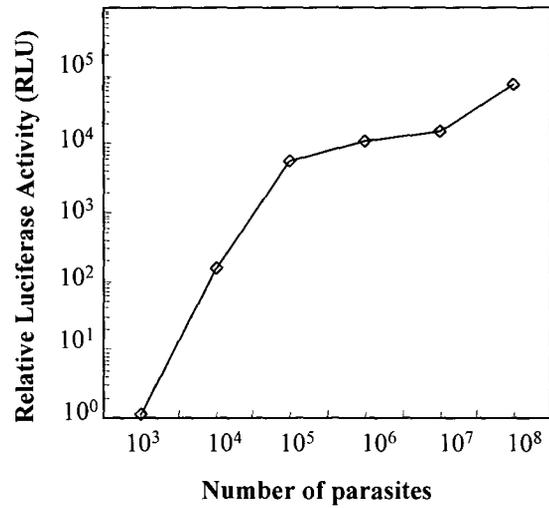


Fig. 2: Correlation between luciferase activity and number of parasites. *L. donovani* were transfected with pGL- α NEO α LUC episomal vector. Parasite cells were counted by the luciferase assay (RLU) (\diamond). Luciferase activity was expressed as RLU (relative light unit). Each point is mean of duplicate samples.

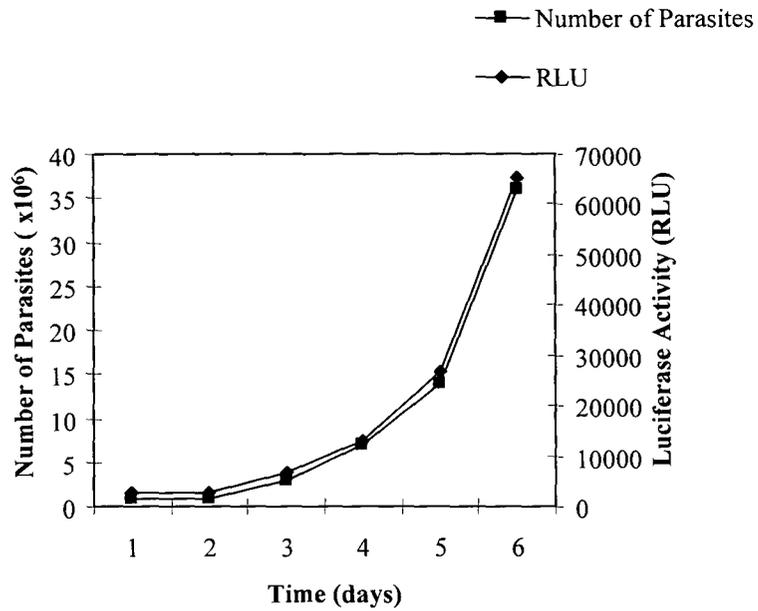


Fig. 3: Comparison of growth prolife of *L. donovani* by Giemsa staining (■) and Luciferase assay (◆). Stationary phase cells were inoculated into the medium at a density of 1×10^6 cells/ml and the cells were counted at 24 h intervals. Parasites were counted by Giemsa staining using hemocytometer. Luciferase activity expressed as RLU (relative light units) was estimated using luminometer. Each point is the mean of duplicate samples.

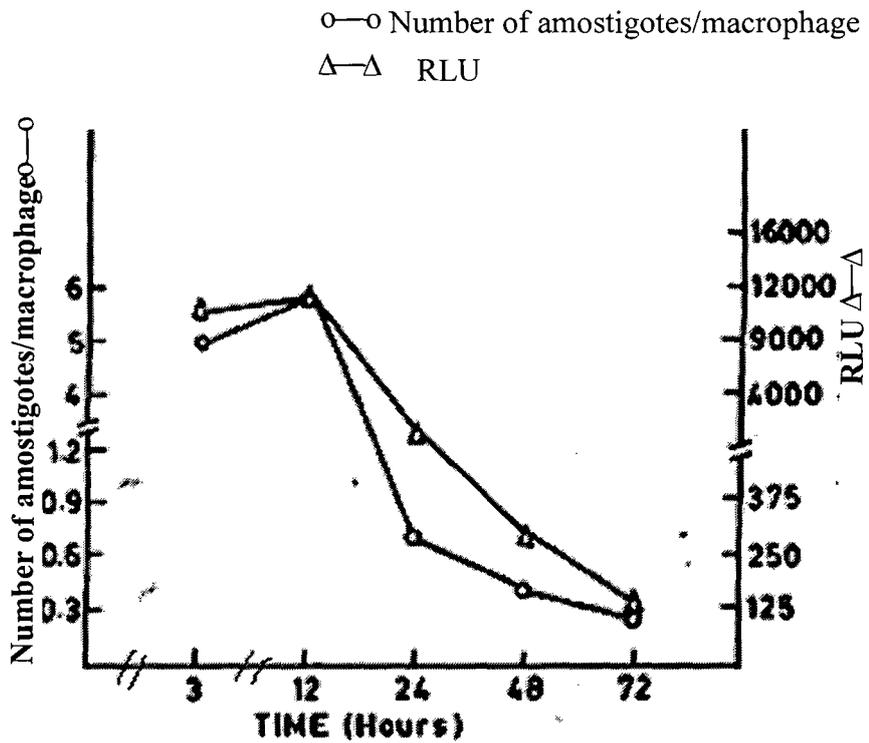


Fig. 4: Quantitation of intracellular *Leishmania* in infected macrophage *in vitro*. Infection of the murine macrophage cell line J774A.1 with *L. donovani* transfected with pGL- α NEO α LUC. Infected cells were counted by the luciferase assay (RLU) (Δ — Δ) and by Giemsa staining followed by microscopic analysis (\circ — \circ).

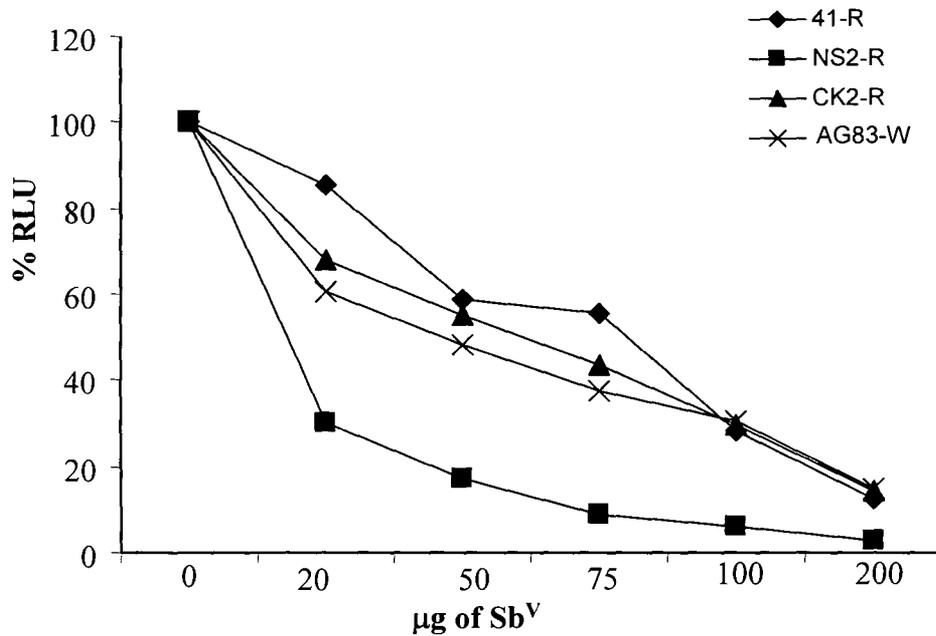


Fig. 5: Effect of pentavalent antimonial compound (Sb^V) on strains 41-R, CK2-R, NS2-R and AG83-W. J774A.1 cells were infected with strains 41-R (\blacklozenge), CK2-R (\blacktriangle), NS2-R (\blacksquare) and AG83-W (\times). These strains were transfected with luciferase episomal vector (pGL- α NEO α LUC) before using them for infectivity assay. Strains 41-R, CK2-R and AG-83 were found to be resistant to pentavalent antimonial compound (Sb^V) whereas, strain NS2-R was found to be sensitive to pentavalent antimonial compound (Sb^V).

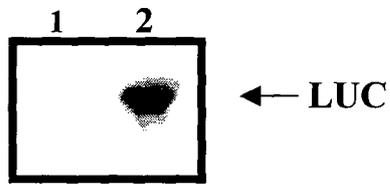


Fig. 6: Northern Blot for Luciferase transfected *L. donovani* (AG83). Northern blot of *Leishmania* LUC-transfectants hybridized with the LUC probe. Lane 1, AG83 (wild type); lane 2, AG83 with episomal LUC (pGL- α NEO α LUC).